Exploring Reactivity and Component Interactions in Toluene/o-Xylene Monooxygenase from *Pseudomonas sp. OX1*

by

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B.A. Chemistry
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Exploring Reactivity and Component Interactions in Toluene/o-Xylene Monooxygenase from *Pseudomonas sp. OX1*

by
Alexandria Deliz Liang

Submitted to the Department of Chemistry on May 08, 2015, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

**ABSTRACTS**

**Chapter 1. Component Interactions in Three- and Four-Component Bacterial Multicomponent Monooxygenases.** Bacterial multicomponent monooxygenases (BMMs) catalyze oxidation of hydrocarbon substrates through activation of dioxygen. Each BMM utilizes a diiron active site housed within a catalytic hydroxylase protein. This diiron active site is responsible for activation of dioxygen and oxidation of hydrocarbons. Additional component proteins modify the structure of the hydroxylase regulating substrate access and pre-organizing the diiron site for reactivity. The relationships between structure and reactivity that provide this control are reviewed for both three- and four-component BMMs, including soluble methane monooxygenase, phenol hydroxylase, toluene 4-monooxygenase, and toluene/o-xylene monooxygenase. Comparisons between three- and four-component BMMs are highlighted to demonstrate how nature preserves the control over reactivity within the BMM superfamily.
Chapter 2. A Flexible Glutamine Regulates the Catalytic Activity of Toluene/o-Xylene Monooxygenase. Toluene/o-xylene monooxygenase (ToMO) belongs to the enzyme superfamily of bacterial multicomponent monooxygenases (BMMs) and is capable of oxidizing aromatic substrates. The carboxylate-rich diiron active site is located 12 Å below the surface of the catalytic hydroxylase component (ToMOH). The shortest opening between the surface of the protein and the diiron active site is a small hydrophilic pore. Here we examine the function of residues lining this pore, N202 and Q228, within ToMOH from *Pseudomonas sp.* OX1. Through characterization of the steady-state turnover of WT ToMOH and three mutant enzymes, N202A, Q228A, and Q228E, we demonstrate that these residues are critical for turnover. Mechanistic analysis reveals that these residues are critical for water egress and efficiently consuming NADH to hydroxylate product. We propose that this activity results from movement of these residues, opening and closing the pore during catalysis. In addition, N202 and Q228 are important for interaction of two component proteins, the diiron-reducing protein and the regulatory protein, suggesting that these two proteins bind competitively to the hydroxylase. The function of the pore region in other BMMs is discussed in light of these results.

Chapter 3. Component Interactions and Electron Transfer in Toluene/o-Xylene Monooxygenase. Toluene/o-xylene monooxygenase (ToMO) activates dioxygen to oxidize aromatic hydrocarbons. Prior to dioxygen activation, the diiron active site must acquire two electrons. This process requires three redox active proteins, a hydroxylase (ToMOH), a Rieske protein (ToMOC), and an NADH oxidoreductase (ToMOF). A fourth, regulatory component with no redox active cofactors is also required to achieve catalysis (ToMOD). Through pre-steady-state kinetics, we demonstrate that ToMOD alters electron transfer from ToMOC to ToMOH. Under steady-state conditions, ToMOD increases the rate of turnover up to one equivalent of ToMOD to ToMOH. At excess ToMOD concentrations, the regulatory protein inhibits steady-state catalysis in a manner that depends upon the concentration of
ToMOC. Protein-binding studies, computational docking, and rapid electron transfer kinetics indicate that this inhibitory function results from competition between ToMOD and ToMOC for binding to ToMOH. These results are discussed in the context of additional proteins in the bacterial multicomponent monooxygenase superfamily.

Chapter 4. Oxygen Activation by the Hydroxylase of Toluene/o-Xylene Monooxygenase in the Presence of its Redox Partners. To hydroxylate arene substrates, toluene/o-xylene monooxygenase (ToMO) utilizes four protein components, a catalytic hydroxylase (ToMOH), a regulatory protein (ToMOD), a Rieske protein (ToMOC), and a reductase (ToMOF). Within ToMOH, this chemistry is achieved through the activation of dioxygen. Previous dioxygen activation studies of ToMO have utilized a simplified protein system comprising ToMOH and ToMOD, but with dithionite and methyl viologen supplying the electrons. Here, we revisit the dioxygen activation experiments but with ToMOC, ToMOF, and NADH. The use of these proteins and NADH dramatically alters dioxygen activation chemistry and subsequent arene hydroxylation in single turnover studies.

Chapter 5. Oxygen Activation in the T201S Variant of Toluene/o-Xylene Monooxygenase. The secondary coordination spheres in diiron proteins influence reactivity at the active site. In the diiron protein toluene/o-xylene monooxygenase, a threonine residue (T201) near the diiron site modulates steady-state turnover and dioxygen activation chemistry in the presence and absence of substrate. Previous oxygen-activation studies, reveal that mutation of this residue to a serine (T201S) yields diiron-O$_2$ adducts different from those observed for of WT ToMOH. These oxygen-activation experiments were conducted using dithionite and methyl viologen as the reducing agents. As in Chapter 4, we re-examine oxygen activation by T201S in the presence of the redox proteins, ToMOC and ToMOF. Stopped-flow UV-visible spectroscopy reveals that the use of these component proteins changes the
number and identity of diiron-O$_2$ adducts formed during the dioxygen activation steps in T201S.

**Appendix A. Heterologous Expression and Purification of Components of Toluene/o-Xylene Monooxygenase from Pseudomonas sp. OX1.** Here we provide the detailed protocols for expression and purification of the component proteins of toluene/o-xylene monooxygenase. Gene sequences and miscellaneous purification and handling notes are also provided.

Thesis Supervisor: Stephen J. Lippard
Title: Arthur Amos Noyes Professor of Chemistry
For my family
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This thesis would not have been possible without the support of several mentors, and colleagues. First and foremost, my thesis advisor, Professor Stephen J. Lippard, has made a tremendous effort to mold me into a better writer, speaker, and scientist. Through stressing both efficiency and clarity, he has taught me to think more deeply about scientific communication. He maintains high expectations of his students, encouraging them to grow from undergraduate observers to experimental chemists. For his effort, guidance, and support, I am immensely grateful.

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meltdowns, data-unrelated meltdowns, emergency coffee runs, wine nights, and endless movie references. After I leave MIT, I will miss being in such close proximity to these amazing women.

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# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>BMM</td>
<td>bacterial multicomponent monooxygenase</td>
</tr>
<tr>
<td>C2,3O</td>
<td>catechol 2,3-dioxygenase</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ET</td>
<td>electron transfer</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>Fd</td>
<td>ferredoxin</td>
</tr>
<tr>
<td>H-Reg</td>
<td>complex between a BMM hydroxylase and regulatory protein</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>$k_{cat}$</td>
<td>catalytic rate constant or turnover</td>
</tr>
<tr>
<td>$k_{cat}/K_M$</td>
<td>catalytic efficiency</td>
</tr>
<tr>
<td>$K_M$</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>KSIE</td>
<td>kinetic solvent isotope effect</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertini</td>
</tr>
<tr>
<td>LMCT</td>
<td>ligand-to-metal charge-transfer</td>
</tr>
<tr>
<td>MMOB</td>
<td>regulatory component of soluble methane monooxygenase</td>
</tr>
<tr>
<td>MMOH</td>
<td>hydroxylase component of soluble methane monooxygenase</td>
</tr>
<tr>
<td>MMOR</td>
<td>reductase component of soluble methane monooxygenase</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>NAD*</td>
<td>nicotinamide adenine dinucleotide, two-electron oxidized form</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide, two-electron reduced form</td>
</tr>
<tr>
<td>OD$_{600}$</td>
<td>optical density at 600 nm</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCET</td>
<td>proton-coupled electron transfer</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PH</td>
<td>phenol hydroxylase</td>
</tr>
<tr>
<td>PT</td>
<td>proton transfer</td>
</tr>
<tr>
<td>RCQ</td>
<td>rapid chemical-quench</td>
</tr>
<tr>
<td>RFQ</td>
<td>rapid freeze-quench</td>
</tr>
<tr>
<td>RNR</td>
<td>ribonucleotide reductase</td>
</tr>
<tr>
<td>sBMO</td>
<td>soluble butane monooxygenase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>sMMO</td>
<td>soluble methane monooxygenase</td>
</tr>
<tr>
<td>T201S</td>
<td>hydroxylase variant of toluene/o-xylene monooxygenase containing the mutation of threonine 201 to serine</td>
</tr>
<tr>
<td>T2MO</td>
<td>toluene 2-monooxygenase</td>
</tr>
<tr>
<td>T3MO</td>
<td>toluene 3-monooxygenase</td>
</tr>
<tr>
<td>T4MO</td>
<td>toluene 4-monooxygenase</td>
</tr>
<tr>
<td>T4moC</td>
<td>Rieske protein of toluene 4-monooxygenase</td>
</tr>
<tr>
<td>T4moD</td>
<td>regulatory protein component of toluene 4-monooxygenase</td>
</tr>
<tr>
<td>T4moF</td>
<td>reductase component of toluene 4-monooxygenase</td>
</tr>
<tr>
<td>T4moH</td>
<td>hydroxylase component of toluene 4-monooxygenase</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>ToMO</td>
<td>toluene/o-xylene monooxygenase</td>
</tr>
</tbody>
</table>
ToMOC  Rieske protein of ToMO
ToMOC$_{ox}$  oxidized, [2Fe-2S]$^{2+}$ form of ToMOC
ToMOC$_{red}$  one-electron reduced, [2Fe-2S]$^{1+}$ form of ToMOC
ToMOD  regulatory protein component of ToMO
ToMOD-Fl  ToMOD labeled with fluorescein
ToMOF  reductase component of ToMO
ToMOF  ToMOF containing the FAD and [2Fe-2S]$^{2+}$ cofactor
ToMOF$_{1e}$  ToMOF$_{ox}$ reduced by one electron
ToMOF$_{2e}$  ToMOF$_{ox}$ reduced by two electrons
ToMOF$_{red}$  ToMOF$_{ox}$ in the presence of one equivalent of NADH
ToMOH  hydroxylase component of ToMO
ToMOH$_{mv}$  mixed valent diiron(II/III) form of ToMOH
ToMOH$_{ox}$  oxidized diiron(III) form of ToMOH
ToMOH$_{peroxo}$  peroxo diiron(III) intermediate of ToMOH
ToMOH$_{red}$  reduced diiron(II) form of ToMOH
TMO  four-component aromatic monooxygenases
Tris  tris(hydroxymethyl)aminomethane
$\delta$  isomer shift in Mössbauer spectroscopy (mm/s)
$\Delta E_Q$  quadropole splitting in Mössbauer spectroscopy (mm/s)
Chapter 1

Component Interactions in Three- and Four-Component Bacterial Multicomponent Monooxygenases
Introduction

Multicomponent enzyme systems are often used in nature to achieve demanding or complex chemical processes. Such enzyme systems include nitrogenase, ribonucleotide reductase (RNR), cytochrome P450, and multicomponent monooxygenases. In all of these systems, an electron transfer (ET) component is separated from the catalytic unit. This arrangement enforces isolation of mechanistic steps, namely ET and substrate activation. Separation of these steps risks wasting energy through deactivation of reactive intermediates. Multicomponent systems, however, have evolved to minimize such losses.

In particular, bacterial multicomponent monooxygenases (BMMs) utilize component interactions to regulate the reduction and oxygen-activation processes required for their reactivity. BMMs catalyze hydroxylation and epoxidation of a wide variety of hydrocarbon substrates. Substrate profiles within this protein family vary from small alkanes to polycyclic aromatics. Three well-studied classes of BMMs include soluble methane monooxygenases (sMMOs), phenol hydroxylases (PHs), and four-component alkene/arene monooxygenases (TMOs). The hydrocarbon substrates and products of these BMMs are shown in Chart 1.1.

Chart 1.1 Select BMMs and their substrates, products, and component proteins.
In all BMMs, oxygen activation and substrate hydroxylation occur at a carboxylate-rich diiron site. A general mechanism for BMM reactivity is shown in Scheme 1.1. The resting state of the hydroxylase (H$_{ox}$) contains a diiron(III) motif. H$_{ox}$ is reduced by two electrons to form the diiron(II) state (H$_{red}$). Following reduction, activation of dioxygen and substrate hydroxylation occur.

**Scheme 1.1.** Diiron chemistry during turnover by BMM hydroxylases.

![Scheme 1.1](image)

For decades, inorganic chemists have attempted to synthesize small-molecule models of the diiron active site within BMMs. This goal, however, has met with only limited success. In part, this failure may result from an inability to model the changes enforced by the component proteins, which are known to alter the diiron site and its ability to attenuate substrate access. Three or four protein components are required to achieve catalytic turnover in all BMMs. These component proteins include a hydroxylase, a regulatory protein, and either one or two ET proteins.

The hydroxylase of all BMMs is dimeric and contains an (αβγ)$_2$ or an (αβ)$_2$ structure. Each alpha domain houses a diiron active site within a four-helix bundle. The regulatory component of BMMs is a small 10-16-kDa protein required for efficient
turnover and coupling. Coupling refers to the ratio of product formation to NADH consumption, where 100% coupling indicates that for every molecule of NADH consumed, a molecule of oxidized hydrocarbon product is formed. Because of its role in coupling, the regulatory protein is sometimes referred to as the coupling protein. Three- and four-component BMMs differ in their ET protein components. In three-component BMMs, a single ET protein is required to transfer electrons from NADH to the hydroxylase. This ET protein is a reductase containing a flavin adenine dinucleotide (FAD) cofactor, a [2Fe-2S] ferredoxin (Fd) cluster, and an NADH binding site. In contrast, four-component BMMs require two ET proteins, an FAD-Fd containing reductase and a Rieske protein, which contains a Rieske-type [2Fe-2S] ferredoxin cluster.

Detailed information about the precise sequence of events during catalytic turnover is incomplete, but recent results have shed new light on the mechanism of BMMs. Here we review the component interactions in BMMs to illustrate how these proteins control the structure and reactivity of the carboxylate-rich diiron center at the active site. This discussion is divided into two sections. In the first, structural features observed by X-ray crystallography and solution-phase techniques are discussed. In the second, the kinetics of ET, oxygen activation, and steady-state turnover are examined in the light of these structural data. The mechanism of oxygen activation in a four-component BMM is discussed in Chapters 4 and 5, and therefore any mention of this topic will be brief. As canonical representatives, the three-component systems sMMO and PH, and the four-component systems toluene/ø-xylene monooxygenase (ToMO) and toluene 4-monooxygenase (T4MO) are considered. Additional BMMs, such as soluble butane monooxygenase (sBMO), are mentioned in cases where relevant data is available.

**Structural Analysis of BMMs**

X-ray crystal structures of five BMM hydroxylases have been reported. In addition, structures of a hydroxylase protein bound to its cognate regulatory protein or ET protein are known for three BMMs. Comparison of these structures provides molecular insight into the component interactions that regulate turnover.
Structures of BMM Hydroxylases. Crystal structures of the BMM hydroxylases are available for sMMO from *Methylococcus capsulatus* (Mc-sMMO)\(^{23}\) and *Methylosinus trichosporium* (Mt-sMMO)\(^{24}\) ToMO from *Pseudomonas* sp. OX1\(^{25}\), PH from *Pseudomonas* sp. OX1\(^{20}\), and T4MO from *Pseudomonas mendocina*\(^{27}\). Each of these hydroxylase proteins contains a dimeric (αβγ)\(_2\) structure. The alpha and beta subunits make up the dimer interface, which contains a depression, termed the canyon region. The αβγ units are related by a C\(_2\) symmetry axis\(^{23}\). The diiron active site is located within the alpha subunit below the canyon region. These active sites are remarkably similar despite the disparate substrate profiles of each protein. The resting state of each hydroxylase contains a diiron(III) site (Figure 1.1).

![Diiron active sites of structurally characterized BMM hydroxylases](image)

Figure 1.1. Diiron active sites of structurally characterized BMM hydroxylases (A) Mc-sMMO, PDB: 1MTY; (B) Mt-sMMO, PDB:1MHY; (C) ToMO, PDB: 2INC; (D) T4MO, PDB: 3DHG; and (E) PH, PDB: 2INP. The iron atoms are shown as orange spheres, where Fe1 is on the left and Fe2 is on the right. The water molecules and hydroxide ions are shown as red spheres. The iron-coordinating ligands are depicted as sticks with carbon, nitrogen, and oxygen atoms colored in grey, blue, and red, respectively.

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In all structurally characterized BMMs, the diiron center is coordinated by two histidine residues and four glutamate/aspartate residues.\textsuperscript{23-27} With the exception of PH, each iron center has a distorted octahedral environment with the remaining coordination sites filled by two bridging hydroxide ions and a single water molecule. Three structures of the PH hydroxylase are known.\textsuperscript{20,26} In all three, the diiron active site deviates from those in other structurally characterized BMMs, in that a carboxylate ligand (E233) coordinated to Fe2 is bidentate (Figure 1.1E). Based on the similarity of this coordination mode to those seen for structures of sMMO,\textsuperscript{9} the authors propose that these PH structures may contain a mixed valent, Fe(II)Fe(III) center, which might account for the differences in coordination.\textsuperscript{20,26} For sMMO, changes at the diiron active site upon reduction, substrate binding, and inhibitor binding have been thoroughly investigated and reviewed.\textsuperscript{9}

In all BMMs hydroxylases, the diiron sites are located approximately 12 Å below the protein surface.\textsuperscript{23-27} How then do the four substrates—oxygen, hydrocarbons, protons, and electrons—access the concealed diiron sites? Analysis of void spaces within these hydroxylases reveals several internal pockets and passages within each protein, depicted in Figure 1.2. Three disconnected, hydrophobic cavities and one 12 Å-deep pore are common to all BMM hydroxylases of known structure (Figure 1.2).\textsuperscript{23-27} The disconnected cavities are labeled by convention cavities 1, 2 and 3, where cavity 1 is closest to the diiron active site. Xenon pressurization crystallographic studies suggested that cavities 1, 2, and 3 might house oxygen or small hydrocarbons.\textsuperscript{28} Because cavities 1 and 2 are disconnected, conformational changes would be necessary to allow full dioxygen access to the diiron active site.

In all BMMs, the 12 Å-deep pore region is solvent exposed and more polar than the cavities. It may, therefore, serve as a product egress pathway for small, polar products, such as methanol in sMMO. The pore region of PH is much wider than that of sMMO, ToMO, and T4MO.\textsuperscript{20,26} This enlarged pore can accommodate aromatic substrates,\textsuperscript{20,26} suggesting that the pore region of PH may serve as the aromatic substrate and product pathway to and from the diiron site and the protein surface. In contrast, the pore regions of ToMO and T4MO are too small to accommodate aromatic substrates or products.
Instead, a channel approximately 6-10 Å wide and 35 Å deep is present in both ToMO\textsuperscript{25} and T4MO\textsuperscript{27}. X-ray crystallographic studies show that aromatic substrate collect within this void space, indicating this channel as their access route to the diiron center.\textsuperscript{17,25} A dynamic picture of the cavities, pores, and channels was discovered following structural characterization of complexes formed between the hydroxylase proteins and their cognate regulatory proteins.

Figure 1.2. Void spaces in BMM hydroxylases. The cavities, channels, pores, and surrounding residues are shown for the hydroxylase proteins from sMMO (A), PH (B), ToMO (C), and T4MO (D). The void spaces were rendered using the cavity-surface mode in PyMol. Cavities 1, 2, and 3 are shown in pink, green, and blue, respectively. The pore region is shown in bright yellow. The channels, present only in ToMO and T4MO, are shown in purple.
Interactions Between BMM Hydroxylases and their Regulatory Proteins. Before any structures were available for a hydroxylase-regulatory protein (H-Reg) complex, the canyon region of the alpha subunit was predicted to be the locus of binding for the regulatory protein. This proposal was based on chemical interaction, cross-linking studies, proteolytic digestion with mass spectrometric analysis, and the sensitivity of the diiron environment to the presence of the regulatory protein. These proposals were validated in 2006 when the first X-ray crystal structure of a H-Reg complex was published for PH. Subsequently, a report of the H-Reg complex of T4MO was reported, and most recently, a structure of the H-Reg complex from sMMO was published. The H-Reg complexes for these protein systems are shown in Figure 1.3.

Figure 1.3. X-ray crystal structures of the H-Reg complexes of BMMs. The dimeric hydroxylase and the bound regulatory proteins are shown for PH (left), T4MO (middle), sMMO (right). The alpha, beta, and gamma subunits are colored as light grey, light blue, and gold, respectively. The regulatory proteins are shown as red spheres, with the N-terminal tails shown as orange spheres.

In the H-Reg complex of PH, the regulatory protein occupancy is only 40 – 50%, resulting in approximately one regulatory protein per hydroxylase dimer instead
of the maximum possible number, two (Figure 1.3A, top view). Regardless, this structure provided the first explicit evidence of a regulatory protein binding to the canyon region of its hydroxylase. This binding mode proved common in all structures of the H-Reg complex. Unlike the PH structure, the ratio of the regulatory protein to hydroxylase dimer is 2:1 in complexes from T4MO and sMMO.

At the binding interface, the regulatory proteins make contact with the alpha and beta subunits of the hydroxylase. The regulatory protein of sMMO contains an extended N-terminal tail, which is not present in other BMMs (Figure 1.4). In solution, this N-terminal tail is disordered in the absence of the hydroxylase. The X-ray crystal structure of the H-Reg complex reveals that this tail assembles onto the surface of the hydroxylase α-subunit forming a remarkable ring structure (Figure 1.3C). Recent double electron-electron resonance (DEER) experiments revealed that this ring structure persists in solutions of the hydroxylase and regulatory proteins.

<table>
<thead>
<tr>
<th>Mc-sMMO</th>
<th>HSYNSNAYDAGIMGLGKDFADQFFADERQVHESDVTVLVLKKSEINT</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mt-sMMO</td>
<td>MSSHNAYDAGIMGLGKDFADQFFADERQVHESDVTVLVLKKSEIDA</td>
<td>50</td>
</tr>
<tr>
<td>PH</td>
<td>-------------------------------------------------</td>
<td>17</td>
</tr>
<tr>
<td>T4MO</td>
<td>IIEIDIVLKKG-KAKKPSIVEDKAGFWIWKAADGAIEDAAEAGELLKPF</td>
<td>99</td>
</tr>
<tr>
<td>ToMO</td>
<td>MTNQ---DNPFAE---MQQPMR1QQAEKRLVINRETMEKL4H4DW</td>
<td>61</td>
</tr>
<tr>
<td>Mc-sMMO</td>
<td>FIEELLLTDYKKNWTVNVEDRAGYWWLIAANGKIEVDCEISEELLGQF</td>
<td>100</td>
</tr>
<tr>
<td>Mt-sMMO</td>
<td>LIEIDIVLKKG-KAKKPSIVEDKAGFWIWKAADGAIEDAAEAGELLKPF</td>
<td>99</td>
</tr>
<tr>
<td>PH</td>
<td>VAEITAEI---DNP9EITVEDRATYRVAEAGELLITRTLSEQLGRPF</td>
<td>72</td>
</tr>
<tr>
<td>T4MO</td>
<td>VEGAAEI---DNP9EITVEDRATYRVAEAGELLITRTLSEQLGRPF</td>
<td>72</td>
</tr>
<tr>
<td>ToMO</td>
<td>VEGAAEI---DNP9EITVEDRATYRVAEAGELLITRTLSEQLGRPF</td>
<td>72</td>
</tr>
</tbody>
</table>

**Figure 1.4.** Sequence alignment of the regulatory proteins for all structurally characterized BMM hydroxylases. The amino acids associated with the N-terminal tail of the sMMO regulatory proteins are colored in orange. The N-terminal tail of the T4MO regulatory protein is colored in violet. Conserved amino acids are colored in blue. The accession numbers for each sequence are as follows: *Mc*-sMMO, AAF04158; *Mt*-sMMO, CAA39070; PH, WP_043313075; T4MO, AAA26002; and ToMO, AAT40434.
Based on these observations, the N-terminal ring was proposed to anchor the regulatory protein to the hydroxylase surface allowing for dissociation of the regulatory protein body while maintaining contact with the larger hydroxylase component. A very short N-terminal tail is present in the regulatory proteins of ToMO and T4MO but not PH (Figure 1.4). Because the regulatory proteins of all other BMM subfamilies are either very short or non-existent, the anchoring mechanism could only operate in the sMMO subfamily, which also includes sBMO. Notably, only these enzyme systems can hydroxylate saturated hydrocarbons.

In each H-Reg complex, the body of the regulatory protein lies above a four-helix bundle within the alpha subunit. Within the four-helix bundle, side-chain reorientation and partial unwinding of the helices occur in the H-Reg complex compared to the hydroxylase alone. This rearrangement in the H-Reg complex results in formation of an extensive hydrogen-bonding network within the hydroxylase. Changes at the four-helix bundle are pronounced and propagate throughout the alpha subunit and some of the beta subunit. Most notably, the internal cavities observed within the hydroxylase undergo rearrangement with respect to both size and connectivity upon binding of the regulatory protein (Figure 1.5). These changes connect the cavities 1 and 2 of the hydroxylase, providing oxygen access to the diiron centers.

The pore regions of the T4MO and sMMO hydroxylases close upon binding of the regulatory protein. This observation is not universal, however. When the regulatory protein binds to the hydroxylase of PH, the pore region remains solvent exposed (Figure 1.5A). The reason for these divergent behaviors is unclear, but may result from the absence of a protruding N-terminal tail in the regulatory protein of PH. A large conformational change upon formation of the H-reg complex of T4MO constricts the aromatic substrate channel trapping substrate within the hydroxylase and preventing further ingress of substrate into the channel. Most impressively, in all three structures, conformational changes link the disconnected cavities of hydroxylases to form pathways for oxygen and/or small hydrocarbons. These results strongly suggest that binding of the regulatory protein controls oxygen access to the diiron center.
Figure 1.5. Access routes to the diiron center within H-Reg complexes of T4MO, sMMO, and PH. The dimeric H-Reg complex of T4MO is shown in panel A. Inset B provides a magnified view of the cavities, channel, and pore, which are shown in mesh. Using CAVER, the possible small-molecule pathways to the diiron center were calculated for H-reg complexes of T4MO (B), sMMO (C), and PH (D). These calculated pathways are shown in solid surface mode. In each panel, the cavities, channels and pores are overlaid in mesh for comparison. The regions of the void spaces are shown in surface mode colored according to their cavity numbers: cavity 1, blue; cavity 2, green; cavity 3, pink; pore, yellow. The iron atoms are shown as orange spheres.

Changes at the diiron centers upon binding of the regulatory proteins were anticipated based on earlier solution-phase methods, including EPR, X-ray absorption (XAS), and magnetic circular dichroism (MCD) spectroscopies. In the H-Reg structures of both PH and sMMO, the diiron site oxidation state is ambiguous, owing to
limited resolution, and could be diiron(III), Fe(II)Fe(III), or diiron(II).\textsuperscript{18,26} Thus, direct comparison of the diiron coordination environments in these systems is compromised because of possible differences in oxidation state assignments. In contrast, higher resolution X-ray structures of the H-Reg complex are known for both the diiron(III) and diiron(II) forms of T4MO, allowing for direct comparison of the diiron active sites (Figure 1.6).\textsuperscript{27}

**Figure 1.6.** Comparison of the diiron(III) and diiron(II) coordination environments of the T4MO hydroxylase in the absence and presence of the regulatory protein. The diiron structures are shown for the diiron(III) hydroxylase (A), the diiron(III) H-Reg complex (B), and the diiron(II) H-Reg complex. The iron atoms are shown as orange spheres with labels for Fe1 and Fe2. The water and hydroxide ligands are shown as red spheres.

Upon binding of the T4MO regulatory protein to its diiron(III) hydroxylase, significant conformational changes occur within the first and second coordination spheres of the diiron center (Figure 1.6 A and B). The Fe1-coordinating residue E231 undergoes a rotameric shift to form hydrogen bonds with residue T201 and a water molecule that enters the active site (HOH5). Residues T201, N202, and Q228 of the pore region also undergo significant changes. In the hydroxylase structure, residue Q228 is solvent exposed. Binding of the regulatory protein pushes residues Q228 into the interior of the
protein, closing the pore and forming hydrogen bonds with residue T201 and HOH5. Within this region, binding of the regulatory protein creates a hydrogen-bonding network organizing the active site as predicted from early XAS studies.\textsuperscript{35}

Comparison of the T4MO H-Reg complexes containing the diiron(III) and diiron(II) hydroxylases indicates different coordination geometries exist in the two forms (Figure 1.6 B and C). Converting the diiron(III) form to the diiron(II) form upon addition of two electrons and two protons results in the loss of the bridging hydroxide ions as water molecules. The loss of water accompanies movement of residue E231 to bridge Fe1 and Fe2 (Figure 1.6C). These structural rearrangements provide an unsaturated coordination environment, priming the diiron site for oxygen activation. Redox-dependent conformational changes have also been reported for BMM hydroxylases in the absence of the regulatory protein,\textsuperscript{9} suggesting that these changes may be similar in all BMMs.

**Interactions Between BMM Hydroxylases and their ET Proteins.** In three- and four-component BMMs, the ET proteins differ. Three-component BMMs, such as sMMO,\textsuperscript{38} sBMO,\textsuperscript{39} and PH,\textsuperscript{40} utilize an approximately 40-kDa reductase to directly transfer electrons from NADH to the diiron centers of their respective hydroxylases. This reductase is also present in four-component BMMs, but in these systems, it cannot directly reduce the diiron active site. These reductase components contain a single polypeptide chain that folds into two distinct domains, and FAD domain and a ferredoxin (Fd) domain.\textsuperscript{41} The FAD domain houses both FAD and an NADH binding site, and the Fd domain houses a single [2Fe-2S] cluster (Figure 1.7). As isolated, the oxidized reductase (Reductase\textsubscript{ox}) contains FAD and [2Fe-2S]\textsuperscript{2+}.\textsuperscript{41,42} The distribution of electrons in these redox-active cofactors upon reduction is discussed below in the section titled *Component Effects on Reactivity of BMMs.*
Figure 1.7. NMR structures of the two domains of the sMMO reductase. The FAD domain (A, PDB: 1TVC) contains an FAD cofactor and an NADH binding site. The FAD cofactor is shown as sticks, and the NADH binding domain is colored in dark green. The remainder of the domain is shown in light green. The Fd domain (B, PDB: 1JQ4) contains a single [2Fe-2S] cluster.

In three-component BMMs, the reductases shuttle electrons directly to the diiron center of the hydroxylase (Scheme 1.2). A single interaction between the two-electron reduced reductase (Reductase$_{2e}$) and the diiron(III) site can produce the diiron(II) state, which is responsible for dioxygen activation. Unlike three-component BMMs, four-component BMMs require a Rieske protein to shuttle electrons from the reductase to the diiron active site of the hydroxylase. The Rieske proteins are only capable of one-electron chemistry cycling between the oxidized Rieske protein (Rieske$_{ox}$) and the one-electron reduced Rieske protein (Rieske$_{1e}$). Thus, in four-component BMMs, the Reductase$_{2e}$ reduces two equivalents of Rieske$_{ox}$ to form Rieske$_{1e}$. After reduction of the Rieske protein, two interactions between the diiron(III) site and Rieske$_{1e}$ are required to form the diiron(II), oxygen-activating species. The thermodynamics and kinetics of these ET steps are discussed below in the section titled Component Effects on Reactivity of BMMs.
Scheme 1.2. Diiron reduction pathway for three- and four-component BMMs.

The term diiron reductant will be used hereafter when referring either to the reductase of three-component BMMs or to the Rieske protein of four-component BMMs. NMR structures (Figure 1.7) are available for the individual FAD$^{43}$ and Fd$^{44}$ domains of the sMMO reductase, but the structure of the full-length protein is unknown. The only available structural information of a full-length diiron reductant is of the Rieske protein of T4MO.$^{47,48}$

The diiron reductants were predicted to bind to the canyon region of their cognate hydroxylases both by chemical intuition and by protein docking models.$^{8,19,21}$ Such a binding mode provides the shortest distance for ET (approximately 12 Å) between the diiron reductant and the diiron active site. This binding mode would require overlapping binding sites on the hydroxylase for the diiron reductant and the regulatory protein. Recently, this model was validated by an X-ray crystal structure of the T4MO hydroxylase bound to its cognate Rieske protein (Figure 1.8).$^{16}$
**Figure 1.8.** An X-ray crystal structure at 2.4-Å resolution of the T4MO hydroxylase bound to its Rieske protein (PDB: 4P1C). The structure of the hydroxylase-Rieske protein complex is shown from the front (A) and the top (B). The alpha, beta, and gamma subunits of the hydroxylase are shown in light grey, light blue, and gold, respectively. The Rieske protein is shown in fuchsia. The iron atoms are shown as orange spheres, and the sulfur atoms of the Rieske cluster are shown in yellow spheres. The void spaces in the hydroxylase-Rieske protein complex are shown in panel C with the same coloring as in Figure 1.2. Magnification of the interface (D) shows the space between the Rieske cluster and the diiron active site of the hydroxylase. Water molecules that participate in the hydrogen-bonding network are shown as red spheres.
In the T4MO structure of the hydroxylase and its diiron reductant, the Rieske protein binds to the canyon region of the hydroxylase. The distance between the $[2\text{Fe}-2\text{S}]$ Rieske cluster and the diiron center is approximately 12 Å, the canonical distance for rapid biological ET. The histidine residues of the Rieske cluster point into the pore region, which is in the closed conformation similar to that seen in the H-Reg complex. Closing of the pore through movement of residue Q228 of the hydroxylase forms a hydrogen-bonding network between the Rieske protein and the diiron active site (Figure 1.8D). This hydrogen-bonding network includes the histidine residues of the Rieske cluster, which may be able to transfer protons, coupling proton transfer to ET. In contrast to the H-Reg structure of T4MO, the aromatic substrate channel remains open upon binding of the Rieske protein to the hydroxylase. This arrangement allows for substrate to enter the active site or product to leave the active site when the Rieske protein is bound to the hydroxylase.

The T4MO hydroxylase-Rieske protein complex provides two important insights into BMM activity. First, the structure provides unambiguous support for competitive binding between a diiron reductant and a regulatory protein. Second, the structure demonstrates that the Rieske and regulatory proteins elicit different conformational changes and chemical events within the hydroxylase despite the shared binding interface.

Docking models indicate that the reductase of sMMO also binds to the canyon region of the hydroxylase, suggesting that competitive binding between the diiron reductant and regulatory protein also occurs with sMMO. Unfortunately, these docking models utilize rigid-body docking techniques and do not account for the surface-exposed ferredoxin cluster of the reductase. Thus, the conformational changes within the hydroxylase remain unknown. We speculate that binding of the sMMO hydroxylase to its reductase will induce rearrangement of the pore and organization of the $[2\text{Fe}-2\text{S}]$ cluster within 12 Å of the diiron active site, similar to that seen in this T4MO structure. Such a conformational change would provide an efficient route for ET, and possibly proton transfer.
Component Effects on Reactivity of BMMs

The effects of the component proteins on catalysis have been studied for many BMMs both during steady-state turnover as well as during the individual steps of catalysis. Included are reduction of the diiron(III) active site, oxygen activation, and hydrocarbon substrate hydroxylation. Here we revisit these results in light of the structural data discussed in the previous section.

Component Effects on Reduction of the Diiron(III) Active Site. Because the ET proteins are separated from the BMM hydroxylases, interprotein interactions are essential for ET. To elucidate the diiron(III) reduction mechanism, the thermodynamics and kinetics governing reduction of the diiron(III) active sites of BMMs have been rigorously examined as a function of the protein components. Here we discuss first the thermodynamics of diiron(III) reduction and second the binding, kinetics, and mechanism of interprotein ET.

For reduction of the active site, two one-electron reduction steps must occur, \( \text{diiron(III)} \rightarrow \text{Fe(II)Fe(III)} \rightarrow \text{diiron(II)} \). For \( Mt\)-sMMO, the relevant midpoint potentials are +76 mV and +21 mV vs. NHE at pH 7 and 4 °C as measured by potentiometric titration with EPR and Mössbauer spectroscopic quantitation.\(^{32}\) For \( Mc\)-sMMO, the midpoint potentials of the hydroxylase alone are more negative, +48 mV and -135 mV vs. NHE at pH 7 and 25 °C as measured by potentiometric titration with quantitation by EPR spectroscopy.\(^ {31}\) With respect to the effects of the component proteins on these midpoint potentials, opposing results are reported for \( Mt\)-sMMO\(^ {32,53}\) and \( Mc\)-sMMO.\(^ {31}\) In the case of \( Mt\)-sMMO, addition of the regulatory protein to the hydroxylase causes the potentials shift by -132 mV vs NHE, but addition of both the reductase and the regulatory protein to the hydroxylase yields midpoint potentials of +109 mV and +75 mV vs. NHE. Inclusion of substrate only minimally perturbs the redox potential of \( Mt\)-sMMO. In the case of \( Mc\)-sMMO, addition of both the reductase and regulatory protein to the hydroxylase yields midpoint potentials of less than -200 mV vs NHE. Inclusion of substrate moves the potential to values greater than 150 mV vs NHE. Because of these conflicting reports, it
is difficult to unambiguously state the effect of the component proteins on the diiron midpoint potentials.

Equilibrium binding studies involving the regulatory protein and hydroxylase also contrast for the two systems, Mt-sMMO and Mc-sMMO. The regulatory protein of Mt-sMMO binds 1000-fold more tightly to the diiron(III) hydroxylase than the diiron(II) hydroxylase. \textsuperscript{29,54} In contrast, the regulatory proteins of Mc-sMMO bind three-fold more tightly to the diiron(II) hydroxylase than the diiron(III) hydroxylase. \textsuperscript{22} For all BMMs, a general binding model in which reduction of the hydroxylase diiron site of initiates H-Reg complex formation is attractive because opening of the oxygen pathway to the diiron site would be prompted exactly when the diiron site is primed for oxygen activation. The multiple differences between Mt-sMMO and Mc-sMMO have yet to be reconciled or sufficiently explained.

In both three- and four-component BMMs, the reductase houses an NADH binding unit, and FAD cofactor, and a [2Fe-2S] cluster (Figure 1.7 and Scheme 1.3). Upon addition of NADH, two electrons are shuttled from NADH and to the FAD cofactor to form Reductase\textsubscript{2e}. These electrons rapidly redistribute between the FAD and [2Fe-2S] cluster. \textsuperscript{55} The FAD cofactor is capable of transferring a single electron to the [2Fe-2S]\textsuperscript{2+} cluster, resulting in the semiquinone (FADH\textsuperscript{-}) and [2Fe-2S]\textsuperscript{+} electron distribution. Reduction of the isolated protein with 1.5 equivalents of NADH produces the three-electron reduced form, which contains FADH\textsubscript{2} and the [2Fe-2S]\textsuperscript{+} cluster. \textsuperscript{41,42} This redox state likely arises from electron exchange between reductase proteins at high concentrations. In cells, the molar ratio of the reductase to the hydroxylase is low, \textasciitilde10\%. \textsuperscript{11} Thus, the biological relevance of the three-electron reduced state is not clear.
Scheme 1.3. The electron flow and cofactors of the BMM reductases.

With respect to interprotein ET mechanism, the ET steps differ significantly for three- and four-component BMMs (Scheme 1.2), largely because of the number of electrons that can be delivered by the diiron reductant. In sMMO, transfer of two electrons in a single interaction of the Reductase_{2e} with the diiron(III) active site is both thermodynamically and kinetically favored. The observed rate constants for ET from the reduced reductase to the diiron(III) center of sMMO (96 s^{-1} and 15 s^{-1}) are faster than both steady-state methane oxidation (0.3 s^{-1}) and association of the regulatory protein with the hydroxylase (~0.9 s^{-1} at 10 μM hydroxylase) at pH 7, and 4 °C. These results strongly suggest that a single interaction between the reductase and diiron(III) active site produces the diiron(II) state.

In four component BMMs, ET from the reductase to the Rieske protein is also rapid, at least two orders of magnitude faster than steady-state turnover (Chapter 3). In contrast, the rate of ET from the Rieske protein to the hydroxylase is much slower, indicating that this step is rate limiting within the ET chain. The Rieske proteins are only capable of one-electron redox chemistry. Reduction of each diiron(III) site could be achieved in one of two ways: (i) sequential interactions of Rieske_{1e} with a diiron site or (ii) formation ternary complex involving Reductase_{2e}-Rieske-H_{ox}. No evidence for the formation of a ternary complex has been reported. Moreover, the reductase of T4MO can
be replaced by a simple spinach ferredoxin reductase, suggesting that a specific complex is not relevant.

Despite these differences, in both three- and four-component BMMs, the regulatory protein and diiron reductant protein compete for binding to the hydroxylase, as can be observed through kinetic analysis of ET. Pre-incubation of the regulatory protein and the hydroxylase yields smaller rate constants for ET from the diiron reductant to the oxidized hydroxylase. Although the Rieske proteins are proposed to evolve by horizontal gene transfer, this competitive mechanism is conserved in both three- and four-component BMMs, suggesting that such a competition is critical for activity. One reason may be to provide a physical barrier against reduction of oxygenated intermediates, such as the high-valent intermediate Q of sMMO. Alternatively, the competitive mechanism, where the diiron reductant is displaced from the canyon region by the regulatory protein, may be indispensible for efficient ET or catalysis. Regardless of its origins, the competitive-binding mechanism represents an important facet of ET chemistry in both three- and four-component BMMs.

**Component Effects on Oxygen Activation.** Oxygen activation by sMMO, ToMO, and mutant T201S of the ToMO hydroxylase has been thoroughly examined by stopped-flow UV-visible spectroscopy and Mössbauer spectroscopy. In the case of sMMO, oxygenated intermediates have also been characterized by resonance Raman spectroscopy and X-ray absorption spectroscopy. In all of these cases, the regulatory protein is critical for accumulation of oxygenated intermediates. Upon increasing the concentration of the regulatory protein from substoichiometric to stoichiometric quantities, oxygenated-diiron intermediates can be observed by UV-visible spectroscopy. These intermediates accumulate much more rapidly in the presence of equimolar concentrations of the diiron active site and the regulatory protein. X-ray structures of the H-Reg complexes strongly suggest that this behavior may result from connection of the cavities upon formation of the H-Reg complex. In mutant T201S of the ToMO hydroxylase, the rate-limiting step during single turnover is oxygen binding to the
diiron site. Using the T201S variant, a systematic mutagenesis study revealed that attenuating the size of the cavities significantly altered the rate of oxygen activation. These results clearly identified the cavities as the pathway for oxygen access to the diiron active site, providing a direct link between the structure and function of the H-Reg complex.

Further supporting its role as an oxygen regulator, mutations of key residues within the regulatory protein alter oxygen-activation kinetics. Mutation H33A of the Mt-sMMO regulatory protein stabilizes the diiron-O₂ intermediate P*, preventing formation of intermediate P (or H₉₀). Because of this altered reactivity, intermediate P* accumulates to nearly 90% during single turnover. H33 of the regulatory protein lies within the N-terminal tail close to the body of the regulatory protein. The H-reg structure of sMMO contains four hydroxylase-regulatory protein interfaces, and predicts that the side chain of H33 is slightly flexible. At three of four crystallographically independent interfaces, H33 forms a hydrogen bond with E28 of the N-terminal tail within the regulatory protein (Figure 1.9a). At the fourth interface, H33 forms a hydrogen bond with Y310 of the hydroxylase (Figure 1.9b). Thus, mutation of the tail may partially alter the N-terminal ring structure found in the H-Reg complex for sMMO, preventing changes enforced by the ring structure or it may alter the conformation of residue Y310 leading to changes throughout the hydroxylase.
Figure 1.9. Residue H33 of the regulatory protein in the H-Reg complex of sMMO. Two different conformations of H33 are observed in the H-Reg complex (PDB: 4GAM). The most common conformation forms internally hydrogen bound to E28 of the N-terminal tail (A). The less common conformation forms a hydrogen bond between H33 and Y310 of the hydroxylase (B). The hydroxylase and regulatory proteins are shown in light grey and yellow, respectively. The oxygen pathway is shown in blue, green, and pink mesh, denoting each cavity as described in Figure 1.2. The iron atoms are shown as orange spheres.

The diiron reductants also affect oxygen activation in sMMO and ToMO. Addition of the reductase of Mt-sMMO in oxygen-activation studies decreases the rate of oxygen activation by 20-fold. The formation of the methane-oxidizing intermediate (Q), however, was unaffected. Based on these data, the reductase was proposed to induce a long-lived conformational change within the hydroxylase, giving rise to a slow-reacting form of the diiron center. In light of the overlapping binding sites for the diiron reductant and the regulatory protein, a model can be envisioned in which the reductase induces a long-lived conformation at the diiron active site, which is slowly transformed into an oxygen-activity species following replacement of the reductase by the regulatory protein. The effects of the diiron reductant during oxygen activation by ToMO are discussed in Chapter 4.
**Component Effects on Hydroxylation: Single Turnover.** Quantitation of hydroxylation during both single turnover reveals that the component proteins play important roles in the efficiency of substrate hydroxylation. Under single turnover conditions, both the regulatory protein and the ET proteins alter the amount of product produced by the diiron active site (Table 1.1).

<table>
<thead>
<tr>
<th>Protein components added to the hydroxylase</th>
<th>% product formed per diiron active site</th>
<th>( \text{sMMO}^{a} )</th>
<th>( \text{PH}^{b} )</th>
<th>( \text{ToMO}^{b} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>40</td>
<td>( n d )</td>
<td>6.9(2)</td>
<td></td>
</tr>
<tr>
<td>+ Regulatory protein</td>
<td>80</td>
<td>( n d )</td>
<td>10(1)</td>
<td></td>
</tr>
<tr>
<td>+ Diiron Reductant</td>
<td>43</td>
<td>8(3)</td>
<td>11(2)</td>
<td></td>
</tr>
<tr>
<td>+ Regulatory protein and Diiron Reductant</td>
<td>88-94</td>
<td>54(1)</td>
<td>46(3)</td>
<td></td>
</tr>
</tbody>
</table>

All reactions reported here used dithionite as the reducing agent. \( a \) Data from ref \(^{53} \) using propylene as a substrate, error in yields reported as approximately 10%. \( b \) Data from ref \(^{66} \) using phenol as a substrate. \( nd \) = none detected.

In sMMO and ToMO, addition of the regulatory protein to the hydroxylase increases the yield of product by two-fold and 1.5-fold, respectively. Surprisingly, addition of the regulatory protein to the hydroxylase of PH did not increase the amount of product formed. The increase in hydroxylation upon addition of the regulatory protein can easily be rationalized through revisiting the X-ray crystallographic data detailed in the structure section above. Binding of the regulatory protein to the hydroxylase causes opening of the oxygen-access route to the diiron center.\(^{18,26,27,65} \) An increase in oxygen access would yield more diiron sites capable of hydroxylating product.

Addition of the diiron reductant to the hydroxylase of sMMO does not increase the yield more than the estimated measurement error. The effects of the diiron reductant
on product yields for ToMO and PH are larger, increasing by 1.6-fold for ToMO and from 0% to 8% for PH.

When both the diiron reductant and regulatory proteins are added to the hydroxylase, the product yield for sMMO increases by approximately 10% over that of the hydroxylase and regulatory protein alone. This result suggests that the diiron reductant modestly affects the activity of sMMO. These results are even more pronounced in PH and ToMO, which undergo more than a six-fold increase in hydroxylation upon addition of the diiron reductant and regulatory protein. Thus, the diiron reductants of PH and ToMO appear to play a significant role in substrate hydroxylation. The physical or chemical changes induced in the presence of the diiron reductants remains unclear. Further structural characterization of the hydroxylase-diiron reductant complex will provide more insight.

Finally, in four-component BMMs, such as ToMO, the reductase appears to play a minimal role in promoting hydroxylation during single turnover. We propose that the lack of involvement of the reductase is because it does not interact directly with the hydroxylase.

**Regulatory Protein Effects on Steady-State Turnover by BMMs.** During steady-state turnover, the effects of the component proteins are much more complex than single turnover. Many facets of steady-state turnover depend on the regulatory protein, including the coupling efficiency, the regiospecificity of hydroxylation, and the rate of catalysis.

A low coupling efficiency indicates that NADH is unproductively consumed to reducing O$_2$. A low coupling efficiency can result from reduction of O$_2$ either by the ET proteins or by the diiron active site of the hydroxylase. Two pathways of uncoupling can be envisioned, the two-electron reduction of O$_2$ to H$_2$O$_2$ or the four-electron reduction of O$_2$ to water. When only the ET proteins are present, the production of H$_2$O$_2$ occurs at rates similar to NADH oxidation, suggesting that the ET proteins produce H$_2$O$_2$ exclusively. Evidence for both two- and four-electron oxidation of O$_2$
by the hydroxylase is reported.\textsuperscript{11,66-68} In the absence of the hydroxylase and regulatory protein, the reductase very slowly reduces $O_2$. Some of the earliest work on BMMs reveals the regulatory protein was identified as a promoter of coupling efficiency.\textsuperscript{67} In the absence of the regulatory protein, NADH is consumed with low rates of product formation.\textsuperscript{11,66-68}

A systematic study of T4MO demonstrates that coupling efficiency maximizes at 0.5 equivalents of the regulatory protein per diiron active site.\textsuperscript{68} Interestingly, EPR studies with sMMO reveal that the regulatory protein induces changes in the EPR signal of the reduced hydroxylase; these changes maximize at approximately 0.3 equivalents of the regulatory protein per diiron active site.\textsuperscript{69} These results hint that a conformational change at the active site may be directly responsible for the increased coupling efficiency. X-ray crystallographic studies show that the regulatory protein rearranges a hydrogen-bonding network at the active site by closing the pore region.\textsuperscript{18,70} As discussed in Chapter 2, mutation of the pore residues of the hydroxylase of ToMO reduces the coupling efficiency even in the presence of excess regulatory protein. Thus, the increase in coupling efficiency upon formation of the H-Reg complex may be linked to conformational changes exerted by movement of residues lining the pore region. A model describing how these changes could affect coupling efficiency is proposed in Chapter 2, where the regulatory protein increases coupling efficiency by protecting the diiron-$O_2$ intermediates formed during oxygen activation.

The regulatory proteins of BMMs also affect the regiospecificity of hydroxylation. In the case of T4MO, hydroxylation in the absence of the regulatory protein yields low regiospecificity, 63\% $p$-cresol, 5\% $m$-cresol, 17\% $o$-cresol, and 15\% benzyl alcohol. In contrast, the reaction in the presence two equivalents of the regulatory protein to the hydroxylase yields higher regiospecificity, 97\% $p$-cresol.\textsuperscript{98} Formation of the T4MO H-Reg complex constricts the active site cavity to fit a single aromatic molecule.\textsuperscript{17} The tight fit may orient the methyl group of toluene, affecting the regiospecificity of hydroxylation.

Although isomers are not possible in the oxidation of methane to methanol, regioisomers can be observed upon hydroxylation by sMMO of larger substrates. In the
absence of the regulatory protein, hydroxylation of nitrobenzene yields 90% \textit{m}-nitrophenol, whereas in the presence of two equivalents of the regulatory protein to the hydroxylase, the product is 89% \textit{p}-nitrophenol.\textsuperscript{69} Many other BMMs hydroxylate substrates with varying regiospecificity, including sBMO,\textsuperscript{39} ToMO,\textsuperscript{71} and alkene monooxygenases.\textsuperscript{72} The role of the regulatory protein in product distributions for these BMMs has not yet been reported.

Early work with sMMO reveals that the regulatory protein acts in a concentration-dependent manner. At substoichiometric concentrations, the regulatory protein serves as a promoter of catalysis.\textsuperscript{56} At excess concentrations of the regulatory protein, it inhibits catalysis.\textsuperscript{56} Steady state analysis of sBMO,\textsuperscript{39} T4MO,\textsuperscript{16} and ToMO\textsuperscript{19} demonstrates that this duality is conserved throughout BMMs. The regulatory protein regulates hydrocarbon access,\textsuperscript{17,28,29} enforces coupling of NADH consumption and product formation,\textsuperscript{11,66-68} controls oxygen access,\textsuperscript{18,20,65,73} and competes with the diiron reductant for hydroxylase binding.\textsuperscript{8,16,19,21} The inhibitor effect of the regulatory protein results from competition between the diiron reductant and the regulatory protein for binding to the hydroxylase (Chapter 3).\textsuperscript{8,16,19,21} Owing to its many functions, the source of the promoter effect is probably multifaceted as described below.

The role of the regulatory protein in attenuating hydrocarbon access differs among BMMs. In sMMO, the hydrophobic cavities of the hydroxylase are the proposed route for hydrocarbon access.\textsuperscript{28} Thus, binding of the regulatory protein increases hydrocarbon access, contributing to the promoter effect. In PH, the pore, which is open in both the hydroxylase and H-Reg structures, is the proposed entry site for hydrocarbon substrates.\textsuperscript{20} Thus, hydrocarbon access is minimally altered by the regulatory protein. In contrast, in T4MO and ToMO, formation of the H-Reg complex closes the top of the aromatic-substrate channel.\textsuperscript{17} If substrate is present in the channel before formation of the H-Reg complex, this action traps substrate at the active site. If substrate is absent from the channel prior to formation of the H-Reg complex, this action prevents substrate access to the diiron site. Thus, one might expect that excess regulatory protein might prevent substrate access to the diiron site in ToMO and T4MO. This model, however, is
ruled out in the studies described in Chapter 3, indicating that attenuation of the arene substrate channel by the regulatory protein does not alter the promoter or inhibitor function of the regulatory protein.

The effect of coupling efficiency and oxygen access on the promoter effect is more uniform throughout BMMs. The maximum rate of turnover occurs at approximately equivalent concentrations of the diiron active site and the regulatory protein.19,39,56 Because coupling efficiency maximizes at substoichiometric equivalents of the regulatory protein,68 the increased coupling efficiency cannot fully account for the promoter effect of the regulatory protein. In contrast, the rates of oxygen activation maximize at or above one equivalent of the regulatory protein per diiron active site.58,63 Therefore, the promoter effect of the regulatory protein must arise both from increased oxygen access and from an independent increase in coupling efficiency. Because optimal coupling efficiency and oxygen activation occur at different ratios of the regulatory protein to the diiron active site, these features of catalysis may be caused by two independent functions of the regulatory protein.

**Diiron Reductant Effects on Steady-State Turnover by BMMs.** The effect of the diiron reductants on steady-state turnover is quite different for three- and four-component BMMs. For clarity, we first discuss the more simple case of three-component BMMs, and then we move to four-component BMMs.

For three-component BMMs, the effects of the reductase on steady-state turnover are most thoroughly characterized for *Mc*-sMMO58 and *Mt*-sMMO.53 Maximum coupling efficiency and hydroxylation of methane occur when 0.2 equivalents of the reductase to the dimeric hydroxylase (or 0.1 equivalents of the reductase to diiron sites).56 The reductase transfers electrons to the hydroxylase and dissociates from the hydroxylase more rapidly than steady-state turnover,56 allowing one reductase to service many hydroxylase proteins. Based on single turnover data, the reductase also plays an indirect role in hydroxylation, increasing the single-turnover product yield and changing the rate of oxygen activation.53 To rationalize this effect, a long-lived conformational change in
the hydroxylase has been evoked to explain the observed effects. Although this theory is compelling and has long been proposed, limited evidence is available for a long-lived conformational change in either Mc-sMMO or Mt-sMMO. Because a structure of the hydroxylase-reductase complex remains elusive, the specific changes that give rise to such a model are unknown.

At high concentrations of the sMMO reductase, uncoupling of NADH consumption and product formation is observed when methane is used as a substrate. In contrast, coupling efficiency is relatively unaffected by reductase concentration when propylene oxidation is examined. Propylene reacts with H\text{peroxo} preventing the formation of the diiron(IV) intermediate Q, which accumulates in the presence of methane. Thus, it was proposed that high concentrations of the reductase can quench intermediate Q. To do so, the reductase would need to displace the regulatory protein, which may only be possible at higher concentrations of the reductase.

In four component BMMs, the two ET proteins are both important for steady-state turnover, but they achieve their maximal function at very different concentrations. For ToMO, the maximum turnover is achieved at approximately 0.1 equivalents of the reductase to the hydroxylase dimer. In experiments with T4MO, approximately 0.1-0.2 equivalents of the reductase per hydroxylase dimer are often used, suggesting that substoichiometric equivalents of the reductase to the hydroxylase suffice for both ToMO and T4MO. With respect to the Rieske protein, the maximum turnover is achieved at two equivalents of the Rieske protein to the hydroxylase dimer for both T4MO and ToMO.

The divergent requirements for the diiron reductants in three- and four-component BMMs suggest that the mechanism of action differs for these proteins. In three-component BMMs, 0.1 equivalents of the reductase per diiron active site is sufficient to elicit full function. Because the Rieske proteins can only transfer one electron at a time, one might expect that approximately 0.2 equivalents of the Rieske protein to diiron active sites would yield the maximum turnover for four-component BMMs. However, one equivalent of the Rieske protein per diiron active site is required to yield maximum
turnover in both ToMO and T4MO. We can envision two mechanisms that might cause this dependence on the Rieske protein: (i) the H-Reg complex is more stable relative to the hydroxylase-Rieske protein complex or (ii) the lifetime of the conformational changes within the hydroxylase are shorter for the hydroxylase-Rieske protein complex of a four-component BMM than for the hydroxylase-reductase complex of a three-component BMM.

In the first scenario, the relative affinities of the H-Reg complex and the hydroxylase-diiron reductant complex may responsible for the different concentrations required for the diiron reductants. If the reductase can displace the regulatory protein of three-component BMMs more efficiently than the Rieske protein can displace the regulatory protein of four-component BMMs, a higher concentration of the Rieske protein might be required. Because the Rieske proteins are smaller than the reductases, the contact interface in the hydroxylase-diiron reductant complex may be smaller in four-component BMMs than three-component BMMs.

In the second scenario, it is assumed that the Rieske protein induces conformational changes in the hydroxylase necessary for turnover. Moreover, it is accepted that that the reductase of three-component BMMs induces a long-lived conformational change within the hydroxylase, which allows for full activity at only 0.1 equivalents of the reductase to the regulatory protein. Accepting these two principles, it is possible that the lifetime of these conformational changes is different. A shorter lifetime for the conformational changes exerted by the Rieske protein might require that high concentrations of the Rieske protein. Because these reductases are much larger than the Rieske proteins, it is possible that formation of the hydroxylase-reductase complex of three-component BMMs give rise to greater structural rearrangement in the hydroxylase than the hydroxylase-Rieske protein complexes. More extensive changes in the hydroxylase may give rise to a longer-lived species in three-component BMMs as compared to four-component BMMs. These two proposals are speculative, but aim to rationalize the differing requirements for the diiron reductants in three- and four-component BMMs.
Working Model for Component and Substrate Interactions in BMMs. Although there are still many outstanding questions regarding the function of BMMs, recent work has allowed for a greater understanding of their mechanisms. Based on the structural and functional studies presented in this chapter and the following chapters, we envision the mechanisms shown in Scheme 1.4, which integrate substrate access, protein interactions, and turnover.

Scheme 1.4. A proposed mechanism for protein interactions during single turnover by sMMO (A) and TMOs (B).
In three-component BMMs, the reductase binds to the hydroxylase closing the pore and initiated electron transfer to form the diiron(II) state. The reduced hydroxylase binds to the hydroxylase closing the pore and opening the pathway for O₂ and methane. Hydroxylation produces water and methanol, which can diffuse from the pore region upon dissociation of the regulatory protein. For both three- and four-component BMMs, the release of water and hydroxylated product may alternatively be induced by re-reduction of the diiron active site. Scheme 1.4 depicts the proposed mechanism for single turnover; thus re-reduction is omitted.

In Scheme 1.4, we assume that the second Rieske protein binds to the hydroxylase before the regulatory protein can bind to the hydroxylase. This sequential binding mode would provide the most effective and efficient sequence of events. During the formation of the hydroxylase-Rieske protein complex, aromatic substrate can enter the active site. Binding of the regulatory protein traps the hydrocarbon substrate and opens the pathway for oxygen access to the diiron site. Hydroxylation chemistry occurs following release of the regulatory protein and products.

**Concluding Remarks**

Multicomponent systems frequently prove complex and difficult to precisely characterize. The reason why nature employs modular units in these systems is often obscure, especially in cases where fewer enzyme components can be evolved in the laboratory to perform similar reactions. Through years of work on both three- and four-component BMMs, significant progress has been made towards characterizing the protein component interactions and substrate interactions that regulate function. In these systems, component competition controls reduction, oxygen activation, and hydrocarbon oxidation to effect high turnover and catalytic efficiency. The combined efforts of X-ray crystallography, solution phase analysis, and kinetic characterization have demonstrated that these events are precisely timed to maximize product formation and prevent loss of electrons and release of reactive oxygen species.
References


Component of Soluble Methane Monoxygenase from *Methylococcus capsulatus* (Bath). *Biochemistry* 41, 15780-15794.


Chapter 2

A Flexible Glutamine Regulates the Catalytic Activity of Toluene/o-Xylene Monooxygenase

This work was published in part:
Introduction

Bacterial multicomponent monooxygenases (BMMs) comprise a family of enzymes capable of hydroxylating and epoxidizing hydrocarbon substrates at carboxylate-rich diiron active sites. Bacteria containing BMMs help regulate the global carbon cycle\(^1\) and are used for bioremediation of environments contaminated with hydrocarbons and halogenated pollutants.\(^2,3\) All BMMs require a hydrocarbon substrate, dioxygen, protons, and electrons acquired through NAD(P)H.\(^4\) Each BMM requires either three or four components that must reversibly bind one another throughout catalysis. Dynamic interactions among these protein components orchestrate substrate delivery to their diiron centers and subsequent catalytic turnover.\(^5-8\) Toluene/o-xylene monooxygenase (ToMO) is a four-component, arene-oxidizing BMM, composed of an NADH-oxidoreductase (ToMOF), a Rieske-type ferredoxin (ToMOC), a cofactorless regulatory protein (ToMOD), and the catalytic hydroxylase protein (ToMOH). Three component BMMs, like soluble methane monooxygenase (sMMO), lack the Rieske-type ferredoxin.

The diiron active sites in the 200-250 kDa hydroxylase components of BMMs are buried ~12 Å from the protein surface within a four-helix bundle.\(^9\) The extensive protein architectures of these hydroxylase components protect intermediates formed during dioxygen activation from undesired side-reactions, but also provide a barrier to direct substrate access. This potential problem is addressed by channels and interconnected cavities that traverse the hydroxylase. These cavities and channels provide access of dioxygen\(^10,11\) and hydrocarbons\(^12,13\) to the diiron active sites, as revealed by X-ray crystallographic studies of four BMMs—ToMO, toluene-4-monooxygenase (T4MO), soluble methane monooxygenase (sMMO), and phenol hydroxylase (PH). A conserved pore comprising the shortest distance from the diiron site to the solvent-exposed surface has been discovered within all crystallographically characterized BMMs but has not yet been functionally investigated. This pore is lined by three hydrophilic residues, a threonine, an asparagine, and either a glutamate or glutamine (Figure 2.1).\(^14-16\)
Figure 2.1. The conformationally flexible pore of T4MO. Binding of the regulatory protein to the hydroxylase of T4MO elicits structural changes within the pore. The unbound, oxidized hydroxylase (PDB: 3DHG) and the complex between the regulatory protein and the oxidized hydroxylase (PDB: 3DHH) are shown in panels A and B, respectively. The regulatory protein, in panel B is depicted as a blue surface. The hydroxylase is colored in grey, cut away to highlight the pore and diiron active site. The active site, drawn as sticks, is colored by atom type: carbon (grey), oxygen (red), nitrogen (blue), and iron (orange). Carbon atoms of the pore appear in green and yellow to emphasize the conformational changes. Hydrogen bonds (varying between 2.7-2.9 Å) between the pore residues, active site ligands, and water molecule HOH5 are indicated by dashed black lines. Water molecules near or within the pore are shown as red spheres.

X-ray crystallographic studies revealed that the pore is hydrophilic, flexible, and can accommodate an ordered water molecule, designated HOH5, when the regulatory protein is bound to the hydroxylase. These structural results strongly suggest a role for the conformationally flexible, surface-exposed pore in the catalytic function of these enzymes.
Proposals for this function include mediating proton transfer (PT)\textsuperscript{9,12} or proton-coupled electron transfer (PCET),\textsuperscript{15} and providing a pathway for water or hydroxylated products to exit the active site.\textsuperscript{13}

In the present study, we evaluate reactivity changes following site-directed mutagenesis of residues within the pore of ToMOH from \textit{Pseudomonas sp.} OX1, the hydroxylase of a canonical four-component BMM that displays low regiospecificity during aromatic hydroxylation.\textsuperscript{17} Steady state and pre-steady-state studies demonstrate that residues N202 and Q228 of ToMOH are critical for efficient turnover, water egress from the active site, protection of intermediates formed during oxygen activation, and binding of the electron transfer protein, ToMOC, to the hydroxylase. We propose that these activities are achieved through movement of residues N202 and Q228 during catalysis.

**Experimental Methods**

**Materials and General Methods.** Wild-type (WT) vectors were kindly provided by Prof. Alberto Di Donato, Naples, Italy. Chromatography was conducted in a cold room maintained at 4 °C. Catechol 2,3-dioxygenase (C2,3O) was used in coupled activity assays as previously described.\textsuperscript{18} Protein images and cartoon representations of mutants were rendered using PyMOL X11/Hybrid.\textsuperscript{19} Tris, Bis-Tris propane, and phosphate salts were purchased from CalBioChem, Santa Cruz Biochemical, and BDH, respectively. NADH was obtained from Roche. Phenol and sodium dithionite were purchased from Sigma-Aldrich.

**Expression and Purification of ToMO Components.** Expression and purification methods for ToMOH, ToMOD, and ToMOC were modified from previously reported procedures, as detailed in the Appendix A. ToMOF was expressed and purified as previously described.\textsuperscript{20} Mutagenesis of ToMOH was carried out using the pET22B(+)\textit{touBEA} plasmid as a template. Quickchange PCR was performed with Pfu Turbo DNA polymerase, dNTPs, and buffers purchased from Stratagene (now Agilent). Primers were purchased from Integrated DNA Technologies, and a standard desalting procedure for these primers was requested. The forward primer sequences were as follows: N202A, 5'-CA TTC GAA ACA GGC TTC AAC GCA ATG CAG TTT CTC GGT TTG-3'; Q228A, 5'-CTG ATT TCA AGC ATA AAC ACG GAC GAA TCA CGT C-3'; Q228E, 5'-CTG ATT TCA AGC ATA GAA ACG GAC GA-3'. All reverse primers used were
the reverse-complement counterparts of the forward primers listed. PCR products were digested
with DpnI for 2 h and assayed on a 1% agarose gel to determine which reactions were most
successful. Those PCR products containing full-length plasmid were transformed into XL1-Blue
cells and plated onto an LB/Agar plate supplemented with 100 µg/ml ampicillin. Three 5-ml
cultures were grown from individual colonies. DNA from each mini-culture was extracted as
indicated by the Qiagen mini-prep kit. DNA sequencing was performed by the MIT Biopolymers
Laboratory.

NADH Consumption Assays. A 350-µL solution comprising 0.15 µM WT or mutant
hydroxylase, 6 µM ToMOD, 6 µM ToMOC, 60 nM ToMOF, and 0-1500 µM phenol was
prepared in 0.1 M Tris, pH 7.3. The protein mixture was allowed to stand at room temperature
for 1 h. The reaction was initiated by addition of NADH ($\epsilon_{340} = 6220 \text{ M}^{-1} \text{ cm}^{-1}$) to a final
concentration of 200 µM. The absorbance change at 340 nm was monitored at 25 °C using a
Hewlett Packard diode array spectrophotometer scanning every 3 s. Initial velocities were
obtained by fitting five time points to a linear function. A minimum of three replicates was
performed for each condition to obtain average values and standard deviations.

Catechol Formation Assays. The rate of catechol formation was determined by a coupled
assay. Reactions were prepared as described above under NADH Consumption Assay except
that excess catechol 2,3-dioxygenase (C2,3O) was added to convert catechol to 2-
hydroxymuconic semialdehyde ($\epsilon_{410} = 12620 \text{ M}^{-1} \text{ cm}^{-1}$). The initial rate of 2-hydroxymuconic
semialdehyde was recorded as a function of absorbance at 410 nm over time. For experiments
carried out in deuterated buffer, D$_2$O was purchased from Icon Isotopes, 99.8% isotope enriched.
Before performing pH readings of D$_2$O-containing buffers, the pH meter probe was soaked in
this solvent. The pD value of each D$_2$O buffer was calculated by adding 0.4 to the pH meter
readings. To vary the viscosity of the reaction buffer, 0-1.25 m sucrose was added to the buffer.
To examine the effect of ToMOC concentration, the rate of turnover with respect to ToMOC
concentration was determined using 1-12 µM ToMOC in the steady-state assays.

Steady-State Data Analysis. Initial velocities were plotted against phenol concentration, and the
resulting curves were fitted in OriginLabs 9.0 to either the Michaelis-Menten equation or a
modified Michaelis-Menten equation accounting for substrate inhibition. Double reciprocal plots were used to calculate the enzymatic efficiencies, \( k_{cat}/K_m \), for each hydroxylase. The kinetic solvent isotope effects (KSIEs) were derived by dividing \( k_{cat} \) or \( k_{cat}/K_m \) obtained in H\(_2\)O by those obtained in D\(_2\)O. Coupling efficiencies were calculated and defined as the rate of product formation divided by the rate of NADH consumption. In assays with varying concentrations of ToMOC, the initial rate was plotted as a function of ToMOC concentration. These plots were fit to the Michaelis-Menten equation with ToMOC as the substrate to determine the \( k_{cat} \) and \( k_{cat}/K_m \) with respect to ToMOC.

**Discontinuous Catechol Formation Assays.** To determine the pH profile of WT ToMOH and mutant Q228A, a discontinuous catechol formation assay was used, monitoring the initial rate of catechol formation as a function of pH. A 300-\( \mu \)L solution of 0.15 \( \mu \)M ToMOH WT or Q228A, 6 \( \mu \)M ToMOD, 6 \( \mu \)M ToMOC, 60 nM ToMOF, and 200-500 \( \mu \)M phenol was prepared in 0.1 M Bis-Tris propane, pH 5.75-7.50. The protein mixture was allowed to stand at room temperature for 1 h. The reaction was initiated by addition of NADH to a final concentration of 200 \( \mu \)M. In 10-30 s increments, 50-\( \mu \)l aliquots of were removed from the reaction and quenched in 50 \( \mu \)l of 0.4 M trichloroacetic acid, the acidified mixture being vigorously pipetted. Five time points were obtained for each condition. The quenched mixture was centrifuged (2000 \( g \)) and the supernatant was diluted 4-fold into buffer containing 500 mM Tris and 50 mM MOPS at pH 7.3. C\(_{2,30} \) (5 U) was added to each of the diluted solutions. The absorbance at 374 nm was graphed as a function of the quenching time (2-hydroxymuconic semialdehyde, \( e_{374} = 33,000 \) M\(^{-1}\) cm\(^{-1}\)). Linear fits were obtained for each time-course. The \( k_{cat} \) and relative coupling efficiency values were plotted as a function of pH and fit to eqs 2.1 and 2.2 for WT ToMOH and mutant Q228A, respectively.

\[
v(s^{-1}) = \frac{y_{max}}{1 + \frac{10^{-pH}}{10^{-pK_{a1}}} + \frac{10^{-pK_{a2}}}{10^{-pH}}} \quad \text{(2.1)}
\]

\[
v(s^{-1}) = \frac{y_{max}}{1 + \frac{10^{-pH}}{10^{-pK_{a2}}}} \quad \text{(2.2)}
\]
The coupling efficiency as a function of pH was determined by dividing the rate of catechol formation obtained from the discontinuous assay by the rate of NADH consumption. Because one method is continuous and the other is discontinuous, the coupling efficiencies were normalized to the value at pH 7.25, the pH closest to those used in other steady-state experiments reported here.

**Preparation of a ToMOD-Fluorescein (ToMOD-Fl).** A 3-ml solution of 100 μM ToMOD in buffer (25 mM MOPS, pH 7.0, 150 mM NaCl, and 10% glycerol) was cycled between vacuum and argon in a Schlenk flask. After 10 such cycles, tris(2-carboxyethyl)phosphine (TCEP: 250.2 g/mol) was added to a final concentration of 1 mM. The flask was again cycled between vacuum and argon. Subsequently, 5-iodoacetamidofluorescein (5-IAF-515.3 g/mol) was added to a final concentration of 1 mM. The reaction was allowed to proceed at room temperature for 2 h under argon with gentle stirring. Throughout the reaction, the flask was covered in aluminum foil to exclude light. After 2 h, the reaction was placed on ice for two additional hours.

In the dark, the solution was loaded onto a PD10 column, and the flow-through was collected in approximately 0.5-ml fractions. The UV-visible spectrum of each fraction was taken. Fractions 3-9 were pooled based on their Abs₄₉₅ and the ratio of their Abs₂₈₀ over Abs₄₉₅ (see data below). The pooled fractions were concentrated in a 3-kDa MWCO centrifugal device. The concentrated protein solution was loaded onto a Superdex 200 column (1.6 cm diameter, 26 cm length, and CV ~140 ml) to remove a high molecular weight impurity. The protein was eluted with an isocratic gradient for 120 ml with buffer containing 25 mM MOPS, pH 7.0, 10% glycerol (v/v), and 150 mM NaCl. Fractions of 3 ml were collected throughout the elution. The absorbance chromatogram contained several peaks, but the fluorescence chromatogram contained only one sharp peak. The fractions corresponding to the fluorescent product were pooled, but not further concentrated owing to aggregation concerns. A Bradford assay was performed with unlabeled ToMOD as the standard.

**Hydroxylase Binding Studies with ToMOD-Fl.** A 1-ml solution of 40 μM the hydroxylase variant and 3 μM ToMOD-Fl was prepared in 25 mM MOPS, pH 7.0, 10% glycerol (v/v), and 150 mM NaCl. The solution was directly injected onto a Superdex 200 column (1.6 cm diameter, 24 cm length, and CV ~140 ml). The protein was eluted from the column with the same buffer.
described above. The total elution volume was 120 ml at a flow-rate of 1 ml/min. Throughout the elution, fractions of 3 ml were collected. The absorbance at 280 nm was monitored throughout. The fluorescence intensity of each fraction was obtained at the end of the elution. The two chromatograms were overlaid and integrated to determine the ratio of free and hydroxylase-bound ToMOD-Fl. The elution patterns of ToMOH and ToMOD-Fl were separately determined to identify the retention times of the individual proteins. The complex of ToMOH with either ToMOD or ToMOD-Fl co-eluted with free ToMOH, such that the absorbance chromatogram was not useful for identifying the percent of complex vs. free protein. Integration of the fluorescence peaks at 65 ml and 90 ml was the only viable method of analysis. The binding studies were performed in triplicate for each hydroxylase variant.

**Electron Transfer from ToMOC$_{\text{red}}$ to Hydroxylase Variants.** ET from reduced ToMOC (ToMOC$_{\text{red}}$) to the oxidized hydroxylase variants was monitored by stopped-flow UV-visible spectroscopy configured with a single-wavelength photomultiplier and a tungsten lamp. All stopped-flow data reported were obtained by using a Hi-Tech Scientific (Salisbury, UK) SF-61 DX2 stopped-flow spectrophotometer. Absorbance changes at 458 and 565 nm were monitored, corresponding to the greatest change of extinction coefficient upon oxidation or reduction, ~4000 and 2000 M$^{-1}$ cm$^{-1}$, respectively. Anaerobic preparation of protein samples and the stopped-flow instrument was carried out by washing the flow lines with dithionite buffer.

The reaction temperatures were maintained at 13 °C with a circulating water bath. The final protein concentrations were 10 μM ToMOC$_{\text{red}}$ and 100 μM of the oxidized hydroxylase variant. All data presented are the result of an average of three or more individual mixes of the ET complex. In OriginLabs 9.0 and Kinetic Studio, the data were fit to a single exponential function, eq 2.3, where $C$ is the initial absorbance, $A$ is the observed absorbance change, $k$ is the rate constant, and $t$ is time. Double exponential fits were also inspected to assure their validity.

$$Abs_{458\text{nm}}(t) = Ae^{-kt} + C$$

(2.3)

**Results**

**General Steady-State Kinetics.** Table 2.1 and Figure 2.2 summarize the results of phenol conversion to catechol for WT ToMOH as well as mutants N202A, Q228A, and Q228E.
Table 2.1. Kinetic parameters of hydroxylase variants

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>N202A</th>
<th>Q228A</th>
<th>Q228E</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_{cat, \text{phenol}} ) (s(^{-1}))</td>
<td>4.1(1)</td>
<td>0.59(3)</td>
<td>0.42(6)</td>
<td>0.058(3)</td>
</tr>
<tr>
<td>( k_{cat}/K_{m, \text{phenol}} ) (mM(^{-1})s(^{-1}))</td>
<td>220(8)</td>
<td>62.7(9)</td>
<td>16(1)</td>
<td>0.72(5)</td>
</tr>
<tr>
<td>KSIE(( k_{cat}/K_{m, \text{phenol}} ))</td>
<td>2.0(1)</td>
<td>1.9(2)</td>
<td>12(1)</td>
<td>0.19(1)</td>
</tr>
<tr>
<td>Coupling Efficiency</td>
<td>0.98(4)</td>
<td>0.57(3)</td>
<td>0.53(1)</td>
<td>0.39(7)</td>
</tr>
<tr>
<td>( k_{cat, \text{ToMOC}} ) (s(^{-1}))</td>
<td>9.8(4)</td>
<td>7.8(3)</td>
<td>4.7(7)</td>
<td>0.8(2)</td>
</tr>
<tr>
<td>( k_{cat}/K_{m, \text{ToMOC}} ) (( \mu \text{M}^{-1})s(^{-1}))</td>
<td>1.04(1)</td>
<td>0.412(8)</td>
<td>0.203(4)</td>
<td>0.019(2)</td>
</tr>
<tr>
<td>Electron Transfer ( k_{\text{obs}} ) (s(^{-1}))</td>
<td>44(2)</td>
<td>21.4(4)</td>
<td>40(10)</td>
<td>6(1)</td>
</tr>
</tbody>
</table>

Figure 2.2. Steady state turnover for hydroxylase isoforms in H\(_2\)O (black) and D\(_2\)O (red). The reactions were performed by the C2,3O coupled assay. In all of these studies, the component concentrations were as follows: 0.15 \( \mu \text{M} \) ToMOH, 6 \( \mu \text{M} \) ToMOD, 6 \( \mu \text{M} \) ToMOC, 0.06 \( \mu \text{M} \) ToMOF, 0-1500 \( \mu \text{M} \) phenol, 200 \( \mu \text{M} \) NADH, and C2,3O.
Compared to that of WT ToMOH, the $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ values were significantly diminished for the mutant proteins. N202A and Q228A retained 15% and 10% of the WT ToMOH $k_{\text{cat}}$, respectively. ToMOH mutant Q228E exhibited the lowest $k_{\text{cat}}$, only 1.5% that of the WT protein. The most deleterious mutations with respect to catalytic efficiency were Q228A and Q228E, which dropped to 7% and 0.3% of the WT values, respectively. The N202A mutant retained 28% of the catalytic efficiency of WT ToMOH.

WT ToMOH and mutant N202A each displayed similar KSIE($k_{\text{cat}}$) values. Mutant Q228A exhibited a much larger KSIE($k_{\text{cat}}$), suggesting that a proton-transfer or viscosity-dependent event is rate-limiting. Conversely, mutant Q228E had a strong inverse KSIE($k_{\text{cat}}$). Inverse KSIE($k_{\text{cat}}/K_m$) values were observed with varying magnitudes for all hydroxylase mutants except that of Q228A.

The rate of hydroxylation versus the rate of NADH consumption (coupling efficiency) for WT ToMOH was near unity. Coupling efficiencies for mutants N202A and Q228A decreased to 50%. ToMOH mutant Q228E exhibited the lowest coupling efficiency, 39%. During the course of these experiments, it was noted that decreasing the reaction temperature led to higher coupling efficiencies for mutant Q228A. The source of this increased coupling efficiency may arise from a decreased rate of water flux to the active site as discussed below, but this effect was not further explored.

**Steady-State Viscosity Dependence.** A plot of $k_{\text{cat}}$ as a function of viscosity is shown in Figure 2.3 for each hydroxylase mutant. The solution viscosity at each sucrose molality is given based on previous reports at 25 °C. Within the viscosity range assayed, the activity did not change significantly for WT ToMOH or mutants N202A and Q228E. With increasing viscosity, the $k_{\text{cat}}$ for mutant Q228A decreased dramatically, such that the activity was lowered by more than a factor of 2 at a sucrose molality of 1 or $\eta/\eta_{\text{rel}}$ of ~3.3.
Figure 2.3. Viscosity dependence of $k_{cat}$ for the hydroxylase isoforms with respect to phenol. The normalized data are shown for WT ToMOH (black, open circles), N202A (red squares), Q228A (blue diamonds), and Q228E (green triangles). Turnover was monitored by the catechol formation assay. The reactions were buffered in 0.1 M Tris buffer, pH 7.3, at 25 °C with sucrose as the viscogen.

**Effect of ToMOC on Steady-State Parameters.** For WT ToMOH and each mutant enzyme system described here, the steady-state parameters as a function of ToMOC concentration are shown in Table 2.1 and Figure 2.4. The enzymatic efficiencies with respect to ToMOC were significantly reduced for all mutants compared to WT ToMOH. The errors associated with the steady-state parameters for mutant Q228E are higher than those for other mutants. Even at 25 μM ToMOC, the curve did not begin to saturate for ToMOC variation experiments with mutant Q228E. It was not possible to use higher concentrations of ToMOC owing to interference of the absorbance features of ToMOC with that of NADH and the catechol degradation product, 2-hydroxymuconic semialdehyde.
Figure 2.4. Turnover at varying concentrations of ToMOC for the hydroxylase isoforms. In these catechol formation assays, the component concentrations were as follows: 0.15 μM ToMOH, 6 μM ToMOD, 1-25 μM ToMOC, 0.06 μM ToMOF, 400 μM phenol, 200 μM NADH, and C2,3O. The reactions were studied by the catechol formation assay.

Steady-State pH Profiles. Figure 2.5 depicts the steady-state pH dependence of both WT ToMOH and mutant Q228A. Concentrations of phenol were varied in these experiments to determine both $k_{cat}$ and $k_{cat}/K_m$ for catechol formation. Although an approximate $k_{cat}$ could be obtained, determination of $k_{cat}/K_m$ was not successful owing to high errors at low concentrations of phenol. The $pK_a$ values derived from fits according to the Experimental Section are indicated in the top panel of Figure 2.5. WT ToMOH exhibits two $pK_a$ values within the assayed region, resulting in a bell-shaped curve. The $pK_{a1}$ of mutant Q228A shifts to a higher value, and the $pK_{a2}$ is either not apparent during catalysis or has moved outside of the accessible pH window. The coupling efficiency as a function of pH is shown in the lower panel of Figure 2.5. Within error, the WT ToMOH coupling efficiency is near unity for all pH values observed. The coupling efficiency for mutant Q228A decreases significantly with decreasing pH.
Figure 2.5. Steady state pH profiles of WT ToMOH (black, open circles) and mutant Q228A (blue triangles). The reactions were buffered in 0.1 M Bis-Tris propane, pH 5.75-7.5 at 25 °C. Reactions were assayed by both the discontinuous catechol formation and NADH consumption methods. (Top) The maximum rate of product formation as a function of pH with fits according to eqs 1 and 2. (Bottom) The coupling efficiency relative to coupling at pH 7.25 of WT ToMOH and mutant Q228A. The dotted-red line indicates full coupling efficiency relative to the coupling efficiency at pH 7.25.

Pre-Steady-State ET Kinetics of Hydroxylase Variants. The ET kinetics of each mutant were measured by stopped-flow UV-visible spectroscopy (Table 2.1 and Figures 2.6). All of the data obtained fit well to a single exponential function based on the quality of the residuals, the adjusted R-squared value, and the error in the fitted parameters. The residuals for the single exponential fit did not oscillate, indicating that a single kinetic event sufficiently described the data. The rate constants for the observed kinetics were similar for WT ToMOH and mutant Q228A. The rate constant decreased by a factor of two upon mutation of N202 to alanine and by approximately eightfold for mutant Q228E.
Figure 2.6. The change of absorbance at 458 nm upon mixing ToMOC\textsubscript{red} with an oxidized hydroxylase isoform. The top panel shows the absorbance changes for WT ToMOH (black), N202A (red), Q228A (blue), and Q228E (green). Fits to the data are shown in light grey. The bottom panel shows the residuals for each fit color-coded the same as indicated for the top panel. The final protein concentrations were 10 \( \mu \)M ToMOC and 100 \( \mu \)M of the ToMOH variant. The fit parameters are shown in Table 1.

**Discussion**

The function of pore residues has generated much speculation since an early Xe-pressurized crystal structure analysis of the hydroxylase of sMMO (MMOH).\textsuperscript{13} Similarly to other BMM hydroxylases, the pore of ToMOH is surrounded by a dynamic hydrogen-bonding network that includes active site water molecules, the shifting carboxylate E231 ligated to the diiron active site, and amino acids T201, N202, and Q228 (Figure 2.1).\textsuperscript{14,24} The role of the conserved threonine residue, T201, has been a subject of extensive investigation.\textsuperscript{20,25-27} The roles of residues N202 and Q228, however, have received far less attention in the literature. To investigate the roles of residues N202 and Q228, we used site-directed mutagenesis and comparative kinetic analysis between WT ToMOH and three hydroxylase mutants, N202A, Q228A, and Q228E. Alanine mutations were selected to definitively disrupt the hydrogen-bonding network surrounding the pore. Mutant Q228E was prepared to mimic the pore of MMOH, which contains a glutamate instead of a glutamine at this key position (Figure 2.7).
Experimental Design. ToMO is capable of hydroxylating a wide variety of arene substrates, including toluene, o-xylene, benzene, halogenated aromatics, and phenol. Hydroxylation of phenol by ToMO yields only one product, catechol, whereas hydroxylation of toluene leads to a distribution of products, o-, m-, and p-cresol. Owing to its solubility and ease of characterization, phenol was used as the substrate in the steady-state reactions described here.

With respect to turnover, there are two competing processes as illustrated in Scheme 2.1, namely, substrate hydroxylation by ToMOH$_{peroxo}$ and loss of H$_2$O$_2$ from ToMOH$_{peroxo}$. In this work, we examined the efficiency of hydroxylation versus uncoupled activity (H$_2$O$_2$ formation).
Two important factors contributing to coupling efficiencies are the concentrations of a hydrocarbon substrate and the function of the regulatory protein, ToMOD. At sub-saturating concentrations of either a hydrocarbon substrate or the regulatory protein, the coupling efficiency decreases for many BMMs. These two factors were kept in mind when designing and interpreting the experiments.

**N202 and Q228 are Critical for Hydroxylation and Coupling Efficiency.** For all three mutants, both the rate of product formation and the coupling efficiency were deleteriously affected. To determine whether a lower affinity for ToMOD was the source of low coupling efficiency, we investigated directly the binding of ToMOH with ToMOD-FI (Table 2.1). Mutants N202A and Q228A each retained similar affinities for ToMOD-FI as that of WT ToMOH. In contrast, mutant Q228E exhibits a diminished binding strength for ToMOD-FI. Because WT ToMOH and mutants N202A and Q228A maintain a similar ToMOD-FI binding affinity, the loss of coupling efficiency in these mutants cannot be attributed to a decreased binding affinity for the regulatory protein. Therefore, residues N202 and Q228 must participate in aromatic hydroxylation by ToMOH$_{peroxo}$ (Scheme 2.1, blue) or down-regulate hydrogen peroxide release (Scheme 2.1, green). This attenuation of activity clearly demonstrates that both N202 and Q228 are critical for efficient hydroxylation.
Residue Q228 Mediates Proton Flux to the Active Site. Identification of HOH5 within the pore of the cognate hydroxylase of T4MO by X-ray crystallography\(^4\) suggests that solvent-mediated PT may occur through the pore. If solvent-derived protons are responsible for a rate-limiting PT step, the observed kinetics will be diffusion limited and highly sensitive to protium/deuterium solvent exchange.\(^3\) To examine the sensitivity of mutants N202A, Q228A, and Q228E to these variables, product formation assays were conducted monitoring KSIEs, viscosity dependence, and pH dependence.

Of the mutants examined here only Q228A exhibited steady-state viscosity dependence and isotope effects greater than two. Because the viscosity of deuterated water (1.081-1.257 mPa·s) is greater than that of protiated water (0.888-1.022 mPa·s),\(^3\) viscosity dependent kinetics can also result in KSIE values greater than 1. At the viscosity of deuterated water (\(n_{rel}, 1.23\) mPa·s\(^3\)), the \(k_{cat}\) of WT and the three mutants varied from 88-108\% of the \(k_{cat}\) in non-sucrose containing buffer. Thus, viscosity can only contribute a maximum KSIE effect of 1.14 for all mutants. The observed KSIE(\(k_{cat}\)) and KSIE(\(k_{cat}/K_m\)) values for mutant Q228A are much larger, however, 12 and 7, respectively. Thus, the rate-limiting reaction of mutant Q228A is sensitive to both viscosity and hydrogen deuterium exchange.

To further examine the proton dependence of mutant Q228A, pH profile studies for catechol formation were carried out with either WT ToMOH or mutant Q228A. The pH profile of WT ToMOH displays a bell-shape (Figure 2.5), indicating that two prototropic groups are critical for catalysis. The \(pK_{a1}\) and \(pK_{a2}\) values of WT enzyme are 6.1 and 7.1, respectively. The functional groups responsible for these \(pK_a\) values are unknown, but may include a histidine side chain (\(pK_a \sim 6.1\)), an iron-aqua species (\(pK_a < 15\), but widely variable),\(^3\) or a diiron-peroxo species (\(pK_a \sim 7.2\)).\(^3\) The coupling efficiency of WT ToMOH did not vary significantly over the pH range investigated (Figure 2.5). Conversely, the pH profile of mutant Q228A is alkaline-shifted (\(pK_a \sim 6.52\)) from that of WT (\(pK_a \sim 6.1\)) and did not exhibit a bell-shape within the accessible pH range. At the lower end of the pH range, mutant Q228A displayed significant uncoupling, in parallel with the diminished \(k_{cat}\) values for conversion of phenol to catechol.

Because the kinetic properties of the Q228A mutant respond to protium-deuterium substitution, the pH of the buffer, and solvent viscosity, we conclude that unregulated solvent derived proton transfer occurs when the carboxamide side chain is removed in mutant Q228A. We propose that movement of the Q228 side chain attenuates proton flux from solvent,
preventing the quenching of oxygenated intermediates in the ToMOH reaction cycle. Figure 2.8 (bottom panel) illustrates a cartoon model that would result in the observed kinetics. Loss of the carboxamide side chain in mutant Q228A may lead to the entry of water molecules into the pore. An unregulated flux of water molecules into the pore exposes the diiron center to more solvent, including H$_3$O$^+$, allowing for deactivation of oxygenated intermediates. In such a model, binding of ToMOD to mutant Q228A would not protect the diiron center from solvent access.

Figure 2.8. Model for explaining the behavior of mutant Q228A during steady-state turnover. The X-ray crystal structure of the reduced hydroxylase-regulatory protein complex of WT T4MO is depicted in the top panel. PyMOL was used to generate representations of the water network for mutant Q228A (bottom panel). The placement of these water molecules was selected to optimize the hydrogen bonding distances between the water molecules and was supported by a previously reported X-ray crystallographic study. In all representations, the hydroxylase is shown as a grey surface and the regulatory protein as a dark blue surface. The side chains of residues T201, N202, Q228X, and E231 are all drawn as sticks color coded according to atoms: carbon (teal), oxygen (red), nitrogen (blue), and iron (orange). Iron atoms and water molecules are shown as orange and red spheres, respectively.

This structural model would explain the sensitivity of mutant Q228A to protium-deuterium substitution, an increase in proton concentration, viscosity, and the diminish efficacy of ToMOD as a coupling protein. An alternative explanation for the observed kinetics is a
change from classical PT in WT ToMOH to tunneling PT in mutant Q228A.36,37 This possibility is highly disfavored owing to the relatively slow rate of turnover for steady-state reactivity. For N202A, there is no significant KSIE or viscosity dependence during steady-state turnover, presumably because Q228 is sufficient to completely block the pore. Unfortunately, no direct evidence regarding proton transfer in WT ToMOH could be obtained through the studies presented here.

**Residue Q228 Regulates Water Dissociation from the Diiron Active Site.** Hydroxylation of substrates by BMMs involves the production of an alcohol or epoxide product and a water molecule. In addition, substrate binding12 and a shifting glutamate residue38 require expulsion of iron-bound water molecules from the active site by diffusion through the protein surface. Water flux through the interior of proteins typically proceeds through defined routes, frequently through hydrophilic channels.39 The BMM pore is the only conserved, hydrophilic channel extending from the diiron center to the solvent exposed surface.10,13,14,24 Water egress cannot be directly monitored to determine whether the pore controls the flux, but indirect evidence is provided by the results of KSIE measurements.

Inverse solvent isotope effects in enzymology have been reported for cysteine protonation/deprotonation during catalysis40,41 and water dissociation from metal ions.42-44 Because there are no cysteine residues in proximity to the active site of ToMOH, the observed inverse isotope effects are assigned to release of water from the diiron active site during turnover. In particular, by protium/deuterium substitution, we observe inverse isotope effects for the KSIE(k_cat) of mutant Q228E and the KSIE(k_cat/K_m) of WT ToMOH and mutants N202A and Q228E.

For mononuclear cobalt,44 iron,45 and zinc,46 the number of aqua or hydroxo ligands dissociating from a metal center can be calculated from the magnitude of the KSIE and fractionation factors (Φ, equilibrium distributions of the two isotopes). Fractionation factors for mononuclear centers have been previously reported (Φ = 0.70).46 However, those factors for dimetallic centers are unreported to our knowledge. If each iron within the diiron active site is treated as a separate mononuclear center, surprising agreement is achieved between the experimental (0.19) and predicted (0.17) values (Scheme 2.2).
In the first step, one bridging hydroxide changes from bridging to singly bound, resulting in a KSIE for this micro-step of 0.70. This singly bound hydroxide becomes protonated resulting in a singly bound water molecule (blue colored water molecule in Scheme 2.2). The bridging hydroxide (pink colored water molecule in Scheme 2.2) is then protonated during the second electron transfer to produce a bridging water molecule. This bridging water molecule dissociates from the diiron core. If we consider the two irons separately, this water dissociation gives rise to a KSIE of 0.49 for dissociation from one iron atom and a second KSIE of 0.49 for dissociation from the second iron atom. The total observed KSIE for this concerted event would then be 0.70 x 0.49 x 0.49 or 0.17. This proposed model is the only possible scheme for water dissociation resulting in (i) an inverse KSIE near 0.19, (ii) avoids high charge accumulation, and (iii) yields a mechanistically relevant final structure. This treatment may be purely coincidental, however, and therefore it is not discussed further.

The KSIE($k_{cat}/K_m$) values for mutants N202A and Q228E, and WT ToMOH are also less than one, indicating that water dissociation from iron also contributes to rate constants comprising the KSIE($k_{cat}/K_m$) values for these hydroxylase variants. For single-substrate enzymes, the $k_{cat}/K_m$ incorporates rate constants for steps prior to the first irreversible one in the reaction pathway. In multi-substrate enzymes like ToMO, however, the order of substrate addition can change the composition of rate constants contributing to $k_{cat}/K_m$. Because of this complexity, changes in KSIE($k_{cat}/K_m$) cannot be interpreted in terms of specific mechanistic changes or specific rate constants.

**Binding of ToMOC to ToMOH is Mediated by Pore Residues.** We studied whether mutation of pore residues might effect a change in the reduction step of the catalytic cycle (Scheme 2.1). Mutants N202A and Q228E exhibited a decrease in rate constant for ET compared to WT
ToMOH. In addition, the rate of interprotein electron transfer from ToMOC\textsubscript{red} to WT ToMOH is limited by the rate of protein association and cannot be saturated even at very high concentrations of ToMOH. Steady state experiments demonstrate that the $k_{cat}$ and $k_{cat}/K_m$ values with respect to ToMOC are lower than WT ToMOH for all N202 and Q228 mutants. Taken together, these results show that N202 and Q228 are important for the function of ToMOC, most probably facilitating protein binding between the hydroxylase and the Rieske protein. If we consider dynamic interactions of the BMM hydroxylases, the regulatory proteins are known to bind over the pore in all X-ray crystallographically characterized BMM hydroxylase-regulatory protein complexes.\textsuperscript{14-16} Thus, if ToMOC were also to bind at the pore, ToMOD and ToMOC must compete for an overlapping binding site on the surface of ToMOH.

**Comparison of Putative Functions of the Pore in ToMOH and MMOH.** Mutation of Q228 to the analogous residue in sMMO, a glutamate, resulted in almost complete loss of steady-state activity and coupling efficiency. The most obvious conclusion is that the negative charge introduced significantly impacts the requisite conformational flexibility of this pore residue. However, the structurally analogous glutamate residue of sMMO, E240, undergoes a conformational rearrangement upon docking of the regulatory protein and hydroxylase, despite its negative charge.\textsuperscript{15} A comparison of crystal structures for ToMOH,\textsuperscript{24} T4moH,\textsuperscript{14} MMOH,\textsuperscript{47} and the hydroxylase-regulatory protein complexes for T4MO\textsuperscript{14} and sMMO\textsuperscript{15} revealed no amino acids surrounding the pore that would selectively stabilize a negatively charged versus a neutral species. The X-ray crystallographic study of mutant Q228E from T4MO offers a possible explanation.\textsuperscript{35} In this investigation, the glutamine residue is able to stabilize HOH5, but mutation to glutamate results in loss of this water molecule.\textsuperscript{35} If HOH5 and Q228 are involved in water release from the diiron active site, mutant Q228E might exhibit low activity and coupling efficiency because its charge might slow down the rate of water egress either by slowing the rate of conformational change or eliminating an essential hydrogen bonding partner. All three of these changes are observed from mutant Q228E, strongly suggesting a role for Q228 and HOH5 in proton transfer. The question of how MMOH functions with a glutamate at this key position remains unanswered. Unlike in ToMOH and T4moH, MMOH must release methanol during each catalytic cycle. It is possible that the pore of MMOH is optimized for release of both water
and methanol whereas, in other BMMs, the pore may be more specifically optimized for water release during catalytic turnover.

Conclusions

The role of a conserved pore near the diiron active site in BMMs, which opens and closes during catalysis,\textsuperscript{14,15} has been a subject of much speculation, largely on the basis of static X-ray crystal structure information. Here we unveil the role of pore residue Q228, through kinetic studies during catalysis. Through investigations of steady-state turnover, coupling efficiency, pH dependence, viscosity effect, and solvent kinetic isotope experiments, we determine that Q228 is critical for mediating water flux and attenuating PT, preventing adventitious attack on activated intermediates formed by the reaction of dioxygen with the reduced, diiron(II) form of the hydroxylase. We postulate that it is the movement of this fluxional glutamine residue, opening and closing the pore, which conveys the observed functionality. Residue N202 of the pore is also critical for catalysis but is more important for protein-protein interaction, possibly stabilizing the conformations of Q228. Finally, we present evidence that the pore is near the binding interface of ToMOH:ToMOC, strongly supporting a competitive binding model for the ToMOC and ToMOD components on the surface of ToMOH. The present work provides the first kinetic evidence regarding the function of a highly conserved pore in BMMs.

References


Chapter 3

Component Interactions and Electron Transfer in Toluene/o-Xylene Monooxygenase

This work was published in part:
**Introduction**

Bacterial multicomponent monooxygenases (BMMs) comprise a family of enzymes that can hydroxylate or epoxidize a variety of hydrocarbon substrates. Enzyme systems in this superfamily contain either three- or four-component proteins that are necessary for catalysis. Included are (i) a catalytic hydroxylase housing two carboxylate-rich diiron active sites; (ii) a 12-16 kDa regulatory protein; (iii) an NADH oxidoreductase containing an NADH binding site, a flavin adenine dinucleotide cofactor, and a [2Fe-2S] ferredoxin cluster; and (iv) a Rieske protein present only in the four-component BMMs. Protein interactions involving three-component BMMs have been thoroughly discussed in the literature. Four-component BMMs are less well investigated and more complicated, owing to their more extended electron transfer (ET) chain. In three-component BMMs, the NADH oxidoreductase directly reduces the diiron(III) centers in the hydroxylase, each by two electrons, without the need for additional proteins. In contrast, the NADH oxidoreductase of four-component BMMs is incapable of directly reducing the hydroxylase. Instead, this reduction is effected by the Rieske protein, designated ToMOC for the four-component BMM toluene/o-xylene monooxygenase (ToMO). Thus, either a ternary complex, involving the hydroxylase (ToMOH), ToMOC, and the NADH-oxidoreductase (ToMOF) proteins, must form, or sequential interactions between ToMOC and ToMOH may occur, as diagrammatically represented for the four-component BMM, ToMO, in Scheme 3.1. This scheme intentionally omits any contribution from the regulatory protein, ToMOD.

**Scheme 3.1.** A model for the intermolecular single ET events within ToMO.
Interactions of the regulatory protein with the hydroxylase have been thoroughly described for the four-component BMM toluene 4-monoxygenase (T4MO) using both equilibrium binding measurements\textsuperscript{10} and X-ray crystallography.\textsuperscript{11,12} Reported functions of the regulatory protein include opening oxygen access to the diiron active site of the hydroxylase\textsuperscript{13,14} and closing the entry point for aromatic substrate within the hydroxylase.\textsuperscript{12,15} The roles of the regulatory protein with respect to ET kinetics and the function of the ET proteins remain unexplored for four-component BMMs, however.\textsuperscript{1} To address this issue, we carried out steady-state and pre-steady-state experiments with the four-component BMM ToMO from \textit{Pseudomonas sp.} OXI. We find that excess ToMOD inhibits turnover and that this inhibition is dependent on the concentration of ToMOC and the temperature of the reaction. ET studies demonstrate that the interaction between reduced ToMOC (ToMOC\textsubscript{red}) and oxidized ToMOH (ToMOH\textsubscript{ox}) occurs with a second order rate constant of 0.42 \textmu{}M\textsuperscript{-1}s\textsuperscript{-1} and that the observed rate constant is diminished in the presence of ToMOD. Finally, chemical cross-linking and protein docking studies support a competitive binding model, where ToMOC and ToMOD compete for binding to the same region of ToMOH. These conclusions provide evidence for a dynamic interaction of ToMOH with both ToMOD and ToMOC. Possible functions for these interactions are discussed in the context of previous reports on three- and four-component BMMs.

\textbf{Experimental Procedures}

\textbf{General Methods.} Plasmids for the component proteins of ToMO were generously provided by the Di Donato laboratory (Naples, Italy). Expression and purification of ToMOC, ToMOD, and ToMOH were carried out as described in Appendix A. ToMOF was expressed and purified as previously described.\textsuperscript{16} NADH and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) were purchased from Roche and Sigma, respectively. ToMOH is an (\alpha\beta\gamma)_2 dimer, with

\textsuperscript{1} During the course of this work, a related study of T4MO appeared, to which we draw the reader's attention: Acheson, J. F., Bailey, L. J., Elsen, N. L., and Fox, B. G. (2014) Structural Basis for Biomolecular Recognition in Overlapping Binding Sites in a Diiron Enzyme System. \textit{Nat Commun} 5. DOI: 10.1038/ncomms6009. These structural studies for T4MO are consistent with the results and conclusions presented here.
carboxylate-bridged diiron centers contained in each alpha subunit. In this work, the concentration of ToMOH is represented as that of the \((\alpha\beta\gamma)_2\) dimer.

**Steady-State Kinetics and Analysis.** Reactions were examined in 400-µl volumes of 0.1 M Tris buffer at pH 7.3. Each reaction contained 2 µM ToMOH (4 µM diiron active sites), 0.5-120 µM ToMOD, 2-16 µM ToMOC, 0.1 µM ToMOF, and saturating levels of toluene (6 mM).\(^7\) Reactions were initiated by addition of NADH to a final concentration of 0.2 mM. The absorbance change at 340 nm, corresponding to conversion of NADH to NAD', was monitored as a function of time. The temperature was held at either 10 or 37 °C using a circulating water bath. The reaction cuvette was held in a thermostated compartment attached to a circulating water bath. The rate of NADH consumption was calculated by fitting the initial absorbance change at 340 nm to a linear function. The negative slope of this line was divided by both the extinction coefficient of NADH (6220 M\(^{-1}\)cm\(^{-1}\)) and the concentration of ToMOH diiron sites (4 µM) to yield the NADH consumption per diiron active site. The resulting values were plotted as a function of ToMOD concentration. Fits to the data were examined for a standard Michaelis-Menten model (eq 3.1), a cooperative model (eq 3.2), and a competitive model (eq 3.3). In all cases, ToMOD was treated as a substrate, a common method for evaluating promoter proteins.\(^8\) The competitive model (eq 3.3) was clearly best as indicated by the lowest adjusted R\(^2\), lowest error of the fitted parameters, and residual values closest to zero.

\[
\frac{v}{[\text{ToMOH diiron sites}]} = \frac{k_{\text{cat}}[\text{ToMOD}]}{K_m + [\text{ToMOD}]} \quad (3.1)
\]

\[
\frac{v}{[\text{ToMOH diiron sites}]} = \frac{k_{\text{cat}}}{1 + \left(\frac{K}{[\text{ToMOD}]}\right)^2} \quad (3.2)
\]

\[
\frac{v}{[\text{ToMOH diiron sites}]} = \frac{k_{\text{cat}}}{1 + \left(\frac{K_m}{[\text{ToMOD}]} + \frac{[\text{ToMOD}]}{K_i}\right)} \quad (3.3)
\]
Colorimetric Reductive Titrations. Anaerobic titrations were performed in sealed cuvettes in 50 mM potassium phosphate, 50 mM NaCl, pH 7.0 at 25 °C. In a nitrogen filled chamber, solutions of 40-50 μM ToMOC and either 40 μM anthraquinone-1,5-sulfonic acid (E_{m}^{o} = -175 mV vs. NHE),\(^9\) 100 μM 2-hydroxy-1,4-napthoquinone (E_{m}^{o} = -137 mV vs. NHE),\(^20\) or 17.5 μM indigo carmine (E_{m}^{o} = -125 mV vs. NHE)\(^21\) were prepared. Each solution was sealed in a quartz cuvette with a screw cap equipped with a rubber septum. A buffered dithionite solution was loaded into a gastight Hamilton syringe with a repeating dispenser. The syringe and cuvette were removed from the anaerobic chamber, and each ToMOC-dye solution was titrated with the buffered solution of dithionite. An HP diode array spectrometer was used to monitor the UV-visible spectrum of the sample throughout the course of the titration. The solution was allowed to reach equilibrium, which was achieved when no further absorbance change was observed (5-30 min). The equilibrium absorbance spectrum for each titration point was fit to a linear combination of oxidized and reduced absorbance spectra of ToMOC and the dye (eq 3.4). The reduction potential of ToMOC was determined from a modified Nernst equation (eq 3.5),\(^22\) where E_{dye}^{o} and E_{ToMOC}^{o} are the midpoint potentials of the dye and ToMOC, respectively; n_{dye} and n_{ToMOC} are the number of electrons that the components can acquire upon reduction; \(F\) is Faraday’s constant; and \(E\) is the equilibrium midpoint potential of the solution.

\[
y = a \ast \text{Abs}_{\text{ToMOC,ox}} + b \ast \text{Abs}_{\text{ToMOC,red}} + c \ast \text{Abs}_{\text{dye,ox}} + d \ast \text{Abs}_{\text{dye,red}}
\]

\[
E = E_{dye}^{o} - \frac{RT}{n_{dye}F} \ln \left( \frac{\text{dye,ox}}{\text{dye,red}} \right) = E_{ToMOC}^{o} - \frac{RT}{n_{ToMOC}F} \ln \left( \frac{\text{ToMOC,ox}}{\text{ToMOC,red}} \right)
\]

Stopped-Flow Kinetics and Analysis for Single-Wavelength Measurements. Single-wavelength kinetic data were obtained using a Hi-Tech Scientific (Salisbury, UK) SF-61 DX2 stopped-flow spectrophotometer equipped with a photomultiplier tube and a tungsten lamp. To remove oxygen from the reaction lines, the stopped-flow was scrubbed overnight with anaerobic buffer containing ~5 mM sodium dithionite. Immediately prior to each experiment, the stopped-flow lines were washed with dithionite-free, anaerobic buffer to remove any excess dithionite. Reactions were carried out in 50 mM potassium phosphate, 50 mM NaCl at pH 7.0 or pD 7.0. The pD was calculated by adding 0.4 to the value reported by the pH meter. The temperature was
held constant using a circulating water bath. Temperatures for all stopped-flow reactions were 13 °C, except where noted otherwise. Protein samples were made anaerobic by cycling between vacuum and argon using a Schlenk line. The anaerobic protein samples were brought into a chamber filled with nitrogen atmosphere for handling prior to stopped-flow analysis. In the nitrogen chamber, the two reactants were loaded into separate Hamilton Sample-Lock syringes equipped with a male luer adapter. The syringes were sealed and removed from the nitrogen atmosphere for loading onto the pre-washed stopped-flow. Protein concentrations listed for stopped-flow experiments are concentrations after rapid mixing of the reaction components. Three or more individual traces were averaged to obtain the final traces shown here. The data were analyzed using OriginLabs 9.0 or 9.1. Single and double exponential fits were compared as models for each of the averaged traces.

For reactions between reduced ToMOC (ToMOC\textsubscript{red}) and oxidized ToMOH (ToMOH\textsubscript{ox}), ToMOC\textsubscript{red} was prepared by titrating ToMOC\textsubscript{ox} with a buffered solution of dithionite under nitrogen atmosphere. When the regulatory protein was included, ToMOD was added to either the syringe containing ToMOH\textsubscript{ox} or ToMOC\textsubscript{red}. Using the stopped-flow, the absorbance change at 458 nm was monitored for each reaction condition, the wavelength having the greatest difference in molar extinction coefficient for the oxidized and reduced forms of ToMOC. Single exponential fits were adequate to fit all experimental traces. In some cases, double exponential fits resulted in lower adjusted R\textsuperscript{2} values. However, in all cases, double exponential fits also produced greater errors of the fitted parameters. The error for double exponential fits was greater than 10x the output parameter. For this reason, double exponential fits were not further considered.

**Stopped-Flow Kinetics and Analysis for Multi-Wavelength Measurements.** Multi-wavelength kinetic data were obtained using a Hi-Tech Scientific (Salisbury, UK) SF-61 DX2 stopped-flow spectrophotometer equipped with a diode array detector and a xenon lamp. Anaerobic preparation of the stopped-flow was the same as indicated for single-wavelength measurements (above). Reactions were carried out in 50 mM potassium phosphate, 50 mM sodium chloride at pH 7.0 at 13 °C. Under a nitrogen atmosphere, reduced ToMOF (ToMOF\textsubscript{red}) was prepared by adding 1.5 equiv of NADH. Oxidized ToMOC (ToMOC\textsubscript{ox}) and ToMOF\textsubscript{red} were loaded into separate Hamilton Sample-Lock syringes equipped with a male luer lock.
Absorbance changes between 380 nm and 750 nm were monitored as a function of time. The reaction was very fast (complete in 15 ms), such only the first three data points displayed a change in absorbance. Owing to the speed of the reaction, the absorbance traces are shown and discussed but were not fit to a kinetic model.

**Cross-linking Experiments.** A solution of 120 μM ToMOC or ToMOF and 60 μM ToMOH was prepared in 25 mM MOPS buffer at pH 7.0. For reactions containing the regulatory protein, ToMOD was added to a final concentration of 120 μM. The zero-atom cross-linking agent EDC was added to the protein solution to a final concentration of 450 mM. The reaction was kept on ice for 1 h. An equal volume of 1 M sodium acetate and 25 mM MOPS at pH 6.8 was added after 1 h to quench the reaction. The products were loaded onto a Sephadex S200 column (1.6 cm x 60 cm). The protein was eluted with 25 mM MOPS, pH 7.0, 10% glycerol (v/v), and 150 mM NaCl. Fractions of 3 ml were collected throughout and were analyzed by UV-visible spectroscopy. The absorbance intensities at 280 and 458 nm were plotted as a function of elution volume.

**Protein Docking Studies.** The docking models were generated with ClusPro. The model required removal of the iron atoms from all structures because the docking software does not have parameters to optimize iron interactions. The input PDB file for the Rieske protein was 1VM9 after removal of the 2Fe-2S cluster and addition of hydrogen atoms. The input PDB file for the hydroxylase bound to two equivalents of the regulatory protein was 3DHH after removal of the diiron cluster and addition of hydrogen atoms. The input PDB file for the hydroxylase bound to one equivalent of the regulatory protein complex was 3DHH after removal of one molecule of the regulatory protein and the diiron cluster. Hydrogen atoms were also added. The docking model is a rigid-body model; thus, removal of the iron site did not change the overall fold of the proteins. The diiron site of 3DHH is buried 12 Å below the surface of the protein, such that removal of the active site does not alter the protein surface for docking studies. The [2Fe-2S] cluster of the ET protein, however, is at the surface of the Rieske protein, thus we are careful here not to over interpret specific contacts in the binding model.
Results and Discussion

Steady-State Inhibition by ToMOD Depends Upon ToMOC Concentration. To assess the effects of ToMOD on catalysis, we performed steady-state reactions with varying concentrations of ToMOD. The regulatory component of three-component BMMs exhibit temperature-dependent effects on steady-state turnover. Influenced by these data with three-component BMMs, we assessed activity at both 10 and 37 °C (Figure 3.1).

![Figure 3.1](chart.png)

**Figure 3.1.** Steady state activity as a function of ToMOD concentration at 10 °C (open circles) and 37 °C (filled squares). Reactions containing 2 μM ToMOH, 4 μM ToMOC, 0.5-120 μM ToMOD, 0.1 μM ToMOF, and saturating amounts of toluene (~6 mM) were initiated by addition of 0.2 mM NADH. The rate of NADH consumption as a function of ToMOD concentration fit well to a ToMOD inhibition model. The vertical, dashed line in the figure demarcates a 1:1 ratio of ToMOD to ToMOH diiron sites.

At substoichiometric concentrations of ToMOD, the rate of NADH consumption increases with increasing ToMOD concentration at both 10 and 37 °C. Under these conditions, ToMOD acts as a promoter protein. X-ray crystallography indicates that promotion by ToMOD arises through conformational changes exerted on ToMOH upon binding of ToMOD. These conformational changes within the hydroxylase enable dioxygen access to the diiron active site and subsequent oxygen activation. At high concentrations of ToMOD, however, we
observed an inhibitory effect, \( K_{i, \text{ToMOD}} \). Importantly, ToMOD is much more effective at inhibiting the reaction at 37 °C vs. 10 °C.

Similar steady-state experiments were performed with varying concentrations of ToMOC, and the results demonstrate that \( K_{i, \text{ToMOD}} \) is dependent on ToMOC concentration (Figure 3.2 and Table 3.1). Upon changing the concentration of ToMOC, there are significant shifts in the apparent \( k_{cat} \), \( K_m, \text{ToMOD} \), and \( K_{i, \text{ToMOD}} \) values as numerically shown in Table 3.1. The most dramatic change is in the \( K_{i, \text{ToMOD}} \), which is much weaker at high concentrations of ToMOC. The dependence of \( K_{i, \text{ToMOD}} \) on ToMOC concentration demonstrates that the inhibitory function is linked to the action of ToMOC.

**Figure 3.2.** Steady state activity under various concentrations of ToMOC graphed as a function of ToMOD concentration. At 37 °C, the rate of NADH consumption as a function of ToMOD concentration was determined for varying concentrations of ToMOC, 4 (black squares), 8 (red circles), 10 (blue triangles), and 16 (pink inverted-triangles) µM. Each reaction contained 2 µM ToMOH, 0.1 µM ToMOF, saturating toluene, and 0.2 mM NADH.

\[ \text{ToMOD : ToMOH diiron sites} \]

\[ \text{ToMOD concentration (mM)} \]

\[ \text{NADH consumption/diiron site (s^{-1})} \]

\[ \text{ToMOD concentration (µM)} \]

\[ 0 \quad 20 \quad 40 \quad 60 \quad 80 \]

\[ 0.6 \quad 0.8 \quad 1.0 \quad 1.2 \quad 1.4 \quad 1.6 \quad 1.8 \quad 2.0 \quad 2.2 \]

\[ \text{ToMOC to diiron sites in ToMOH is 1:1 for the data in Figure 1. At this ratio of ToMOC to ToMOH, the maximum NADH consumption occurs at a ratio of one-to-one ToMOD to ToMOH diiron sites, which was the first steady-state indication of binding competition or allostery.} \]
X-ray crystallographic data indicate that binding of the T4MO regulatory protein to its hydroxylase covers the entrance to the aromatic substrate channel and shrinks its diameter.\textsuperscript{11,12} Attenuation of substrate access may therefore also contribute to inhibition at the higher ToMOD concentrations. To assess this possibility directly, steady-state experiments with varying concentrations of ToMOD were performed at 0.75 and 5 mM toluene concentrations (Figure 3.3).

**Table 3.1. Steady state parameters as a function of ToMOC concentration**

<table>
<thead>
<tr>
<th>ToMOC (µM)</th>
<th>( k_{cat} ) (s(^{-1}))</th>
<th>( K_{m,ToMOD} ) (µM)</th>
<th>( K_{i,ToMOD} ) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>1.88(5)</td>
<td>0.57(6)</td>
<td>44(4)</td>
</tr>
<tr>
<td>6</td>
<td>2.00(5)</td>
<td>0.75(8)</td>
<td>60(5)</td>
</tr>
<tr>
<td>10</td>
<td>2.5(2)</td>
<td>0.8(1)</td>
<td>110(30)</td>
</tr>
<tr>
<td>16</td>
<td>2.34(6)</td>
<td>0.87(9)</td>
<td>330(50)</td>
</tr>
</tbody>
</table>

**Figure 3.3.** Turnover as a function of toluene and ToMOD concentrations. At 37 °C, the rate of NADH consumption as a function of ToMOD concentration was determined at 0.75 mM (black squares) and 5.0 mM (open circles) toluene. Each reaction contained 2 µM ToMOH, 2 µM ToMOC, 0.1 µM ToMOF, toluene, and 0.2 mM NADH.

The \( k_{cat,ToMOD} \) increases from 2.1(1) s\(^{-1}\) at the 0.75 mM toluene concentration to 2.7(4) s\(^{-1}\) at 5 mM toluene. In contrast, \( K_{i,ToMOD} \) is unaltered, yielding values of 14(2) µM and 17(5) µM for
0.75 and 5 mM toluene, respectively. Thus, covering of the aromatic substrate channel by ToMOD does not affect \( K_{i,\text{ToMOD}} \) under the present conditions. Instead, this inhibition constant depends directly on ToMOC concentration. The data provide further support for the model involving direct competition between ToMOC and ToMOD for binding to the hydroxylase, either at the same site by one protein triggering allosteric changes that alter the conformation of ToMOH and affect the binding of the other protein.

The site of the interaction between BMM regulatory proteins and their respective hydroxylases are well documented.\(^{11,12,27,28}\) The regulatory proteins bind to the hydroxylases in a region originally termed the ‘canyon’ for the first BMM hydroxylase characterized by X-ray crystallography, MMOH,\(^{29}\) the hydroxylase from soluble methane monooxygenase (sMMO). Subsequent X-ray crystallography demonstrated that the canyon region occurs in all members of the BMM family.\(^{11,15,28}\) The canyon is defined by a significant depression at the surface of these hydroxylases formed between the \( \alpha \) and \( \beta \) subunits. The shortest distance between the diiron active sites and the surface of the hydroxylases, \(~12\ \text{Å} \), exits at one of the canyon walls, the locale of regulatory protein binding.\(^{11,27,28}\) Although short distances are not required for biochemical ET,\(^{30-32}\) binding of ToMOC to ToMOH in the canyon would provide the most efficient distance for ET.\(^{33}\) If ToMOC were to bind in the canyon, which we consider to be quite likely for efficient ET, ToMOD would compete with ToMOC for binding to ToMOH.

**Electron Transfer.** To assess competition between ToMOD and ToMOC for binding to ToMOH, we evaluated intermolecular ET. Monitoring ET provides a direct assessment of inhibition without the need to unravel the complex kinetics of steady-state turnover involving four protein components and multiple substrates. In these investigations, we examined the thermodynamic and kinetic parameters of ET from ToMOC\(_{\text{red}}\) to ToMOH by exploiting redox-dependent absorbance features of ToMOC. Upon oxidation of ToMOC\(_{\text{red}}\), the absorbance at 458 nm increases two-fold (Figure 3.4), allowing for kinetic characterization of ET to and from ToMOC as well as facile redox determination by colorimetric methods.
Figure 3.4. Redox-dependent extinction coefficients of ToMOC. The extinction coefficients as a function of wavelength are shown for ToMOC\(_{\text{ox}}\) (solid, maroon) and ToMOC\(_{\text{red}}\) (dotted, grey).

The midpoint potential of ToMOC was determined by a colorimetric reductive titration to yield -130(30) mV vs. NHE. This midpoint potential is near that reported for T4moC (-173 mV vs. NHE)\(^3\)\(^4\) and lies between the potentials expected for the [2Fe-2S] cluster of ToMOF (-205 mV vs. NHE at 25 °C)\(^3\)\(^5\) and ToMOH (+48 mV vs. NHE at 4 °C).\(^5\) These results indicate that the Rieske cluster of ToMOC is thermodynamically suitable for shuttling electrons from the redox centers of ToMOF to the redox centers of ToMOH. ET from the reduced ToMOF (ToMOF\(_{\text{red}}\)) to ToMOC\(_{\text{ox}}\) is rapid (complete in less than 15 s) and much faster than steady-state turnover (Figure 3.5).

Figure 3.5. ET from ToMOF\(_{\text{red}}\) to ToMOC\(_{\text{ox}}\). The absorbance traces for the reaction between 12.5 \(\mu\)M ToMOF\(_{\text{red}}\) and 25 \(\mu\)M ToMOC\(_{\text{ox}}\) at pH 7.0 and 13 °C. The first three time points at 0 (solid, red line), 1 (dashed, orange line), and 16 (dotted, gold line) ms are shown. Longer time points, 46 and 300 ms, are shown to demonstrate the completeness of the reaction.
Owing to the rapidity of electron transfer from ToMOFr to ToMOCr, we were not able to fit the kinetic traces with any degree of confidence. Despite limitation with respect to kinetic quantitation, these results demonstrate that ET from ToMOFr to ToMOCR is rapid even in the absence of ToMOH, indicating that ET between ToMOF and ToMOC is not rate limiting within the ET pathway and a ternary complex between ToMOF, ToMOC, and ToMOH is not necessary for ET from ToMOF to ToMOC.

We next assessed ET to ToMOHox from ToMOCR as monitored by stopped-flow UV-visible spectroscopy. The data were obtained using limiting concentrations of ToMOCR, such that only a single electron would be transferred to the hydroxylase to form one-electron reduced, mixed-valent ToMOH (ToMOHmv). Figure 3.6 shows the observed rate constants derived from single exponential fits of the absorbance change at 458 nm.

![Figure 3.6](image)

**Figure 3.6.** ET from ToMOCR to ToMOHox as a function of ToMOH concentration. The $k_{\text{obs}}$ for the reaction between 5 μM ToMOCR and varying concentrations of ToMOHox at pH 7.0 and 13 °C. The data fit well to a linear function with a slope of 0.42(5) μM$^{-1}$s$^{-1}$.

At excess ToMOH, the observed rate constant depends linearly upon the concentration of ToMOH, from which a second order rate constant of 0.42(5) μM$^{-1}$s$^{-1}$ could be derived (Figure 3.6), consistent with a bimolecular reaction between the two proteins. The observed absorbance change corresponded to complete oxidation of ToMOCR. At concentrations of ToMOH ≥ 200 μM, the solution viscosity began to affect the instrument mixing time, and it was not possible to
obtain meaningful data in this range. Although these data are do not permit the evaluation of the true ET reaction, they suffice to evaluate the effects of ToMOD on this ET step.

**Effect of ToMOD on ET.** With the use of pre-steady-state single-mixing stopped-flow experiments, we examined the effect of increasing concentrations of ToMOD, pre-mixed with ToMOH\textsubscript{ox}, on electron transfer from ToMOC\textsubscript{red}. Under these conditions, the observed rate constant for ET decreased significantly as the concentration of ToMOD increased. Figure 3.7 shows the average absorbance change at increasing concentrations of ToMOD. A control experiment revealed that pre-incubation of ToMOD with ToMOC\textsubscript{red} did not change the results from those obtained in the absence of ToMOD (Figure 3.8). Thus, ToMOD slows the reaction between ToMOC\textsubscript{red} and ToMOH\textsubscript{ox} through its interaction with ToMOH.

![Graph showing absorbance change and rate constants](image)

**Figure 3.7.** ET from ToMOC\textsubscript{red} to ToMOH\textsubscript{ox} pre-incubated with ToMOD. The absorbance at 458 nm was monitored for the reaction between ToMOC\textsubscript{red} and ToMOH\textsubscript{ox} pre-incubated with 0 (black), 25 (green), 50 (orange), and 125 (violet) µM ToMOD. The absorbance change at 458 nm over time is plotted on a logarithmic scale (top), and the observed rate constants obtained from single exponentials fits of the data are shown as a function of ToMOD to ToMOH diiron sites (bottom).
An inhibitory effect upon ET could be a consequence of competitive binding, non-cooperative allosteric effects including conformational changes, or an alteration of the ToMOH redox potential in the presence of ToMOD. The last possibility is suggested by the finding that, for the three-component BMM sMMO, addition of the regulatory protein to the hydroxylase shifts the redox potential to a more negative value. Thus, alteration of the redox potential of ToMOH by ToMOD binding would not be unprecedented. Attenuation of the redox potential of ToMOH by ToMOD, however, would not give rise to a $K_{i,\text{ToMOD}}$ dependent upon ToMOC concentration as we report here based on steady-state data. Therefore, we propose that either ToMOD and ToMOC compete for binding to the same location on ToMOH or ToMOD enforces allosteric effects on ToMOH inhibiting ET from ToMOC$_{\text{red}}$ to ToMOH$_{\text{ox}}$ throughout turnover. To assess these possibilities binding interactions and protein docking models were explored.

**Binding Interactions.** To verify further that ToMOC and ToMOH interact and that this interaction is perturbed by ToMOD, we studied the binding of these proteins by use of the zero-atom cross-linking agent EDC. The cross-linking reaction was analyzed by gel chromatography to separate the products by size without denaturation. Absorbance measurements (Figure 3.9) were obtained for isolated fractions from the column flow-through. At 280 nm, the absorbance intensity is a function of ToMOH ($\varepsilon = 600,000$ M$^{-1}$cm$^{-1}$), ToMOC ($\varepsilon = 34,400$ M$^{-1}$cm$^{-1}$), and ToMOD ($\varepsilon = 2860$ M$^{-1}$cm$^{-1}$). Because only ToMOC absorbs at 458 nm ($\varepsilon = 6870$ M$^{-1}$cm$^{-1}$), the...
absorbance at 458 nm was used as a way to determine the ToMOC retention time. The elution times for ToMOC and ToMOH alone are 80 and 60 ml, respectively (Figure 3.9, black traces). When ToMOC and ToMOH were incubated with EDC, the elution point of ToMOC shifted to 55 ml, overlapping with the peak of ToMOH alone (Figure 3.9, red traces). The reduction of retention time demonstrates that cross-linking of the two proteins increases the hydrodynamic radius of both ToMOH and ToMOC, showing that the two proteins bind in solution. In the presence of ToMOD, the absorption intensity at 428 nm was much stronger in the low MW fraction than the high MW fraction. This result indicates that cross-linking of ToMOC and ToMOH is less efficient in the presence of ToMOD (Figure 3.9, purple traces), consistent with competition between ToMOD and ToMOC for a binding site on ToMOH. This result, however, cannot distinguish between allosteric vs. direct competition for such a location.

![Figure 3.9](image)

**Figure 3.9.** Cross-linking of ToMOC and ToMOH in the presence and absence of ToMOD. Absorbance at 458 nm (top) and 280 nm (bottom) as a function of elution volume are shown. Elution of ToMOC alone is shown with a solid black line; elution of ToMOH alone is shown with a dotted black line. Elution of the reaction of ToMOC, ToMOH, and the cross-linking agent EDC is shown as a red trace with red dots. Elution of the reaction of ToMOC, ToMOH, ToMOD, and the cross-linking agent EDC is shown as a purple trace with open squares. Absorbance measurements at 458 nm were performed for each fraction, whereas absorbance measurements at 280 nm were an output from the Ákta FPLC system.
ToMOF, on the other hand, did not cross-link with ToMOH under the same reaction conditions (Figure 3.10). This result demonstrates that ToMOF would be incapable of transferring electrons to ToMOH because the two proteins cannot bind one another, a conclusion that is supported by the fact that there is no steady-state activity in the ToMO system in the absence of ToMOC.9

![Figure 3.10](image)

**Figure 3.10.** Cross-linking between ToMOF and ToMOH. Comparison between the absorbance traces at 280 nm and 458 nm indicate that ToMOF, which absorbs at both 280 and 458 nm, elutes separately from ToMOH, which has negligible absorbance at 458 nm.

Finally, to distinguish between direct competition vs. indirect allosteric inhibition, we used a computational approach involving an automated protein-docking program.23–25 Guided by X-ray crystal structure information,11,15,26,36 we examined binding between T4moC and either ToMOH, T4moH, or the T4moHD complex.5 The T4MO proteins used for this docking model have high homology with ToMO, display similar chemistry,9,37 and have known structures from

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5 The Rieske, regulatory, and hydroxylase proteins of T4MO are designated T4moC, T4moD, and T4moH, respectively. The complex of T4moH with two regulatory proteins is termed T4moHD.
X-ray crystallography. When T4moC was docked to the hydroxylase alone, the preferred binding site was conclusively at the canyon regions of the proteins (Figure 3.11A).

![Figure 3.11](image)

**Figure 3.11.** Binding predictions for T4moC to ToMO and the complex of T4moH with the regulatory protein. Shown above are representative models for the predicted binding interactions between T4moC and (A) ToMOH, (B) the complex of two equivalents of the regulatory protein and the hydroxylase of T4MOH, and (C) the complex of one equivalent of the regulatory protein and the hydroxylase of T4MO. The hydroxylases are represented in surface mode with the two halves of the dimer distinguished in yellow and grey. T4moC and the regulatory protein are shown in red and green, respectively.

This binding mode was similar for all ten of the lowest energy predictions (Chart 3.1). When the T4moHD complex was used, the best predicted T4moC binding site was nonsensical for efficient ET (Figure 3.11B), being far removed from the hydroxylase diiron center, and not conserved among the ten lowest energy structures provided by the docking model (Chart 3.1). Upon removal of one of the two regulatory proteins from the hydroxylase complex, T4moC bound to the canyon region on the other side of T4moH (Figure 3.11C). In this case, the ten lowest energy structures all revealed binding of the T4moC in a canyon region of the hydroxylase (Chart 3.1). Use of the docking program necessitated removal of the [2Fe-2S] Rieske cluster, which limits our ability to compute a distance for ET. The model nonetheless is consistent with a competitive binding model supported by both ET and steady-state turnover.
Chart 3.1. The ten lowest energy structures predicted from docking of the Rieske protein (red) to the hydroxylase (grey) or complexes of the hydroxylase and regulatory protein (green).
Comparison of Three- and Four-Component BMMs – Conserved Control of ET. The BMM regulatory proteins have been shown to (i) couple NADH consumption with hydrocarbon oxidation,\(^3\) (ii) gate hydrocarbon substrate\(^1\) and dioxygen\(^1\) access to the diiron active sites, and (iii) alter of the redox potential of the catalytic diiron active site.\(^3\)\(^3\)\(^9\) Competition between the reductase and regulatory protein of the three-component BMM, sMMO, has been described in detail.\(^3\)\(^4\)\(^4\) Recently, studies of soluble butane monooxygenase (sBMO) also reveal a dual promotion and inhibition effect of the regulatory protein on turnover.\(^4\) Because the Rieske protein of the four-component BMMs is much smaller than the NADH oxidoreductase of three-component BMMs, the Rieske protein might bind to the hydroxylase in a ternary complex with the regulatory as was previously proposed.\(^10\) The present results, however, indicate that competitive binding between the electron-transfer partner of the hydroxylase and the regulatory proteins is retained even in four-component BMMs. Additional evidence for competitive binding is available (see footnote 1),\(^4\)\(^2\) which agrees with our conclusions. Conservation of this competition in both three and four-component BMMs reveals that nature has preserved this mechanism despite radically altering the ET partners, suggesting that this feature of catalysis is indispensible. Competitive binding between the ET and regulatory proteins provides a mechanism for controlling unwanted ET during dioxygen activation protecting the oxygen-activated intermediates from unwanted reduction.

References


Structure of Xenon-Pressurized Phenol Hydroxylase from *Pseudomonas sp.* OX1. *Biochemistry* 50, 11058-11069.


Chapter 4

Oxygen Activation by the Hydroxylase of Toluene/o-Xylene Monooxygenase in the Presence of its Redox Partners
Introduction

Oxygen activation by non-heme diiron enzymes generates reactive intermediates capable of hydrocarbon hydroxylation, epoxidation, desaturation, radical formation, and N-oxygenation.¹ The protein environments surrounding the diiron active sites of these enzymes control reactivity and can dictate the types of oxygenated intermediates formed during single turnover conditions. Because structurally similar active sites catalyze diverse chemical transformations, the mechanisms by which these diiron enzymes control reactivity are of particular interest.

Toluene/o-xylene monooxygenase (ToMO) belongs to such a family of carboxylate-rich diiron proteins termed bacterial multicomponent monooxygenases (BMMs), which activate O₂ to oxidize hydrocarbon substrates.²³ ToMO catalyzes the hydroxylation of toluene to form o-, m-, and p-cresol, and can also catalyze the regiospecific hydroxylation of phenol to form catechol.⁴ Four protein components are required to carry out efficient catalysis, a dimeric 210-kDa hydroxylase (ToMOH),⁵ a 12-kDa regulatory protein (ToMOD), a 38-kDa reductase (ToMOF), and a 12-kDa Rieske protein (ToMOC).² Previous oxygen-activation studies with ToMO utilized a simplified protein system comprising ToMOH, ToMOD, dithionite, and methyl viologen,⁵⁻⁹ where dithionite and methyl viologen serve as the electron donor and the electron-transfer agents, respectively. Under these conditions, a colorless intermediate (ToMOHₚerox⁻) having Mössbauer parameters δ = 0.54 mm/s and ΔE_Q = 0.67 mm/s is generated.⁵ This intermediate was proposed to have a μ-1,1-hydroperoxo structure, in which the peroxodiiron(III) charge-transfer band is quenched by protonation of the pendant oxygen atom.⁸⁻⁹ ToMOHₚerox⁻ differs from all four oxygenated intermediates generated in the BMM, soluble methane monooxygenase (sMMO), each of which has broad absorbance features at 420 or 720 nm corresponding to Fe-O charge-transfer bands.¹⁰⁻¹²

¹ The concentrations of ToMOH reported refer to the dimer; the concentration of the diiron active sites being twice that value. Sometimes, both the hydroxylase and diiron concentrations are noted for clarity.
Unlike sMMO, ToMO requires a Rieske protein, ToMOC, which binds to the hydroxylase and delivers electrons to the diiron active site during turnover.\(^2\) We were interested to determine whether oxygen activation and substrate hydroxylation by ToMO may be different when carried out in the presence of the natural reduction system, which includes ToMOC, ToMOF, and NADH. Here we report the results of single turnover studies of ToMO$_{\text{red}}$ (ToMOH, ToMOD, ToMOC, ToMOF, and NADH) with O$_2$-saturated buffer and arene substrate.

Results and Discussion

Rapid chemical-quench (RCQ) experiments were performed to monitor the rate of hydroxylation of phenol under single turnover conditions. The conversion of phenol to catechol by ToMO$_{\text{red}}$ was measured at two concentrations of ToMOC, corresponding to 0.01 or 1 equivalents of ToMOC per diiron active sites (Figure 4.1). Both high and low concentrations were selected to determine if ToMOC concentration affects single turnover yields. At the lower concentration of ToMOC, approximately 36(3)% of the diiron sites form catechol by 2 s. After 10 min, approximately 84(4)% of the diiron sites produce catechol. The rate of catechol production is biphasic with rate constants 8(1) s$^{-1}$ and 0.0090(9) s$^{-1}$, respectively. In contrast, at 1 equivalent of ToMOC per the diiron active site, approximately 70(4)% of the diiron sites produce catechol by 3 s. After 10 min, approximately 83(6)% of the diiron sites produce catechol.

These results suggest that two phases of product formation occur, a fast phase and a slow phase. Using low concentrations of ToMOC, only 36% of the hydroxylation occurs in the fast phase. At high concentrations of ToMOC, the percent of product formed in the fast phase increases significantly. Therefore, ToMOC must promote hydroxylation chemistry by increasing the amount of product formed in the fast phase. Importantly, the rates of each phase are not significantly affected by increasing the ToMOC concentration. This suggests that ToMOC does not directly participate in hydroxylation chemistry, but may induce a more active hydroxylase.
Figure 4.1. Hydroxylation of phenol to catechol at 4 °C during single turnover by ToMO. The concentrations of each component are as follows: 50 μM ToMOH (100 μM diiron sites), 100 μM ToMOD, 1 μM (▲) or 100 μM (○) ToMOC, 0.05 μM ToMOF, 90 μM NADH, approximately 625 μM O₂, 2 mM phenol.

Because RCQ experiments precipitate the protein and release any product bound at the active site, these experiments indicate that ToMOC is critical for steps prior to product release. Steps leading to hydroxylation include reduction, conformational changes, oxygen activation, aromatic substrate binding, and direct hydroxylation chemistry. Reduction of the hydroxylase can be achieved by even substoichiometric equivalents of the Rieske protein, which indicates that one of the other four mechanisms must be responsible for the observed activity.

To further evaluate the role of ToMOC, we studied the oxygen-activation chemistry by stopped-flow UV-visible and Mössbauer spectroscopies. Rapid UV-visible spectroscopy measurements reported here were conducted with 0.01 equivalents of ToMOC per diiron active site. Both ToMOC and ToMOF absorb strongly in the visible range, and their extinction coefficients depend on oxidation states of their internal chromophores. Minimal concentrations of ToMOC (1 μM) and ToMOF (0.05 μM) were,
therefore, used in these reactions, such that the combined absorbance changes for redox cycling were only 3 mAU at 420 nm and 0.25 mAU at 675 nm.

In the absence of hydrocarbon substrates, the reaction of ToMO$_{\text{red}}$ with O$_2$-saturated buffer** produces distinctive absorbance changes at 420 and 675 nm (Figure 4.2). The absorbance changes in this reaction are much larger than those possible for redox cycling of the ET proteins, ToMOC and ToMOF. Moreover, in the absence of either the hydroxylase or O$_2$, these absorbance features do not appear (Figure 4.2), revealing that the bands correspond to ToMOH- and O$_2$-dependent intermediates arising from O$_2$ activation at the diiron center.

Figure 4.2. Oxygen activation by ToMO$_{\text{red}}$ at 5 °C as measured by stopped-flow UV-visible spectroscopy. Absorbance changes at 420 (orange) and 675 (grey) nm upon rapidly mixing ToMO$_{\text{red}}$ with O$_2$-saturated buffer. Control experiments omitting either O$_2$ or ToMOH are shown in violet and blue, respectively. The component concentrations are 50 μM ToMOH (100 μM diiron sites), 100 μM ToMOD, 1 μM ToMOC, 0.05 μM ToMOF, 90 μM NADH, and approximately 625 μM O$_2$. Fits to the data are shown in black.

** O$_2$–saturated buffer contains approximately 1.25 mM O$_2$ at 5 °C. In these experiments, one-to-one mixing yields 625 μM O$_2$ in the final reaction.
The simplest interpretation of these kinetics invokes the accumulation of four species: 675A ($t_{\text{max}} \approx 2 \text{ ms}$), 420A ($t_{\text{max}} \approx 200 \text{ ms}$), 420B ($t_{\text{max}} \approx 70 \text{ s}$), and $\text{H}_\text{ox}$. Derived rate constants for formation and decay of these species are shown in Table 4.1. Varying the concentration of $\text{O}_2$ reveals that these kinetics are independent of oxygen concentration between 375 – 625 $\mu$M $\text{O}_2$ (Table 4.1 and Figure 4.3).

<table>
<thead>
<tr>
<th>[O₂] ($\mu$M)</th>
<th>675A Decay (s⁻¹)</th>
<th>420A Formation (s⁻¹)</th>
<th>420B Formation (s⁻¹)</th>
<th>$\text{H}_\text{ox}$ Formation (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>625</td>
<td>40(5)</td>
<td>23(1)</td>
<td>0.023(2)</td>
<td>0.0018(2)</td>
</tr>
<tr>
<td>500</td>
<td>34(2)</td>
<td>23(2)</td>
<td>0.027(2)</td>
<td>0.0017(3)</td>
</tr>
<tr>
<td>375</td>
<td>39(2)</td>
<td>25(1)</td>
<td>0.026(1)</td>
<td>0.0025(2)</td>
</tr>
</tbody>
</table>

Figure 4.3. $\text{O}_2$-dependence of the reaction between ToMO$_\text{red}$ and oxygenated-buffer at 5 °C. Absorbance changes at 420 nm (left) and 675 nm (right) upon reaction of ToMO$_\text{red}$ with 375 $\mu$M (red), 500 $\mu$M (purple), and 675 $\mu$M (blue) $\text{O}_2$. The concentrations of all other component are the same as those reported in Figure 4.2.

Because formation of these intermediates is zero-order with respect to $\text{O}_2$ concentration, the interaction of ToMOH with $\text{O}_2$ must be irreversible and faster than the
decay of 675A. Therefore, 675A represents an oxygenated intermediate rather than a simple diiron(II) state. We note that 675A could be a direct diiron-O₂ adduct or a Michaelis-type complex between O₂ and ToMOH. In the latter case, O₂ is not directly coordinated to the diiron center but interacts with the hydroxylase near the active site. Michaelis-type complexes between O₂ and a BMM hydroxylase are reported for intermediates O and P* of sMMO from Methylosinus trichosporium (Mt-sMMO).

Extensive kinetic analyses indicate that Michaelis-type complexes are not formed in Mc-sMMO.

The maximum accumulation of 675A occurs before the mixing time of the instrument, 2 ms, which limits the ability to fully characterize this species. The decay of 675A is slightly faster than the formation of 420A, even accounting for experimental error, suggesting the possibility of a colorless intermediate, possibly similar to ToMOH_peroxid.

Intermediate 420A accumulates with a rate constant of 23 s⁻¹, similar to that observed for ToMOH_peroxid (~26 s⁻¹), the colorless intermediate previously reported when dithionite and methyl viologen were used as the diiron reductant. Much like ToMOH_peroxid, 420A is stable over a long period of time (> 6 s). Because of its long lifetime, we initially questioned whether this intermediate is a true oxygen-derived species. Long-lived, diiron-O₂ adducts are not rare, however. A peroxo species formed by human deoxyhypusine hydroxylase decays slowly over approximately 3 h in the presence of dithionite; a variant of sMMO increases the longevity of intermediate P* on the order of seconds; and even the high-valent diiron(IV) intermediate, Q, of sMMO is stable in the absence of substrate for > 5 s.

Generation of the final intermediate, 420B, is significantly slower than steady-state turnover (toluene hydroxylation, 0.5 s⁻¹), suggesting that it represents a decay pathway not relevant to steady-state kinetics. The final species has negligible absorbance at 420 and 675 nm, consistent with the diiron(III) resting state, H₀.

Rapid freeze-quench methods were employed to analyze intermediates 420A and 420B by ⁵⁷Fe-Mössbauer spectroscopy (Figure 4.4). In these experiments, ⁵⁷Fe-enriched
ToMOH was used, but neither ToMOC nor ToMOF were enriched with $^{57}$Fe. The natural abundance of $^{57}$Fe is 2%$,^{16}$ therefore the spectra result exclusively from the iron atoms of ToMOH. Only 0.1 equivalents of ToMOC per the diiron sites of ToMOH were used in these experiments. Experiments with 1 equivalent of ToMOC per the diiron sites of ToMOH displayed parameters within error of those reported here.

![Mössbauer spectra](image)

**Figure 4.4.** $^{57}$Fe-Mössbauer spectra of samples freeze-quenched at 200 ms (top) and 70 s (bottom) in the reaction of ToMO$_{red}$ with O$_2$-saturated buffer. The data are shown in grey. The simulated spectra are shown for the sum of the two sites (purple), site 1 (blue), and site 2 (blue). The concentrations of each component are as follows: 250 $\mu$M $^{57}$Fe-ToMOH (500 $\mu$M diiron sites), 500 $\mu$M ToMOD, 5 $\mu$M ToMOC, 0.5 $\mu$M ToMOF, 500 $\mu$M NADH, and approximately 625 $\mu$M O$_2$.

At 200 ms, quantification of the iron oxidation states indicates that approximately 80% of the sites are iron(III) with Mössbauer parameters $\delta = 0.54$ mm/s and $\Delta E_Q = 0.67$ mm/s. These Mössbauer parameters are identical to those reported for ToMOH$_{peroxo}$.\textsuperscript{5} The
remaining 20% is iron(II) with Mössbauer parameters $\delta = 1.31$ mm/s and $\Delta E_Q = 2.88$ mm/s, corresponding to unreacted diiron(II).\textsuperscript{5,17} Previous oxygen activation experiments reported approximately 50% accumulation of ToMOH\textsubscript{peroxo}, which was attributed to half-sites reactivity with respect to the hydroxylase dimer.\textsuperscript{5,8} Our data, however, clearly exhibit greater than 50% reactivity. In the current work, the ratio of $O_2$ to the diiron active sites is 1.25, whereas in previous studies\textsuperscript{5,8} this ratio was near 0.6. The limiting concentration of $O_2$ in previous experiments is most likely responsible for the observation of 50% reactivity.

At 70 s, the Mössbauer spectra reveal the presence of only diiron(III). The sample fits well to a two-site model in which the 87% of the iron sites correspond to Mössbauer parameters $\delta = 0.52$ mm/s and $\Delta E_Q = 0.65$ mm/s. The remaining 13% of the iron sites have Mössbauer parameters $\delta = 0.45$ mm/s and $\Delta E_Q = 1.56$ mm/s. Therefore the increase in absorbance at 420 nm results from a change in only 13% of the iron atoms. This change may represent slow oxidation of the unreacted diiron(II) sites remaining at 0.4 s. We cannot identify the exact nature of this species, but we conclude that 420B represents only a small fraction of the iron sites in ToMOH.

Given the significant difference in the absorption profiles of 420A and ToMOH\textsubscript{peroxo}, these intermediates must be distinct. Differing hydrogen-bonding or protonation states of the peroxo species might attenuate absorption within the visible range but result in indistinguishable changes in the Mössbauer parameters.\textsuperscript{12} The latter of which are a function of the electron density around the iron nuclei.\textsuperscript{16} Simply changing the pH of the buffer did not quench the absorbance corresponding to 420A (Figure 4.5, left), indicating that excess protons alone cannot induce formation of ToMOH\textsubscript{peroxo} rather than 420A. Thus, conformational changes may also be required. The rate constants corresponding to the formation and decay of these intermediates are pH dependent (Figure 4.5, right).
Figure 4.5. pH dependence of oxygen activation by ToMO\textsubscript{red}. Left: The absorbance changes at 420 nm (top) and 675 nm (bottom) for the reaction of O\textsubscript{2} with ToMO\textsubscript{red}. Right: The rate constants for decay of 675A (top), formation of 420A (middle), and formation of H\textsubscript{ox} (bottom) are shown as a function of pH. The protein concentrations are as reported in Figure 4.2. Simulations of these data are shown for each rate constant based on equations described in Chapter 2.

Decay of 675A is promoted at high pH, whereas formation of 420A is optimal near neutral pH. Because pH changes in this reaction may alter protein-protein interactions or hydrogen bonding distal to the active site, it is not possible to assign the protic groups responsible for these pH dependences.

To assess which of the intermediates are competent for hydroxylation, the reaction between ToMO\textsubscript{red} and O\textsubscript{2} was monitored by stopped-flow UV-visible spectroscopy in the presence of toluene (Figure 4.6). The absorbance changes at 420 nm are significantly altered by the addition of toluene, but only minor changes are observed at 675 nm. The decay of the first intermediate, 675A, is unchanged in the presence of
substrate (Figure 4.6, bottom panel), indicating that this intermediate is not responsible for oxidizing aromatic substrates.

**Figure 4.6.** Oxygen activation by ToMO\textsubscript{red} in the presence of toluene. Absorbance changes at 420 nm (top) and 675 nm (bottom) upon reaction of ToMO\textsubscript{red} with O\textsubscript{2}\textsuperscript{-} saturated buffer in the presence of 0.2 mM toluene. The protein concentrations are as reported in Figure 4.2. Fits to the data are shown in black.

The absorbance changes at 420 nm are best described by three exponential phases. In the first phase, the absorbance increases at 420 nm with a rate constant similar to that of intermediate 420A. Because the rate of formation and maximum absorbance wavelength are similar, we assign this species to intermediate 420A. The decay of 420A occurs more rapidly in the presence of substrate (15 s\textsuperscript{-1}), consistent with 420A being responsible for substrate oxidation. Instead of directly forming H\textsubscript{ox}, 420A reacts to form a species with t\textsubscript{max} \sim 0.5 s (420-Ar), corresponding to toluene oxidation. Species 420-Ar is not observed in the absence of substrate and absorbs at both 420 and 675 nm. The formation rate constant of 420-Ar is similar to that observed for hydroxylation of phenol.
in RCQ experiments. Binding of phenolic derivatives to the diiron active site increases the absorbance at 420 and 675 nm (Figure 4.7).

**Figure 4.7.** Absorbance changes at 420 nm upon titration of 50 μM of oxidized ToMOH (100 μM ToMOH) with a solution of phenol or catechol. The solutions were buffered at pH 7.0 with 50 mM potassium phosphate, 50 mM NaCl at room temperature.

Based on these observations, we assign 420-Ar to a cresol-diiron adduct resulting from hydroxylation of toluene. In the final phase, the absorbance at 420 nm decays with a rate constant of 0.69 s⁻¹. This absorbance decrease may result from product release from the active site. This rate constant is similar to the rate of steady-state turnover (Chapter 2). Thus, release of product may be rate limiting for steady-state turnover, as previously proposed for the analogous BMM toluene 4-monooxygenase (T4MO) based on X-ray crystallographic data. Oxygen activation in the presence of phenol was also examined, but proved to be much more complex than that observed with toluene (Figure 4.8). The increased complexity in the presence of phenol probably results from formation of both diiron-phenol and diiron-catechol adducts during single turnover.
Figure 4.8. Oxygen activation by ToMO<sub>red</sub> in the presence of phenol at 5 °C. Absorbance changes at 420 nm upon rapidly mixing ToMO<sub>red</sub> with O<sub>2</sub>-saturated buffer in the presence of 5 mM phenol. The concentrations of each component are as indicated in Figure 4.2. Fits to the data are shown in black.

The difference in reactivity of the dithionite-methyl viologen reduced hydroxylase and ToMO<sub>red</sub> could result either from ToMOC-induced activation of 420A or dithionite-induced quenching of 420A. Because hydroxylation efficiency increases with ToMOC concentration, it is more probable that ToMOC induces formation of the activated intermediate 420A. In the X-ray crystal structure of the hydroxylase and Rieske protein complex for T4MO, residues E104 and E231 adopt different conformations than those reported in previous structures of the same BMM. Movement of E104 or E231, which participate in hydrogen bonding with water, peroxo, and residues within the secondary coordination sphere, may change the protonation state of the peroxodiiron species or a carboxylate ligand. Computational analysis of the peroxo intermediates formed in the diiron protein ribonucleotide reductase (RNR) indicates that the transition near 420 nm corresponds to LMCT from both the carboxylate ligands and the peroxo ligand to the diiron site. Thus, it is possible that the protonation of a carboxylate ligand or the peroxo ligand may be responsible for the observation of 420A.
Irrespective of mechanistic details, the work presented here demonstrates the importance of natural ToMO components, particularly ToMOC, for efficient hydroxylation and oxygen activation by ToMO. The use of the natural system yields 420A, a diiron-O$_2$ species with features in the visible range, never previously identified. Species 420A is sensitive to substrate concentration and decays to form a diiron-cresol adduct indicative of hydroxylation. A proposed scheme to account for the observed reactivity is given in Scheme 4.1.

Scheme 4.1. Proposed mechanism for oxygen activation by ToMO$_{red}$.

The difference in reactivity between the natural system and the dithionite/methyl viologen simulation is surprising, given that sMMO undergoes single turnover very well using the dithionite is used as the reductant.$^{23,24}$ For oxygen-activating diiron proteins, impaired reactivity upon replacement of a reductase with dithionite and methyl viologen is not unprecedented, however. In the case of stearoyl-acyl carrier protein desaturase, dithionite reduction also produces an inactive peroxo species.$^{25,26}$

The structure of 420A may correspond to any one of many conformations, including $\mu$-1,2-(hydro)peroxo, $\mu$-1,1-(hydro)peroxo, or $\mu$-$\eta^1:\eta^1$-peroxo species. Because 420A is long-lived and has Mössbauer properties similar to those observed and calculated for $\mu$-1,2-peroxodiiron intermediates,$^{12,22}$ we tentatively assign 420A as such a species.

Comparison between ToMO and sMMO. Comparison of reactivity between ToMO and sMMO provides insight into the differences in mechanism for these two proteins. In sMMO, a 38-kDa reductase analogous to ToMOF binds to the hydroxylase, directly
reducing the diiron sites. Under single turnover conditions, addition of the reductase modestly increases the product yield from 80 to 90%. The maximum effects of the reductase on product formation, however, are observed at only 0.1 equivalents of the reductase to the diiron sites of the hydroxylase. In contrast, here we find that much higher equivalents of ToMOC per ToMOH only yield 70% of the potential product. The chemical or physical changes responsible for this promotion of substrate hydroxylation activity are unknown for both sMMO and ToMO. Additional structural studies are needed to elucidate the effects of the reductase and ToMOC.

The intermediates observed in ToMO and sMMO from *Methylococcus capsulatus* (Bath) are distinct. The time-dependent absorbance changes corresponding to these intermediates are shown in Figure 4.9. Here, we compare the absorbance change at 675 nm for ToMO to the absorbance change at 720 nm for sMMO. Each of these wavelengths represents a local maximum for a very broad absorbance feature.

**Figure 4.9.** Comparison of oxygen activation by ToMO$_{red}$ (red) and Mc-sMMO (black). The top two panels show the stopped-flow UV-visible traces in the absence of substrate, and the bottom two panels show the stopped-flow UV-visible traces in the presence of substrate.
In the absence of substrate (Figure 4.9, top two panels), the formation of the oxygenated intermediates is much faster in ToMO than Mc-sMMO. Under steady-state turnover conditions, Mc-sMMO also oxidizes methane more than two-fold slower than ToMO oxidizes toluene. The intensity of the absorbance change at 420 nm is much greater for Mc-sMMO than for ToMO. In sMMO, the species with the highest extinction coefficient is a diamond-core diiron(IV) \((\epsilon_{420}\sim 7500-8415 \text{ M}^{-1}\text{cm}^{-1})\). This intermediate was not observed for ToMO, which is most likely the reason for the weaker absorbance changes. Unlike \(H_{\text{peroxo}}\) of sMMO, 420A does not exhibit strong absorbance above 600 nm. Quenching of this lower energy transition may result from the presence of a coordinating water molecule or opening of a bridging carboxylate ligand in 420A as compared to \(H_{\text{peroxo}}\).

**Conclusion**

Our results clearly demonstrate that ToMOC is essential for efficient single turnover. The effect of ToMOC must include chemistry subsequent to ET, possibly through inducing conformational changes in the hydroxylase. Using this knowledge, we were able to identify a previously unobserved species, 420A, during oxygen activation by the diiron protein ToMO. Unlike the previously observed intermediate of ToMO, 420A decays rapidly in the presence of substrate, indicating that this species is relevant both to single turnover and to catalysis. Species 420 contains a distinct chromophore from those apparent during oxygen activation by sMMO and mutant forms of ToMO. Towards understanding this activity, we have proposed chemical structures for 420A and a scheme for its formation and decay. To understand the molecular effects of ToMOC on catalysis, high-resolution structural data of the oxidized and reduced systems may be required.

**Experimental Methods**

**General Methods.** Expression and purification of ToMOH, ToMOD, and ToMOC were carried out as reported in Appendix A. Expression and purification of ToMOF was
conducted as previously reported. All kinetics experiments were buffered at pH 7.0 using 50 mM potassium phosphate and 50 mM sodium chloride at 5 °C.

**Anaerobic Sample and Instrument Preparation.** The protein samples were prepared in the 50 mM potassium phosphate, 50 mM sodium chloride, at pH 7.0. The samples were degassed on a Schlenk-line by cycling between vacuum and argon for a minimum of ten cycles. The samples were brought into an anaerobic chamber under a nitrogen atmosphere. Anaerobic buffer was prepared by purging a sealed vessel with argon for 1 h. The buffer was stirred in the anaerobic chamber for a minimum of 5 h before use. To obtain reduced hydroxylase samples, NADH was added to the reconstituted ToMO sample inside the anaerobic chamber.

The stopped-flow UV-visible apparatus was washed with buffer containing ~15 mM dithionite for a minimum of 3 h to remove oxygen from the system. Immediately before use, the stopped-flow lines were washed with anaerobic phosphate buffer without dithionite to remove excess dithionite from the lines.

**Stopped-Flow UV-visible Spectroscopy and Data Analysis.** Oxygen activation chemistry was monitored using a Hi-Tech Scientific (Salisbury, UK) SF-61 DX stopped-flow UV-visible spectrophotometer equipped with a photomultiplier tube. The protein samples were loaded into airtight Hamilton luer-lock syringes inside an anaerobic chamber. Oxygen-saturated buffer was prepared by bubbling pure O₂ through a sealed falcon tube of buffer; a needle outlet was used to relieve pressure. The protein and the buffer were loaded onto the stopped-flow sample-handling unit immediately after removal from the anaerobic chamber. The final component concentrations were as follows: 50 μM ToMOH (100 μM diiron sites), 100 μM ToMOD, 1 μM ToMOC, 0.05 μM ToMOF, and 90 μM NADH. A slightly limiting concentration of NADH is used to prevent multiple turnover. Two control experiments were also performed omitting either ToMOH or O₂. For assays containing substrate, either toluene or phenol was added to the oxygen-saturated buffer.
The absorbance was monitored at 420, 675 and 720 nm. The data were collected on a logarithmic timescale. At least three individual reactions were averaged to obtain standard deviations. Because the absorbance changes in the presence of toluene were very small, over 10 separate reactions were averaged to obtain reasonable signal-to-noise. The stopped-flow data were analyzed in OriginLabs 9.0 or 9.1. The averaged absorbance traces were fit with functions containing three or four exponents (eq. 4.1 and 4.2).

\[ y = y_0 + A_1e^{(k_1t)} + A_2e^{(k_2t)} + A_3e^{(k_3t)} \]  
(4.1)

\[ y = y_0 + A_1e^{(k_1t)} + A_2e^{(k_2t)} + A_3e^{(k_3t)} + A_4e^{(k_4t)} \]  
(4.2)

The best fit was determined by the following standards: Adj. R^2 near unity, non-oscillatory residuals, low error of the fitted parameters, and good overlap of the simulated curve with the experimental data.

**Isolation of \(^{57}\)Fe-reconstituted ToMOH.** \(^{56}\)Fe-ToMOH was prepared as reported in Appendix A. Purified \(^{56}\)Fe-ToMOH was buffer exchanged into 100 \(\mu\)M MOPS at pH 7. To chelate the \(^{56}\)Fe ions from ToMOH, a solution of 50 \(\mu\)M ToMOH, 0.5 mM 3,4-dihydroxybenzaldehyde, and 5 mM 8-hydroxyl-5-quinoline sulfonic acid was prepared. Upon addition of ToMOH, the solution changed from colorless to light red. The reaction was stored on ice for 4 h. The color of the reaction changed from red to green. A gel filtration column (PD10) was used to isolate ToMOH. The resulting apo-ToMOH was concentrated using a 50-kDa MWCO centrifugal device. The concentrated protein was colorless to very pale blue. The iron content was less than 0.5 Fe ions per ToMOH dimer as measured by the ferrozine assay.\(^{30,31}\)

A solution of 2 mM dithiothreitol (DTT), 200 \(\mu\)M \(^{57}\)Fe(SO\(_4\))\(_2\) was prepared in 100 \(\mu\)M MOPS at pH 7. The solution was placed under argon by cycling between vacuum and argon in a schlenk flask. Apo-ToMOH was added to the solution to a final concentration of 50 \(\mu\)M. In an argon atmosphere, the sample was incubated on ice for 2 h. The sample was exposed to air, and the protein was concentrated using a 50-kDa MWCO
centrifugal device. The final iron content was 3.8 Fe ions per ToMOH dimer as measured by the ferrozine assay. The specific activity was 1150 mU/mg as measured by the C2,3O-coupled assay.²

**RFQ-Mössbauer.** An anaerobic solution of reconstituted ToMO and NADH was prepared in 50 mM potassium phosphate, 50 mM NaCl at pH 7. The solution was sealed and removed from the anaerobic chamber. Using a rapid mixing apparatus from Update Instruments, the protein solution was rapidly mixed with O₂-saturated buffer. Each sample was rapidly quenched in a -130 °C isopentane bath. Samples corresponding to 40 ms, 200 ms, and 70 s were prepared. The final reactant concentrations were 250 μM ToMOH (500 μM diiron sites), 500 μM ToMOD, 5 μM ToMOC, 0.5 nM ToMOF, 490 μM NADH, and approximately 625 μM oxygen. The samples were packed into Mössbauer cells and stored in liquid nitrogen for a maximum of two weeks before data collection. Each sample was analyzed at 78 °C by Mössbauer spectroscopy. The zero field velocity is referenced to the centroid of a room-temperature spectrum of metallic iron foil. The data were fit by using the WMOSS software package (WEB Research). Single, double, and triple site models were compared for each spectrum. The best model was selected based on the Adj.-R² and the residual traces.

**RCQ.** An anaerobic solution of reconstituted ToMO and NADH was prepared. The solution was sealed and removed from the anaerobic chamber. The protein solution was rapidly mixed with oxygen-saturated buffer. For time points less than 5 s, a rapid chemical apparatus from Update Instruments was used to mix the two solutions. For reactions longer than 5 s, the two solutions were mixed using a pipette. The final reactant concentrations were 50 μM ToMOH, 100 μM ToMOD, 1 μM ToMOC, 10 nM ToMOF, 90 μM NADH, and approximately 625 μM oxygen. The reaction was rapidly quenched in 100 μl of 1 M trichloroacetic acid (TCA). The acidified solution was neutralized with 1 M Tris-base to pH 7. The pH of each sample was verified by spotting the solution on pH paper. To quantify the amount of catechol formed, catechol-2,3-dioxygenase (C2,3O)
was added to convert catechol to 2-hydroxymuconic semialdehyde ($\epsilon_{375\text{nm}} = 33,000$). The reactions were performed with three different batches of ToMOH.

References


Chapter 5

Oxygen Activation in the T201S Variant of Toluene/o-Xylene Monoxygenase
Introduction

The secondary coordination spheres of non-heme diiron proteins often provide control over catalytic function, substrate specificity, and enzymatic efficiency. In the diiron enzymes toluene monooxygenases (TMOs), a threonine residue near the diiron active site effects enzymatic function in the presence and absence of substrate. TMOs are multicomponent enzymes that catalyze the oxidation of arene substrates through activation of O₂. Mutation of the conserved threonine residue to a serine (T201S) alters the mechanism of oxygen activation and doubles the rate of steady-state turnover. This threonine residue participates in a hydrogen-bonding network that fluctuates upon binding of the regulatory protein, the Rieske protein, or substrate analogs to the hydroxylase (Figure 5.1).

Figure 5.1. The dynamic hydrogen-bonding network at the diiron active site of the TMO, toluene 4-monooxygenase (T4MO). The hydrogen-bonding network at the active site is shown for the diiron(III) hydroxylase-regulatory protein complex (A), the diiron(II) hydroxylase-regulatory protein complex (B), the diiron(III) hydroxylase-regulatory protein complex with phenol (C), and the hydroxylase-Rieske protein complex (D).
Oxygen activation chemistry in this enzyme subfamily is most thoroughly described for toluene/o-xylene monoxygenase (ToMO). ToMO catalyzes hydroxylation of a wide variety of aromatic substrates and exhibits high catalytic efficiency for hydroxylation of both toluene and phenol. ToMO contains four component proteins required for catalysis, a 210-kDa hydroxylase (ToMOH), a 12-kDa regulatory protein (ToMOD), a 38-kDa reductase (ToMOF), and a 12-kDa Rieske protein (ToMOC). ToMOH houses the diiron center and the strictly conserved threonine residue.

During oxygen activation, the hydrogen-bonding network involving T201 is proposed to stabilize O₂ intermediates formed at the diiron active site. In previous oxygen activation studies, a simplified ToMO system was used in which ToMOF and ToMOC were replaced by dithionite and methyl viologen. In these experiments, oxygen activation by mutant T201S of ToMOH differed from that of wild-type (WT) ToMOH. In light of results reported in Chapter 4, we re-examined oxygen activation by mutant T201S of ToMOH using the reduced, multicomponent system, which includes the T201S variant of ToMOH, ToMOD, ToMOC, ToMOF, and NADH (hereafter T201S-ToMOrd). Our results reveal that oxygen activation by T201S-ToMOrd differs from oxygen activation by T201S in the presence of ToMOD, dithionite, and methyl viologen. Moreover, oxygen activation by T201S-ToMOrd also differs from oxygen activation by ToMOred (Chapter 4). Here we report the single turnover kinetics for T201S-ToMOrd and compare these results with those obtained for ToMOred and systems using dithionite-methyl viologen reduction.

Results and Discussion

In the absence of hydrocarbon substrate, oxygen activation by T201S-ToMOrd was examined in the presence of saturating O₂ by stopped-flow UV-visible spectroscopy. Upon rapidly mixing T201S-ToMOrd with O₂-saturated buffer, the absorbance changes in the visible range were recorded as a function of time (Figure 5.2). Two broad absorbance features arise within this range with local maxima at 420 and 675 nm. An isosbestic point occurs at 500 nm. The absorbance changes at 420 and 675 nm as a function of time are also shown in Figure 5.2.
Figure 5.2. Oxygen activation by T201S-ToMO_red as measured by stopped-flow UV-visible spectroscopy. Left: The absorbance changes as a function wavelength and time. Right: The absorbance changes at 420 (orange) and 675 (grey) nm. Fits to the data are shown in black. The concentrations of each component are as follows: 50 μM mutant T201S (100 μM diiron sites), 100 μM ToMOD, 1 μM ToMOC, 0.05 μM ToMOF, 90 μM NADH, and approximately 625 μM O₂. The solutions were buffered at pH 7.0 with 50 mM potassium phosphate, 50 mM NaCl.

The simplest explanation for the observed changes invokes the accumulation of three intermediates, T201S-675A (t_max ~ 11 ms), T201S-420A (t_max ~ 30 ms), and T201S-B (t_max ~ 0.4 s). Intermediate T201S-675A accumulates and decays with rate constants of 190(10) s⁻¹ and 26(2) s⁻¹. Intermediate T201S-420A accumulates with a rate constant of 150(10) s⁻¹, but the decay of this intermediate cannot be confidently established because the absorbance change between intermediates T201S-420A and the subsequent intermediate are very small. Intermediate 420B is the final intermediate that absorbs in the visible range and decays with a rate constant of 0.17(5) s⁻¹. The decay of intermediate T201S-675A is much slower than the formation of intermediate T201S-420A, which strongly suggests that the mechanism is bifurcated rather than linear.

The lifetime of all three intermediates is much shorter than that of intermediate 420A observed with WT ToMOH (Chapter 4). The decay of the oxygenated intermediates to form the final resting state (H_ox) is 100-fold faster for mutant T201S than WT ToMOH (0.0018 s⁻¹). In the
absence of aromatic substrate, the reduced lifetime of the oxygenated intermediates may be the reason for the faster generation of hydrogen peroxide for mutants of T201.  

Unlike intermediate 420A observed for WT ToMOH (Chapter 4), the intermediates observed with T201S-ToMO_red are sensitive to O₂ concentration. The absorbance changes in the presence of 100-625 μM O₂ are shown in Figure 5.3. Upon increasing the amount of O₂, intermediates T201S-675A and T201S-B accumulate to higher concentrations. The formation rate constants for intermediate T201S-675A increase linearly with respect to the concentration of O₂, exhibiting a second-order rate constant of 0.38(3) μM⁻¹s⁻¹. This result is similar to the second order rate constant derived for intermediate T201_peroxo (k₂ = 0.22 μM⁻¹s⁻¹) when dithionite and methyl viologen are used to reduce the mutant T201S.

![Figure 5.3](image.png)

**Figure 5.3.** O₂-dependence of the reaction between T201S-ToMO_red and oxygenated buffer at 5 °C. The concentration of O₂ was varied from 100 – 625 μM. The concentrations of all other reaction components are as indicated in Figure 5.2.

The pH dependence was also examined to determine how pH affects intermediate accumulation and the rates of formation and decay (Figure 5.4). The minimum accumulation of all three intermediates occurs at pH 6.0, and the maximum accumulation of all three intermediates occurs at pH 7.0. This pH sensitivity suggest that two protic groups participate in formation of these intermediates. Because the more hydrophobic interior of the protein can significantly alter the pKₐ values of amino acids, peroxo, and hydroxo species, it is not possible to identify the protic groups related to this reactivity.
Figure 5.4. pH dependence of oxygen activation by T201S-ToMO$_{red}$. The pH was varied from 6.0-8.0 in 50 mM potassium phosphate buffer with 50 mM sodium chloride. The concentrations the reaction components are as indicated in Figure 5.2.

All three intermediates are only slightly sensitive to buffer composition. At the same pH and temperature, two buffer systems were evaluated, (i) 25 mM MOPS at pH 7 and (ii) 25 mM potassium phosphate, 25 mM NaCl at pH 7. The comparison between these reactions is shown in Figure 5.5. Only moderate buffer effects were observed. The differences between the two buffers could be a result of the ionic strength of each buffer. The ionic strength of the MOPS and potassium phosphate buffers are 0.05 M and 0.15 M, respectively.

Figure 5.5. Buffer dependence of oxygen activation by T201S-ToMO$_{red}$. The concentrations the reaction components are as indicated in Figure 5.2. The buffer solutions contained either 25 mM MOPS at pH 7.0 or 50 mM potassium phosphate, 50 mM NaCl at pH 7.0.
Lastly, we examined oxygen activation by T201S-ToMO\textsubscript{red} in the presence of substrate (Figure 5.6). Two hydrocarbon substrates were examined, toluene and phenol. Neither T201S-675A nor T201S-420A were affected by these hydrocarbon substrates. In contrast, T201S-B did not accumulate in the presence of substrate. These results suggest that T201S-B is responsible for substrate hydroxylation. In each spectrum, we note that the reactions in the presence of substrate result in a higher final absorbance. This change is most likely due to the formation of a diiron-phenol or diiron-catechol adduct. Each of which gives rise to charge-transfer bands absorbing at 420 and 675 nm (Chapter 4).

![Graphs showing absorbance over time for different substrates](image)

**Figure 5.6.** Reactivity of T201S-ToMO\textsubscript{red} with O\textsubscript{2}-saturated buffer in the presence and absence of aromatic substrate. The reactions with and without toluene (left, top and bottom) were performed in 25 mM MOPS at pH 7.0. The reactions with and without phenol (right, top and bottom) were conducted in 50 mM potassium phosphate and 50 mM sodium chloride at pH 7. In the reactions containing substrate, 0.2 mM toluene or 2.5 mM phenol was added to the O\textsubscript{2}-saturated buffer. The concentrations the reaction components are as indicated in Figure 5.2.
When dithionite and methyl viologen were used to reduce T201S, a single intermediate was observed (T201\textsubscript{peroxo}) with absorbance at 675 nm. The maximum accumulation T201\textsubscript{peroxo} occurred at \(~\)40 ms. This intermediate was sensitive to the concentration of O\textsubscript{2} and phenol.\textsuperscript{5,6} Although T201S-675A and T201S-420A accumulate with similar rate constants to T201\textsubscript{peroxo} and are sensitive to O\textsubscript{2} concentration, neither of T201S-675A nor T201S-420A are sensitive to aromatic substrates. In contrast, T201S-B is sensitive to both O\textsubscript{2} and substrate, but the maximum accumulation is much later, approximately 400 ms. Finally, T201\textsubscript{peroxo} accumulates more at low pH (~6.5).\textsuperscript{6} The maximum accumulation for T201S-675A, T201S-420A, and T201S-B occurs at pH 7, dropping off significantly at lower pH values. Based on this comparison, it appears that T201\textsubscript{peroxo} is not identical to intermediates observed using T201S-ToMO\textsubscript{peroxo}. From these data, we conclude that use of the natural reduction system (ToMOC, ToMOF, and NADH) gives rise to new intermediates during oxygen activation by mutant T201S. A proposed mechanism for the reactivity of T201S-ToMO\textsubscript{red} is shown in Scheme 5.1.

Scheme 5.1. Proposed mechanism for the reactivity of T201S-ToMO\textsubscript{red}.

Three major differences exist between oxygen activation by T201S-ToMO\textsubscript{red} and ToMO\textsubscript{red}: (i) for T201S-ToMO\textsubscript{red} two intermediates absorb at 675 nm, T201S-675A and T201S-B, (ii) the rates of reactivity and decay are significantly faster for T201S than WT, and (iii) the mechanism is bifurcated between formation of T201S-675A and T201S-420A. In the analogous protein system T4MO, X-ray crystallographic analysis of a T201 mutant suggests that mutation of T201 may decrease the propensity of E231 to bridge Fe1 and Fe2 during turnover.\textsuperscript{9} Computational analysis of the diiron protein ribonucleotide reductase (RNR) indicates that the coordination modes of the carboxylate ligands strongly impact the intensity of the transitions in the visible range.\textsuperscript{13} Thus, the difference between oxygen activation by WT ToMOH and T201S
may result from different $O_2$ adducts and/or from different carboxylate coordination modes. RFQ-resonance Raman spectroscopy may be able, in future experiments, to distinguish between these possibilities.

**Experimental Methods**

**General Methods.** The plasmid for mutant T201S was prepared as previously reported. Expression and purification of mutant T201S, ToMOD, and ToMOC were carried out as reported in Appendix A. Expression and purification of ToMOF were conducted as previously reported.

**Anaerobic Sample and Instrument Preparation.** The protein samples were prepared in buffer containing 50 mM potassium phosphate and 50 mM sodium chloride at pH 7.0. The samples were degassed on a Schlenk-line by cycling between vacuum and argon for a minimum of ten cycles. The samples were brought into an anaerobic chamber under a nitrogen atmosphere. Anaerobic buffer was prepared by purging a sealed vessel with argon for 1 h. The buffer was brought into the chamber of nitrogen atmosphere and was stirred in the anaerobic chamber for a minimum of 5 h before use. To obtain reduced hydroxylase samples, NADH was added to the reconstituted ToMO sample inside the anaerobic chamber. The stopped-flow UV-visible apparatus was washed with buffer containing $\sim15$ mM dithionite for a minimum of 3 h to remove oxygen from the system. Immediately before use, the stopped-flow lines were washed with anaerobic phosphate buffer without dithionite to remove excess dithionite from the lines.

**Stopped-Flow UV-visible Spectroscopy and Data Analysis.** Oxygen activation chemistry was monitored by stopped-flow UV-visible spectroscopy equipped with either a diode array or a photomultiplier tube. The protein samples were loaded into airtight Hamilton luer-lock syringes inside an anaerobic chamber. Oxygen-saturated buffer was prepared by bubbling pure $O_2$ through a sealed falcon tube of buffer; a needle outlet was used to relieve pressure. The protein and the buffer were loaded onto the stopped-flow sample-handling unit immediately after removal from the anaerobic chamber. The final component concentrations were as follows: 50 $\mu$M ToMOH, 100 $\mu$M ToMOD, 1 $\mu$M ToMOC, 0.05 $\mu$M ToMOF, and 90 $\mu$M NADH. Two control experiments were performed omitting either ToMOH or $O_2$. 

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All of the reactions were carried out in 50 mM potassium phosphate, 50 mM sodium chloride, pH 7.0, at 5 °C. For assays containing substrate, either toluene or phenol was added to the oxygen-saturated buffer.

The absorbance was monitored at 420 and 675 nm. The data were collected on a logarithmic timescale. At least three individual reactions were averaged to obtain standard deviations. Because the absorbance changes in the presence of toluene were very small, over 10 separate reactions were averaged to obtain higher signal-to-noise. The stopped-flow data were analyzed in OriginLabs 9.0 or 9.1. The averaged absorbance traces were fit with functions containing three or four exponents (eq. 4.1 and 4.2). The best fit was determined by the following standards: Adj. R² near unity, non-oscillatory residuals, low error of the fitted parameters, and good overlap of the simulated curve with the experimental data.

References


Appendix A

Heterologous Expression and Purification of Components of Toluene/o-Xylene Monooxygenase from Pseudomonas sp. OX1

Genes Sequences for Expression of WT proteins. The genes used for expression of ToMOH, ToMOD, ToMOC, and ToMOF are listed below.

touBEA: Dark purple = gamma; Green = beta; Blue = Alpha; Spacers = black, bold

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ATGGCGACGTTCCTCCATTTATGCTCATAATTTTGAGCTGATTTTTGTATCCAGTTGGTGCCCGTGACACGG
AGGATACTGAGATGACGTGACGAAATGTGCTGTTACCGAAGGATGAGGTGATTTGATCGACTCTCGAACC
GGAAAGATTTTACGTTTGCCTCGGCAAGGAGATTTGCTGTTGCTCCGAGGCCATGATCGTATCGGAT
GCACGTGGCAGTGGCAGAATTTTATGAGGTTGTATCGACCAATCTTACATCTTACGGATAATCCGGAAAGACCATGGGAGTTAGACTCAAACCTCCCAATGCAAACATGGTATAAAAATATTGCTTTGATAGCCCACTCAAACACGATGACTGGAATGCTTTTCGCGATCCTGACCAGCTCGTCTACAGAACTTATAATCTACTTCAAGACGGTCAAGAGTCGTACGTTCAGGGATTATTTGATCAACTCAATGACCGCGGCCACGATCAGATGCTGACTCGTGAATGGGTAGAAACATTAGCCCGTTTTTATACACCAGCAAGGTATCTTTTTCAGCTCAGTATACCTCAATGAAATACCGATGATGCTGAGGTGACTCCAAACTCCTAGCATATCCAGGCAAGGAGATTTTATGAGGTTGTATCGACCAATCTTACATCTTACGGATAATCCGGAAAGACCATGGGAGTTAGACTCAAACCTCCCAATGCAAACATGGTATAAAAATATTGCTTTGATAGCCCACTCAAACACGATGACTGGAATGCTTTTCGCGATCCTGACCAGCTCGTCTACTGTTAATCTGCTATGGGAAGCTTGGGAAAAATACGATGAACCGTACAAGATACTTATCCGGAATACGTCAGTATTCAGCGGCACGCCAAGGTGATACTCTGCTCGGTCTCTTGGCTCAGGCACAAAAACGTGACGCTGAGCGGCATCGGAGATGGTCTTCTGCACTGGTCGGAATATGGCTCTGGAGAAAGAAGGCAACCGTGAAGTGTTGCAGAAATGGGTTGCCAAATGGGAAACCATTAGCCGAACAAAGCCATAGAGGCATATTGCAGCGCATTACCTGACGGTGAAAACGCCATCGTCGAA
```
Note: the gene *touC* differs from that in the NCBI data bank (accession number: AY621080). Within the gene used in this work, codon 74 is CAT; this codon in the NCBI sequence is CAC. Because both code for histidine, the codon difference does not affect the resulting amino acid at this position.

*touF*:

ATGAGCAATAAGATAAAAAATTTGCCGATACAGATGCAGATTACCATCTCCGACAGGGATACGATTCTGA
GGGCGGCTTTTACGTGATGGAAATACCAATCTCATACGAGTGCAACTCCGCGGTCGCTTAAGCCCAAGGGGCAAAAGGA
AAAAGCAGAAAAACTTGCGCTGAGCTGTTGGGACCCGGTCTCGTGTGATGGAATACCAATCTCATACGAGTGCAATTCCGCGGA
TATAAACTACCCGAAATACGCCCTTTTGCTGTCGTTGGGTTTTCGGAATGAGAGACCGCTCGCTACCTTCTTCGGAACAAATATGCGATTGTGACT
GTCCCCGGTTATTGAGGAGATAGGGTTATATTCCATGGTCCATGTGGTTTCTTACGATTCGAGCTGGAGCTGTT
TTATTATAAACGCAATGCGCGAGTAAGGCATTCAACGTGGCTCTGCGATCGAATTGCACGATTACGAGGATCCACGCTCTCCG
AAGCAGATGGGAGGCCCTTTTGCTGCTGCTACCTTCTTCGGAACAAATATGCGATTGTGACT
TGGAATTGAGTACTATTCTGCGCCGCCGCGGACTGAGGGTACGCATGCCATGCTGATGATCGA
TGCAAAAGATCGCCGTTCGATTTCGATCGGTTTTTCTGTA

**Protein Concentration and Handling.** For ToMOH and ToMOD, the protein concentrations were measured as a function of their $\varepsilon_{280}$, 600,000 M$^{-1}$cm$^{-1}$ and 2,860 M$^{-1}$cm$^{-1}$, respectively. For ToMOC, the protein concentration was determined as a function of the $\varepsilon_{458}$, 6870 M$^{-1}$cm$^{-1}$. For ToMOF, the protein concentration was determined as a function of the $\varepsilon_{454}$, 48,100 M$^{-1}$cm$^{-1}$. To measure these values, the stock solutions were diluted by 1:10 in series to achieve absorbance between 0.1-1 for the wavelength of interest. Purified, frozen proteins were always thawed on ice. Loss of activity for ToMOH and loss of the iron clusters of ToMOC and ToMOF were often observed upon multiple freeze-thaw cycles. Because of this, freeze-thawing these components was avoided when possible. To concentrate the proteins described here, centrifugal filter devices from Millipore were used. This step was always carried out at 4 °C. Before and after use, the
filtration devices were cleaned by thoroughly rinsing with sterile water. Between uses, these filters were stored in sterile water.

**General Chromatography Notes.** All column chromatography was conducted in a cold room at 4 °C. The buffers used for chromatography were filtered through 0.2 μm membrane filters prior to use. All buffers were pH adjusted at room temperature, cooled to 4 °C, and the pH of the cooled buffer was re-adjusted if necessary. The DEAE-sepharose and Q-sepharose columns were cleaned by washing with 2 M NaCl(aq) for 3 column volumes and then wash with 2 column volumes of buffer containing 25 mM MOPS and 10 % glycerol at pH 7. Rarely, the top of the column resin remained grey or green after washing with 2 M NaCl(aq). When this occurred, the following more extensive protocol was carried out: (i) wash with 3 column volumes of water; (ii) wash with 3 column volumes of 1 M NaOH(aq); (iii) wash with 3 column volumes of water; and (iv) wash with 2 column volumes of buffer containing 25 mM MOPS and 10 % glycerol at pH 7.

**ToMOH Expression.** *Escherichia coli* (E. coli) BL21(Gold)-DE3 cells were transformed with pET22B(+)/touBEA, which encodes the three subunits of ToMOH from *Pseudomonas sp.* OX1. A 500-ml flask containing 300 ml of LB media was autoclaved and cooled. Ampicillin was added to the culture to a final concentration of 100 μg/ml. The LB-ampicillin media was inoculated with one colony from the transformation plate, and the inoculated flask was allowed to shake at 37 °C for approximately 10 h at 150 rpm.

In baffled flasks, 8 L of LB media were autoclaved and cooled to room temperature. To the autoclaved solution were added 0.8 g of ampicillin and 200 ml of the incubated 300-ml starter culture. The inoculated cultures were set to shake at 150 rpm with a temperature of 37 °C until the O.D.₆₀₀ reached 0.6 (~2.5 h). At this time, the temperature was reduced to 18 °C. The temperature of the incubator stabilized after approximately 15 min. The flasks were shaken for an additional 15 min to allow the media temperature to fully reduce to 18 °C.

Expression was induced with 50-125 μM IPTG (isopropyl-β-D-1-thiogalactopyranoside) at 18°C. Ferrous ammonium sulfate hexahydrate was added in 5 aliquots over a period of 5 h to a final concentration of 625 μM. The cultures were left to shake at 18°C for a total of 21 h. The cells were harvested by centrifugation at 11,325 g for 10 min at 4 °C. The resulting cell paste was
dropped in dollops into liquid nitrogen and stored at -80 °C. The average yield of wet cells was 7 g per liter of culture media.

**ToMOH Purification.** Approximately 25 g of wet cell pellets were weighed out for protein extraction and purification. The pellets were thawed at room temperature and resuspended in a chilled (4 °C) solution of 100 ml of 25 mM MOPS, pH 7.0, and 10% glycerol (v/v). Once the mixture became homogeneous, magnesium chloride was added to a final concentration of 10 μM; 100 μL of DNAse (1 unit/μl) was added; lysozyme was added to 1 mg per ml of mixture; and PMSF (phenylmethylsulfonyl fluoride) was added to a final concentration of 0.1 mM. The mixture was stirred very slowly on ice for 3-4 h. The mixture was then sonicated on ice for 5 min with 0.2 s pulses resting for 0.5 s between each pulse. During sonication, the ice was refreshed twice to prevent the sample from warming. The lysate was then centrifuged at 95,000 g for 10 min at 4 °C. The supernatant was decanted, filtered through a 0.2 μm membrane filter, and immediately loaded onto a DEAE-Sepharose column (2.6 x 58 cm) at a rate of 2 ml/min. The column was washed with 150 ml of 25 mM MOPS, pH 7.0, 25 mM NaCl, and 10% glycerol (v/v). The column was then washed with 400 ml of 25 mM MOPS, pH 7.0, 150 mM NaCl, and 10% glycerol (v/v). ToMOH was eluted with 25 mM MOPS, pH 7.0, 10% glycerol (v/v) with a 1200-ml linear gradient from 150 mM NaCl to 350 mM NaCl. The flow-rate was 3 ml/min. A representative chromatogram is shown below.

![Chromatogram](image)

ToMOH reproducibly eluted between 700–900 ml into the linear gradient, corresponding to a NaCl concentration of 260-275 mM. Fractions within this range were analyzed by SDS-
PAGE for further identification. From these fractions, those that exhibited at least half of the maximum absorbance at 280 nm were pooled. The pooled fractions were concentrated to ~5 ml in 50-kDa MWCO centrifugal tubes at 4000 g and 4 °C. The concentrated sample was applied to a Sephacryl S300 column (2.5 x 80 cm). ToMOH was eluted from the column with 25 mM MOPS, pH 7.0, 10% glycerol (v/v) and 150 mM NaCl at a rate of 1.25 ml/min. A representative chromatogram and a representative gel are shown below.

Fractions were analyzed by means of a UV-visible spectroscopy, SDS-PAGE, and a specific activity assay (see below). For WT ToMOH, fractions containing protein with a specific activity of 1000-1300 nmol/min/mg were pooled and concentrated. For ToMOH variants, fractions containing 75-100% of the most active fraction assayed were pooled. The pooled fractions were concentrated as described above and flash-frozen in liquid nitrogen. The frozen pellets were stored at -80 °C. The frozen protein concentrations varied from 300 μM to 900 μM. The yields for ToMOH varied from 20-50 mg per L of cell culture (or 3-8 mg ToMOH per g of cell paste).

In a few cases, WT ToMOH was not of adequate specific activity after the DEAE-sepharose and S300 columns. When this occurred, the crude protein from the S300 column was diluted with an equal volume of 25 mM MOPS, 10% glycerol at pH 7. This step reduced the concentration of NaCl to 75 mM prior to loading a Q-sepharose column (2.6 x 14 cm). ToMOH was eluted from the Q-sepharose column in buffer containing 25 mM MOPS, pH 7.0, 10% glycerol (v/v) with a 600-ml linear gradient from 75 mM NaCl to 350 mM NaCl. The flow-rate was 3 ml/min. The final protein was selected based on the C2,3O activity assay.
Specific Activity Assay. The specific activity of each fraction was determined by a coupled assay using the protein catechol 2,3-dioxygenase (C2,3O). The absorbance at 280 nm for each fraction was measured. This absorbance was divided by 600,000 M⁻¹cm⁻¹ to estimate the concentration of ToMOH assuming 100% purity. Using this estimate, a solution was prepared comprising 0.15 μM of ToMOH from a single fraction, 6 μM ToMOD, 6 μM ToMOC, 60 nM ToMOF, and 2.5 mM phenol. The dilution buffer contained 0.1 M Tris, pH 7.3. This step was conducted for reactions containing a ToMOH concentration of higher than 8 μM. The reaction was initiated by addition of NADH to a final concentration of 200 μM. The absorbance change at 410 nm was monitored at 25 °C using a Hewlett Packard diode array spectrophotometer scanning every 3 s. This absorbance change corresponds to conversion of catechol to 2-hydroxymuconic semialdehyde (ε₄₁₀ = 12620 M⁻¹ cm⁻¹). The initial rate of 2-hydroxymuconic semialdehyde was recorded as a function of absorbance at 410 nm over time. The specific activity was calculated by the following equation.

\[
\text{specific activity (nmol/min/mg)} = \frac{\text{rate (AU/min)}}{1 \text{ cm}^{-1}} \cdot \frac{1 \text{ cm}^{-1}}{12620 \text{ M}^{-1} \text{ cm}^{-1}} \cdot \frac{\text{Rxn volume (L)}}{\text{ToMOH mass (mg)}} \cdot 10^9
\]

ToMOD Expression. *E. coli* BL21(Gold)-DE3 cells were transformed with pET22B(+)/*touD*. The starting culture was prepared as indicated in ToMOH Expression above. In baffled flasks, 4 L of LB media were autoclaved and cooled. To the autoclaved media was added 0.4 g of ampicillin and 50 ml of the incubated 300-ml starter culture were added. The inoculated cultures were set to shake at 150 rpm with a temperature of 37 °C until the O.D.₆₀₀ reached 0.6 (~2.5 h). Expression was induced with 200 μM IPTG. The cultures were left to shake at 37 °C for 3 h. The cells were harvested by centrifugation at 11,325 g for 10 min at 4 °C. The resulting cell pellet was dropped piecewise in liquid nitrogen and stored at -80 °C. The average yield of wet cells 5 g per liter of culture media.

ToMOD Purification. Approximately, 25 g of wet cell pellet were weighed out for protein extraction and purification. The cells were lysed as indicated above under ToMOH Purification.
After centrifugation and filtration, the crude protein solution was immediately loaded onto a Q-Sepharose column (2.6 x 14 cm) at a rate of 2 ml/min. The column was washed with 50 ml of 50 mM MOPS, pH 7.0, 50 mM NaCl, and 10% glycerol (v/v). ToMOD was eluted with 25 mM MOPS, pH 7.0, 10% glycerol (v/v) with a 500-ml linear gradient from 50 mM NaCl to 350 mM NaCl. The flow-rate throughout the NaCl gradient was 3 ml/min. ToMOD reproducibly eluted between 300 – 390 ml into the linear gradient, corresponding to a NaCl concentration of 230-290 mM. ToMOD has a very low absorbance at 280 nm, making it difficult to identify the peak in the UV-visible chromatogram. A representative chromatogram and a representative gel are depicted below with a brief commentary.

A gel and the absorbance trace throughout the elution were used to analyze the contents of selected fractions. Gel lanes: 7, 14, ladder, 20, 28, 32, 40, 48, 63, 70, ladder. Based on the gel, fraction 48 contains ToMOD. Fractions 42-53 were pooled and concentrated in 3-kDa MWCO amicon tubes. Fractions were analyzed by SDS-PAGE to identify ToMOD in the eluent. From these fractions, those shown to contain ToMOD by gel analysis were pooled. The pooled fractions were concentrated to ~3 ml in 3-kDa MWCO centrifugal tubes at 4000 g. When concentrating ToMOD, care was taken to not exceed 2 mM ToMOD. At high concentrations, ToMOD forms an inactive oligomer. This oligomer can be separated by size exclusion chromatography. The concentrated sample was applied to a Sephacryl S75 column (2.6 x 90 cm). ToMOD was eluted from the column with 25 mM MOPS, pH 7.0, 10% glycerol (v/v) and 150 mM NaCl at a rate of 1.25 ml/min. Fractions were analyzed by UV-visible spectroscopy and SDS-PAGE. ToMOD
reproducibly eluted between 275 – 315 ml into the column isocratic gradient. A representative chromatogram is shown below.

These fractions were analyzed by SDS-PAGE. Fractions containing minimal impurities were pooled and carefully concentrated as described above. The final, colorless protein was flash-frozen in liquid nitrogen and stored at -80 °C. The frozen ToMOD concentrations varied from 500 μM to 1.5 mM. A typical yield of ToMOD was 12 mg per L of cell culture (or 3 mg of ToMOD per g of cell paste).

**ToMOC Expression.** *E. coli* BL21(Gold)-DE3 cells were transformed with pET22B(+)/touC. The starting culture was prepared as indicated in ToMOH Expression above. In baffled flasks, 8 L of LB media were autoclaved and cooled. To the autoclaved media was added 0.8 g of ampicillin and 200 ml of the incubated 300-ml culture. The inoculated cultures were set to shake at 150 rpm with a temperature of 37 °C until the O.D.₅₀₀ reached 0.6 (~2.5 h). Expression was induced with 200 μM IPTG. Ferrous ammonium sulfate hexahydrate was added over a period of 3 h to a final concentration of 500 μM. The cells were harvested 4 h after induction with IPTG. The resulting cell pellet was dropped piecewise in liquid nitrogen and stored at -80 °C. The average yield of wet cells was 6 g per liter of culture media.

**ToMOC Purification.** Purification of ToMOC was conducted as rapidly as feasible. At 4 °C, the [2Fe-2S] cluster is slowly lost from the protein. Minimizing the time between lysing the cells and freezing the final protein results in higher yields of holo-ToMOC. The following protocol was
typically completed in under 24 h. Approximately 35 g of wet cell pellet were weighed out for protein extraction and purification. The cells were lysed as indicated in ToMOH Purification above. After centrifugation, the crude protein solution was immediately loaded onto a Q-Sepharose column (2.6 x 14 cm) at a rate of 2 ml/min. The column was washed with 50 ml of 50 mM MOPS, pH 7.0, 50 mM NaCl, and 10% glycerol (v/v). ToMOD was eluted with 25 mM MOPS, pH 7.0, 10% glycerol (v/v) with a 500-ml linear gradient from 100 mM NaCl to 500 mM NaCl. The flow-rate throughout the NaCl gradient was 3 ml/min. ToMOC reproducibly eluted between 350 – 400 ml into the linear gradient, corresponding to a NaCl concentration of 380-420 mM. The ratio of the absorbance at 458 nm and 280 nm [Abs(458/280)] was measured for all red-colored fractions. Fractions with an Abs(458/280) ratio of greater than 0.12 were pooled. The pooled fractions were concentrated to ~3 ml in 3-kDa MWCO centrifugal tubes at 4000 g. The concentrated sample was applied to a Sephacryl S75 column (2.6 x 90 cm). ToMOC was eluted from the column with 25 mM MOPS, pH 7.0, 10% glycerol (v/v) and 150 mM NaCl at a rate of 1.25 ml/min. ToMOC reproducibly eluted around 300 ml into the column isocratic gradient.

![Graph](image)

Fractions with an Abs(458/280) of greater than 0.17 were pooled and concentrated as described above. The final protein was flash-frozen in liquid nitrogen and stored at -80 °C. Prior to size exclusion chromatography, the primary impurity appears to be apo-ToMOC, thus fractions from the S75 column were routinely analyzed by Abs(458/280) but not by SDS-PAGE.
**ToMOF Expression and Purification.** ToMOF was expressed and purified as previously described. A few additional notes on the purification are provided here for completeness. When purifying ToMOF, the color of ToMOF on the column may change to purple due to the presence of DTT in the running buffer. Once the protein has been exposed to air off of the column, the color will change back to orange. For the S300 column purification, DTT was omitted. The cutoff values used for determining which fractions to concentrate for the final, purified ToMOF were as follows: $\text{Abs}(334/280) \geq 0.2$ and $\text{Abs}(454/280) \geq 0.25$. Below the following representative data is provided: a gel from the Q-sepharose column (in which the left-most lane is ToMOH for a standard), a chromatogram from the S300 column, and the UV-visible analysis of fractions from the S300 column.
References

BIOGRAPHICAL NOTE

Alexandria Deliz Liang was born in Tallahassee, FL to parents Jeffrey Santos Liang and Jaime Llewellyn Doyle. She was raised with younger sister Nicole Santos Liang. In 2006, she graduated from Leon High School and matriculated to New College of Florida, where she worked for Professor Susan E. Sherman. Her undergraduate thesis focused on mimicry of oxalate degrading enzymes through synthesis and metalation of triazacyclononane derivatives. In 2010, Alexandria began her graduate work at the Massachusetts Institute of Technology (MIT) in the laboratory of Prof. Stephen J. Lippard. During her graduate work, she interned at Biogen Idec in partial fulfillment of her fellowship on the NIGMS/MIT Biotechnology Training Grant. After graduation, Alexandria plans to pursue postdoctoral studies in the laboratory of Prof. Jason W. Chin at MRC Laboratory of Molecular Biology, Cambridge, UK. Alexandria enjoys traveling, the outdoors, cooking, wine, and music composition.
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Publications


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“Steady-State and Pre-Steady-State Characterization of Toluene Monooxygenase”
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