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

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

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Supporting Information

ABSTRACT: Toluene/o-xylene monoxygenase (ToMO) is a bacterial multicomponent monoxygenase capable of oxidizing aromatic substrates. The carboxylate-rich diiron active site is located in the hydroxylase component of ToMO (ToMOH), buried 12 Å from the surface of the protein. A small, hydrophilic pore is the shortest pathway between the diiron active site and the protein exterior. In this study of ToMOH from Pseudomonas sp. OX1, the functions of two residues lining this pore, N202 and Q228, were investigated using site-directed mutagenesis. Steady-state characterization of WT and the three mutant enzymes demonstrates that residues N202 and Q228 are critical for turnover. Kinetic isotope effects and pH profiles reveal that these residues govern the kinetics of water egress and prevent quenching of activated oxygen intermediates formed at the diiron active site. We propose that this activity arises from movement of these residues, opening and closing the pore during catalysis, as seen in previous X-ray crystallographic studies. In addition, N202 and Q228 are important for the interactions of the reductase and regulatory components to ToMOH, suggesting that they bind competitively to the hydroxylase. The role of the pore in the hydroxylase components of other bacterial multicomponent monoxygenases within the superfamily is discussed in light of these conclusions.

Bacterial multicomponent monoxygenases (BMMs) comprise a family of enzymes capable of hydroxylating and epoxidizing hydrocarbon substrates at carboxylate-rich diiron active sites. Bacteria containing BMMs help to regulate the global carbon cycle and are used for bioremediation of environments contaminated with hydrocarbons and halogenated pollutants. All BMMs require a hydrocarbon substrate, molecular oxygen, protons, and electrons acquired through NAD(P)H. 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. Toluene/o-xylene monoxygenase (ToMO) is a four-component, arene-oxidizing BMM composed of an NADH-oxidoreductase (ToMOP), a Rieske-type ferredoxin (ToMOC), a cofactorless regulatory protein (ToMO), and the catalytic hydroxylase protein (ToMOH). Three component BMMs, like soluble methane monoxygenase (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. The extensive protein architectures of these hydroxylase components protect intermediates formed during dioxygen activation from undesired side reactions, but they 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 and hydrocarbons to the diiron active sites, as revealed by X-ray crystallographic studies of four BMMs: ToMO, toluene-4-monooxygenase (T4MO), soluble methane monoxygenase (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 1). X-ray crystallographic studies revealed that the pore is hydrophilic and flexible and can accommodate an ordered water molecule, designated HOHS, 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) or proton-coupled electron transfer (PCET) and providing a pathway for water or hydroxylated products to exit the active site.

In the present study, we evaluate reactivity changes following site-directed mutagenesis of residues within the pore of ToMOH from Pseudomonas sp. OX1, the hydroxylase of a canonical four-component BMM that displays moderate regiospecificity during aromatic hydroxylation. Steady-state and pre-steady-state studies demonstrate that residues N202 and Q228 of ToMOH are critical for efficient turnover, water...
Details for the preparation of mutants N202A, Q228A, and Q228E are included in the Supporting Information.

**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 (ε_max = 6220 M⁻¹ cm⁻¹) to a final concentration of 200 μM. The absorbance change at 340 nm, corresponding to NADH consumption, 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 standard deviations.

**Catechol Formation Assays.** The rate of catechol formation was determined by a coupled assay. Reactions were prepared as described for the NADH consumption assay except that excess C23O was added to convert catechol to 2-hydroxymuconic semialdehyde (ε_max = 12 620 M⁻¹ 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, we use D₂O purchased from Icon Isotopes, 99.8% isotope enriched. Before performing pH readings of D₂O-containing buffers, the pH meter probe was soaked in this solvent. The pD value of each D₂O 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. Graphical representations of the data analysis are shown in Figure S2. The kinetic solvent isotope effects (KSIEs) were derived by dividing k_cat or k_cat/K_m obtained in H₂O by those obtained in D₂O. 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 rates were 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 (Figure S2). A 300 μL solution of 0.15 μM ToMOH WT or Q228A, 6 μM ToMOD, 6 μM ToMOC, 60 nM ToMOF, and 200–500 μM 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 μM. In 10–30 s increments, aliquots of 50 μL were removed from the reaction and quenched in 50 μL of 0.4 M trichloroacetic acid, with the acidified mixture being vigorously pipetted. Five time points were obtained for each condition. The quenched mixture was centrifuged (2000g), and the supernatant was diluted 4-fold into buffer containing 500 mM Tris and 50 mM MOPS at pH 7.3.

![Figure 1. 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 gray, cut away to highlight the pore and diiron active site. The active site, drawn as sticks, is colored by atom type: carbon (gray), 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 and 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.](image-url)
Table 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>$K_{SIE}(k_{cat, \text{phenol}})$</td>
<td>2.0(1)</td>
<td>1.9(2)</td>
<td>12(1)</td>
<td>0.19(1)</td>
</tr>
<tr>
<td>$K_{SIE}(k_{cat}/K_m, \text{phenol})$</td>
<td>0.48(9)</td>
<td>0.24(9)</td>
<td>7(2)</td>
<td>0.018(2)</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>$k_{cat}/K_m, \text{ToMOC}$ (μM$^{-1}$ s$^{-1}$)</td>
<td>1.04(1)</td>
<td>0.41(8)</td>
<td>0.20(4)</td>
<td>0.019(1)</td>
</tr>
<tr>
<td>electron transfer $k_{\text{elec}}$ (s$^{-1}$)</td>
<td>44(2)</td>
<td>21.4(4)</td>
<td>40(10)</td>
<td>6(1)</td>
</tr>
</tbody>
</table>

7.3. C230 (S 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, $\varepsilon_{374} = 33 000$ 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 1 and 2 for WT ToMOH and mutant Q228A, respectively.

$$v (s^{-1}) = \frac{v_{max}}{1 + \frac{10^{-pH}}{10^{pK_a}}}$$

(1)

$$v (s^{-1}) = \frac{v_{max}}{1 + \frac{10^{-pH}}{10^{pK_a}}}$$

(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 that used in other steady-state experiments reported here.

**Electron Transfer (ET) from ToMOC 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 are detailed in the Supporting Information.

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 3, where C is the initial absorbance, A is the overall absorbance change, k is the rate constant, and t is time.

$$A(t) = Ae^{-kt} + C$$

(3)

**RESULTS**

**General Steady-State Kinetics.** Table 1 and Figures S2–S4 summarize the results of phenol conversion to catechol for WT ToMOH as well as mutants N202A, Q228A, and Q228E. Compared to that of WT ToMOH, the $k_{cat}$ and $k_{cat}/K_m$ values were significantly diminished for the mutant proteins. N202A and Q228A retained 15 and 10% of the WT ToMOH $k_{cat}$, respectively. ToMOH mutant Q228E exhibited the lowest $k_{cat}$, 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 $K_{SIE}(k_{cat})$ values. Mutant Q228A exhibited a much larger $K_{SIE}(k_{cat})$, suggesting that a proton-transfer or viscosity-dependent event is rate-limiting. Conversely, mutant Q228E had a strong inverse $K_{SIE}(k_{cat})$. Inverse $K_{SIE}(k_{cat}/K_m)$ 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 (data not shown).

**Steady-State Viscosity Dependence.** A plot of $k_{cat}$ as a function of viscosity is shown in Figures 2 and S5 for each hydroxylase mutant. Within the viscosity range assayed, the activity did not change significantly for WT ToMOH or mutants N202A and Q228E. With increasing viscosity, the $k_{cat}$ 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 $\eta/\eta_0$ of ~3.3.

**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 1 and Figure S6. The enzymatic efficiencies with respect to ToMOC were significantly reduced for all mutants compared to that of 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.

**Steady-State pH Profiles.** Figures 3 and S7 depict the steady-state pH dependence of both WT ToMOH and mutant
Q228A. Concentrations of phenol were varied in these experiments to determine both $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ for catechol formation. Although an approximate $k_{\text{cat}}$ could be obtained, determination of $k_{\text{cat}}/K_m$ was not successful owing to high errors at low concentrations of phenol. The $pK_a$ values derived from fits according to the Materials and Methods are indicated in the top panel of Figure 3. 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 3. 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.

**Pre-Steady-State ET Kinetics of Hydroxylase Variants.**

The ET kinetics of each mutant were measured by stopped-flow UV–visible spectroscopy (Table 1, lower panel and Figures S8 and S9). 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 kinetic patterns were similar for WT ToMOH and mutant Q228A. The rate constant decreased by a factor of 2 upon mutation of N202 to alanine and by approximately 8-fold for mutant Q228E.

**DISCUSSION**

The function of pore residues has generated much speculation since an early Xe-pressurized crystal structure analysis of the hydroxylase of sMMO (sMMOH). Similar to that of other BMM hydroxylases, the pore of ToMO 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 1). The role of the conserved threonine residue, T201, has been a subject of extensive investigation. 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 sMMOH, which contains a glutamate instead of a glutamine at this key position (Figure S1).

**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 1, namely, substrate hydroxylation by ToMOH$_{\text{perox}}$ and loss of H$_2$O$_2$ from ToMOH$_{\text{perox}}$. 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 subsaturating concentrations of either a hydrocarbon substrate or the regulatory protein, the coupling efficiency decreases for many BMMs. These 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 detrimentally affected. To determine whether a lower affinity for...
ToMOD was the source of low coupling efficiency, we investigated directly the binding of ToMOD with fluorescein-labeled ToMOD (ToMOD-Fl, see Supporting Information protocols and Figure S10 for details). Mutants N202A and Q228A each retained similar affinities for ToMOD-Fl as that of WT ToMOD. In contrast, mutant Q228E exhibits a diminished binding strength for ToMOD-Fl. Because WT ToMOH and mutants N202A and Q228A maintain a similar ToMOD-Fl 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 1, blue) or downregulate hydrogen peroxide release (Scheme 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 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. 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 a steady-state viscosity dependence and isotope effects greater than two (Figure 2). Because the viscosity of deuterated water is greater than that of protiated water, viscosity-dependent kinetics can also result in KSIE values greater than 1. At the viscosity of deuterated water (n_{prot} 1.23 mPa s^{−1}), the k_{cat} of WT and the three mutants varied from 88 to 108% of the k_{cat} in non-suerocontaining buffer. Thus, viscosity can contribute only a maximum KSIE effect of 1.14 for all mutants. The observed KSIE(k_{cat}) and KSIE(k_{cat}/k_{cat}) 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 3), indicating that two prototropic groups are critical for catalysis. The pK_{1} and pK_{2} values of WT enzyme are 6.1 and 7.1, respectively. The functional groups responsible for these pK_{i} values are unknown but may include a histidine side chain (pK_{H} ~ 6.1), an iron-aqua species (pK_{H} < 15, but widely variable), or a diiron-peroxo species (pK_{H} ~ 7.2). The coupling efficiency of WT ToMOH did not vary significantly over the pH range investigated (Figure 3). Conversely, the pH profile of mutant Q228A is alkaline-shifted (pK_{H} ~ 6.52) from that of WT (pK_{H} ~ 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 (Figure 3).

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 4 (bottom panel) illustrates in cartoon form a 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_{2}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. 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 proton tunneling in
mutant Q228E. 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 binding and a shifting glutamate residue 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. The BMM pore is the only conserved, hydrophilic channel extending from the diiron center to the solvent-exposed surface. 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 catalysis and water dissociation from metal ions. 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($k_{cat}/K_m$) of mutant Q228E and the KSIE($k_{cat}/K_m$) of WT ToMOH and mutants N202A and Q228E.

For mononuclear cobalt, iron, and zinc, the number of aqua or hydroxo ligands dissociating from a metal center can be calculated from the magnitude of the KSIE and fractionation factors ($\Phi$, equilibrium distributions of the two isotopes). Fractionation factors for mononuclear centers have been previously reported. 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 and predicted values (see the Supporting Information). From these data we conclude that water dissociation is rate-limiting for steady state turnover in mutant Q228E.

The KSIE($k_{cat}/K_m$) values for mutants N202A and Q228E and for WT ToMOH are also less than 1, 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 multisubstrate enzymes like ToMO, however, the order of substrate addition can change the composition of rate constants contributing to $k_{cat}/K_m$ (Schemes S2 and S3 in the Supporting Information). 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 affect change in the reduction step of the catalytic cycle (Scheme 1). Mutants N202A and Q228E exhibited a decrease in rate constant for ET compared to WT ToMOH. The rate of interprotein electron transfer from ToMOC$_{red}$ 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 those of 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. 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 sMMOH. 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. A comparison of crystal structures for ToMOH, T4moH, sMMOH, and the hydroxylase–regulatory protein complexes for T4MO and sMMO 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. In this investigation, the glutamine residue is able to stabilize HOHS, but mutation to glutamate results in loss of this water molecule. If HOHS and Q228 are involved in water release from the diiron active site, then mutant Q228E might exhibit low activity and coupling efficiency because its charge might retard the rate of water egress either by slowing the rate of conformational change or eliminating an essential hydrogen-bonding partner. The question of how sMMOH functions with a glutamate at this key position remains unanswered. Unlike in ToMOH and T4moH, sMMOH must release methanol during each catalytic cycle. It is possible that the pore of sMMOH 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, 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 release during catalytic turnover. It is possible that the pore of sMMOH 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.
petitive 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.

■ ASSOCIATED CONTENT

 Supporting Information

Protein preparation details; proposed model for water dissociation from the diiron active site of Q228E; sequence alignment of alpha subunits of the hydroxylase proteins within BMMs; graphical descriptions of the NADH consumption, catechol formation, and quenched-activity assays; contributions to catalysis and the concentrations necessary to reach optimal catalytic activity of the component proteins used in the ToMO reactivity studies; reactions of each of the hydroxylase isoforms studied in H2O and D2O by the catechol formation assay; non-normalized data of the hydroxylase isoforms in varying concentrations of sucrose; reactions of each of the hydroxylase isoforms studied with varying concentration of ToMOC by the catechol formation assay; results from the quenched-activity and NADH assays; change in the extinction coefficient of ToMOC upon reduction with phosphate buffered dithionite; change of absorbance at 458 nm upon mixing ToMOCred with NADH assays; change in the extinction coefficient of sucrose; reactions of each of the hydroxylase isoforms used in the ToMO hydroxylase component assays; contributions to regiospecificity in the diiron enzyme toluene 4-yl monooxygenase. A hypothetical diagram depicting catalytic turnover of A of a bisubstrate reaction; and mathematical representation of the bisubstrate system. This material is available free of charge via the Internet at http://pubs.acs.org.

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 Notes

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■ ABBREVIATIONS USED

BMM, bacterial multicompartment monooxygenase; NAD(P)H, reduced nicotinamide adenine dinucleotide (also NADH) or reduced nicotinamide adenine dinucleotide phosphate; PT, proton transfer; ET, electron transfer; ToMO, toluene/o-xylene monooxygenase; ToMOH, hydroxylase component of ToMO; ToMOC, cofactorless regulatory component of ToMO; ToMOD, flavin-labeled ToMOD; ToMOD, Rieske-type ferredoxin of ToMO; ToMOF, NADH-oxidoreductase of ToMO; sMMO, soluble methane monooxygenase; sMMOH, hydroxylase component of sMMO; WT, wild type; C23O, catechol-2,3-dioxygenase; T4MO, toluene-4-monoxygenase; T4mO, hydroxylase component of T4MO; T4mOD, cofactorless regulatory component of T4MO; ToMOD, flavin-labeled ToMOD; Tris, tris(hydroxymethyl)-aminoethane; Bis-Tris, propane, bis(tris(hydroxymethyl)methylamino)-propane; ToMOCh, reduced ToMOC; ToMOOx, oxidized ToMOC; KSIE, kinetic solvent isotope effect.

■ REFERENCES


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