Supporting Information for

A Flexible Glutamine Regulates the Catalytic Activity of Toluene *o*-xylene Monooxygenase

Alexandria Deliz Liang, Alexandra T. Wrobel, and Stephen J. Lippard
Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139

Index

Protein Expression and Purification Methods	2-6
Preparation of ToMOD-Fl and Binding Studies	6-7
Anaerobic Stopped-Flow Preparation	8
Inverse Kinetic Solvent Isotope Effects, Q228E, Scheme S1	9-10
Figure S1. BMM Sequence Homology	11
Figure S2. Steady State Assays	12
Figure S3. Steady State Component Dependence of WT ToMOH	13
Figure S4. Steady State Phenol Dependence: H_2O vs. D_2O	14
Figure S5. Steady State Viscosity Dependence	15
Figure S6. Steady State ToMOC Dependence	16
Figure S7. Steady State pH Profiles	16
Figure S8. Redox Dependent Extinction Coefficients of ToMOC	17
Figure S9. Stopped-Flow Electron Transfer	17
Figure S10. Size-exclusion chromatograms for binding between ToMOD-Fl and hydroxylase variants	18
Scheme S2. Representative Catalytic Cycle of a Bisubstrate	19
Scheme S3. Mathematical Analysis of a Bisubstrate Enzyme	20

Protein Expression and Purification Methods

ToMOH Expression. BL21(Gold)-DE3 cells were transformed with pET22B(+)/touBEA, which encodes the three subunits of ToMOH from *Pseudomonas stutzeri*. 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.

Four 4-L flasks each containing two liters of LB media were autoclaved and cooled. To each of these flasks were added 0.4 g of ampicillin and 50 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 $O.D._{600}$ reached 0.6 (~2.5 h). At this time, the temperature was reduced to 18 °C. After 30 min, the temperature stabilized at 18 °C.

Expression was induced with 50 µM IPTG (isopropyl-β-D-1-thiogalactopyranoside) at 18 ºC. Ferrous ammonium sulfate hexahydrate was added over a period of 5 h to a final concentration of 625 µM. The cultures were left to shake at 18 ºC for 21 h. The cells were harvested by centrifugation at 11,325 g for 10 min. The resulting cell pellet was dropped piecewise in liquid nitrogen and stored at -80 ºC. 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 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 was added; lysozyme was added to 1 mg per ml of mixture; and PMSF

(phenylmethylsulfonyl fluoride) was added to a final concentration of 1 mM. The solution was stirred very slowly on ice for 3-4 h. The lysate was then centrifuged at 95,000 g for 45 min. The supernatant was decanted 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. 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 gel electrophoresis 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. The concentrated sample was applied to a Sephacryl S200 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. Fractions were analyzed by means of a UV-vis detector, SDS-page gel electrophoresis, and a catechol 2,3-dioxygenase-coupled activity assay. 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 ºC.

ToMOD Expression. BL21(Gold)-DE3 cells were transformed with pET22B(+)/touD. The starting culture was prepared as indicated in *ToMOH Expression* above. Two 4-L flasks each containing two liters of LB media were autoclaved and cooled. To each of these flasks, 0.4 g of ampicillin and 50 ml of the incubated 300 ml culture were added. The inoculated

cultures were set to shake at 150 rpm with a temperature of 37 ºC until the $O.D._{600}$ reached 0.6 (~2.5 h). Expression was induced with 50 µM IPTG. The cultures were left to shake at 37 ºC for 3 h. The cells were harvested by centrifugation at 11,325 g for 10 minutes. The resulting cell pellet was dropped piecewise in liquid nitrogen and stored at -80 ºC. 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, 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-vis chromatogram. Fractions between 250 and 450 ml into the elution were analyzed by SDS-page gel electrophoresis 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 3kDa MWCO centrifugal tubes at 4000 g. 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 through a UV-vis detector and SDS-page gel electrophoresis. ToMOD reproducibly eluted between 275 – 315 ml into the column isocratic gradient. These fractions were analyzed by SDS-page gel electrophoresis. Fractions

containing minimal impurities were pooled and concentrated as described above. The final protein was flash-frozen in liquid nitrogen and stored at -80 ºC.

ToMOC Expression. BL21(Gold)-DE3 cells were transformed with pET22B(+)/touC. The starting culture was prepared as indicated in *ToMOH Expression* above. Four 4-L flasks each containing two liters of LB media were autoclaved and cooled. To each of these flasks were added 0.4 g of ampicillin and 50 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 $O.D._{600}$ reached 0.6 (~2.5 h). Expression was induced with 50 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. 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 3kDa 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 between 300 – 350 ml into the column isocratic gradient. Fractions with an Abs(458/280) of greater than 0.17 were pooled. These fractions were analyzed by SDS-page gel electrophoresis. Fractions containing minimal impurities were pooled and concentrated as described above. The final protein was flash-frozen in liquid nitrogen and stored at -80 °C.

Preparation of a ToMOD-Fluorescein Construct (ToMOD-Fl) and Binding Studies

Reaction: 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.

Workup: 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-vis spectrum of each fraction was taken. Fractions 3-9 were pooled based on their Abs_{495} and the ratio of their Abs_{280} over Abs_{495} (see data below). The pooled fractions were concentrated in a 3K 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.

Binding Studies: 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. Representative fluorescence chromatograms and final binding data are shown in Figure S10.

Anaerobic Stopped-Flow Preparation

Instrument Preparation. To remove oxygen from the stopped-flow instrument, the system was flushed with degassed buffer containing ca. 5 mM sodium dithionite. The dithionite-containing buffer was incubated in the reaction lines overnight. Prior to each experiment, the stopped-flow lines were washed with dithionite-free, degassed buffer to remove any excess dithionite.

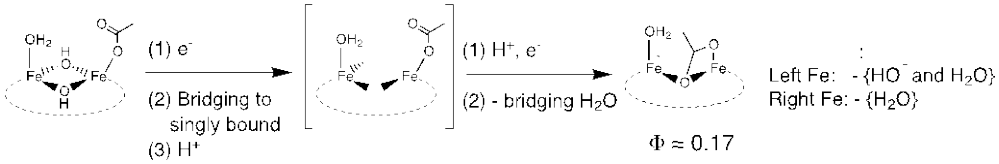
Sample Preparation. All protein samples were prepared in 50 mM potassium phosphate, pH 7.0, and 50 mM NaCl. Buffers were filtered through 0.22 µm membranes prior to use. Anaerobic buffer was prepared by purging a sealed solution with argon gas for 1-2 h. Protein solutions were degassed by cycling between vacuum and argon gas on a Schlenk-line for a minimum of ten cycles. The sealed protein samples and anaerobic buffers were immediately transferred into an anaerobic chamber under nitrogen atmosphere.

$ToMOC_{red}$ was prepared for stopped-flow experiments by titrating a concentrated solution of $ToMOC_{ox}$ with a solution ca. 0.5 mM sodium dithionite in buffer. Complete reduction was confirmed by watching the absorption change at 490 nm using an Eppendorf Biophotometer within the anaerobic, nitrogen atmosphere chamber. This wavelength was chosen based on those available using the Biophotometer. Prior to these experiments, the expected absorbance change within the visible range was determined by anaerobic colorimetric titration of ToMOC with phosphate buffered dithionite (Figure S8). The absorbance changes over time are shown for WT ToMOH and the three mutants in Figure S9.

Inverse Kinetic Solvent Isotope Effect with Respect to Q228E

For mononuclear cobalt,[1] iron,[2] and zinc,[3] dissociation of a hydroxide ion corresponds to a fractionation factor of 0.70. The magnitude of the observed isotope effect is a multiplicative function of total number of protons attached to the oxygen dissociating from the metal. If more than one water or hydroxide ion is dissociating, the total number of protons on all of the dissociating water molecules and hydroxide ions is multiplicative. For dissociation of one, two, and three water molecules from a mononuclear site of iron, inverse isotope effects of 0.49 (two protons: 0.70^2), 0.24, and 0.12, respectively, are expected.[1,2] If we adopt the mononuclear fractionation factors as a first approximation for water release from the diiron site in ToMOH, we get surprising agreement between the experimental (0.19) and predicted (0.17) values for the KSIE. The sequence of reactions depicted in Scheme S1 is proposed as a model for the chemistry underlying the observed inverse KSIE(k_{cat}) for Q228E, and a description of the predicted KSIE follows.

Scheme S1. Proposed Model for Water Dissociation from the Diiron Active Site of Q228E



In the first step, one hydroxide changes from bridging to singly bound, resulting in a KSIE for this micro-step of 0.70. In the process, the singly bound hydroxide becomes protonated, resulting in a singly bound water molecule (blue colored water molecule in Scheme S1). The remaining, bridging hydroxide (pink colored water molecule in Scheme S1) is protonated during the second electron transfer step to produce, transiently, a bridging water that dissociates from the diiron core. If we consider the two iron atoms 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 that results in (i) an inverse KSIE near 0.19, (ii) avoids high charge accumulation, and (iii) yields a mechanistically relevant final structure.

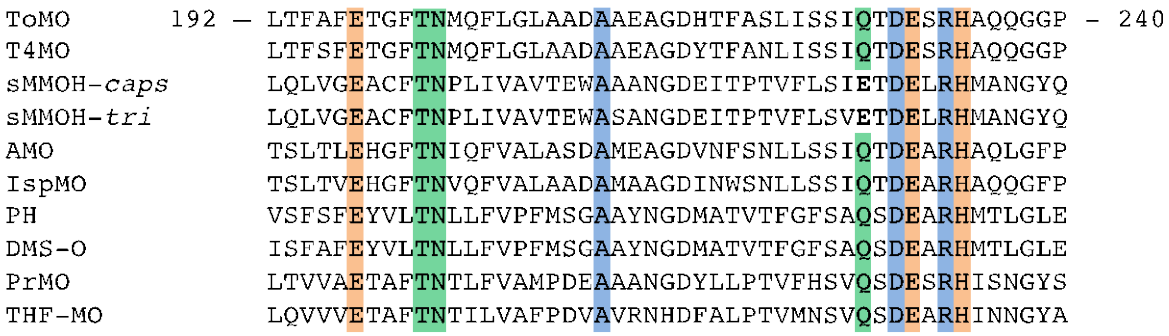


Figure S1. Sequence alignment of alpha subunits of the hydroxylase proteins within BMMs was completed with Clustal Omega. Amino acids responsible for coordination to the diiron site are highlighted in orange; highly conserved amino acids not involved in iron coordination are highlighted in blue. The three residues of the pore region—threonine, asparagine, and glutamine/glutamate—are highlighted in green.
ToMO = toluene/*o*-xylene monooxygenase of *Pseudomonas sp.* (AY621080)
T4MO = toluene-4-monooxygenase of *Pseudomonas mendocina* (AAA25999)
sMMO-*caps* = soluble methane monooxygenase of *Methylococcus capsulatus* (P22869)
sMMO-*tri* = soluble methane monooxygenase of *Methylococcus trichosporium* (AAZ81968)
AMO = alkene monooxygenase of *Xanthobacter autotrophicus* Py2 (CAA07366)
IspMO = isoprene monooxygenase of *Rhodococcus sp*. AD45 (CAB55825)
PH = phenol hydroxylase of *Pseudomonas putida* (AAA25942)
DMS-O = dimethylsulfide oxygenase of *Acinetobacter sp.* (BAA23333)
PrMO = propane monooxygenase of *Gordonia sp*. (BAD03956)
THF-MO = tetrahydrofuran monooxygenase of *Pseudonocardia tetrahydrofuranoxydans* (CAC10506)

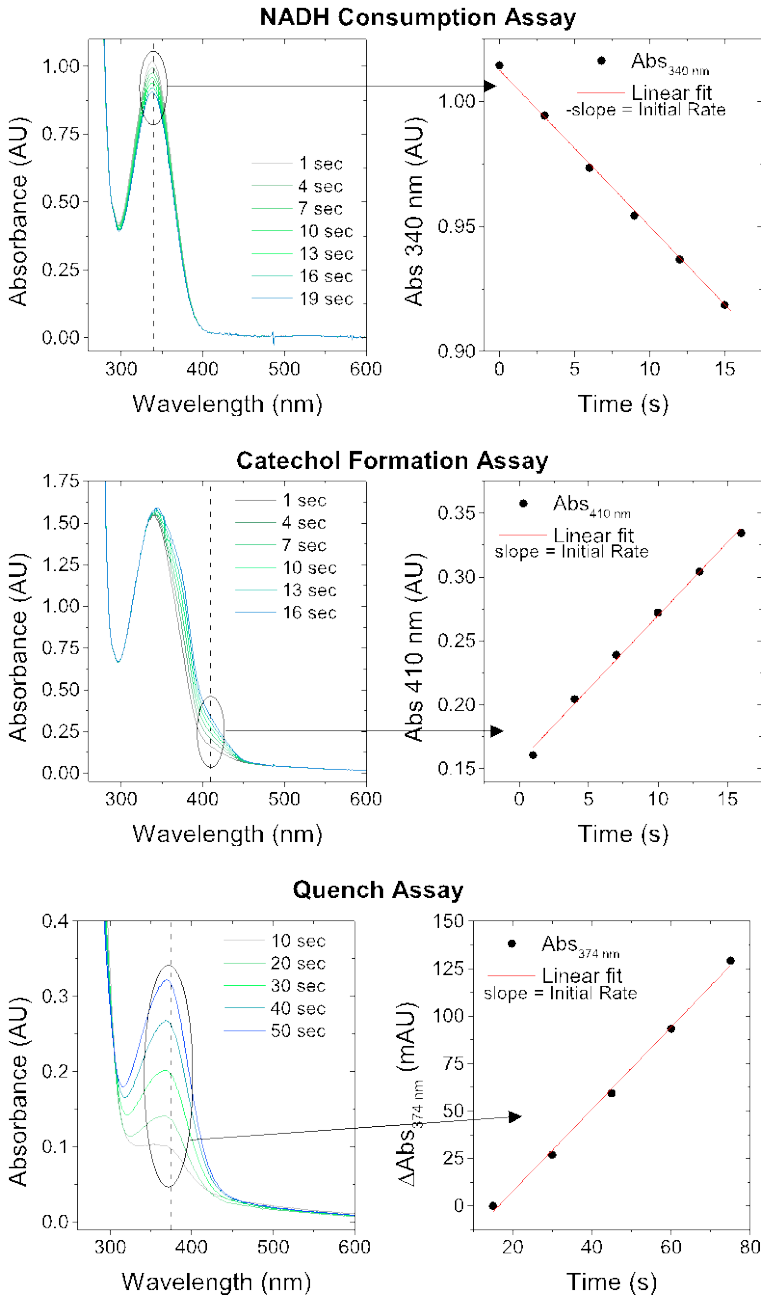


Figure S2. Graphical description of the NADH consumption (top), catechol formation (middle), and the quenched-activity (bottom) assays. The graphs on the left-hand side depict a typical absorbance profile observed during reactivity. These traces are transferred to a time-domain for either 340, 410, or 374 nm. The time-domain plots are used to derive the initial rate or velocity of reactivity. The absorbance points monitored are selected to minimize the contribution from other analytes in the reactions.

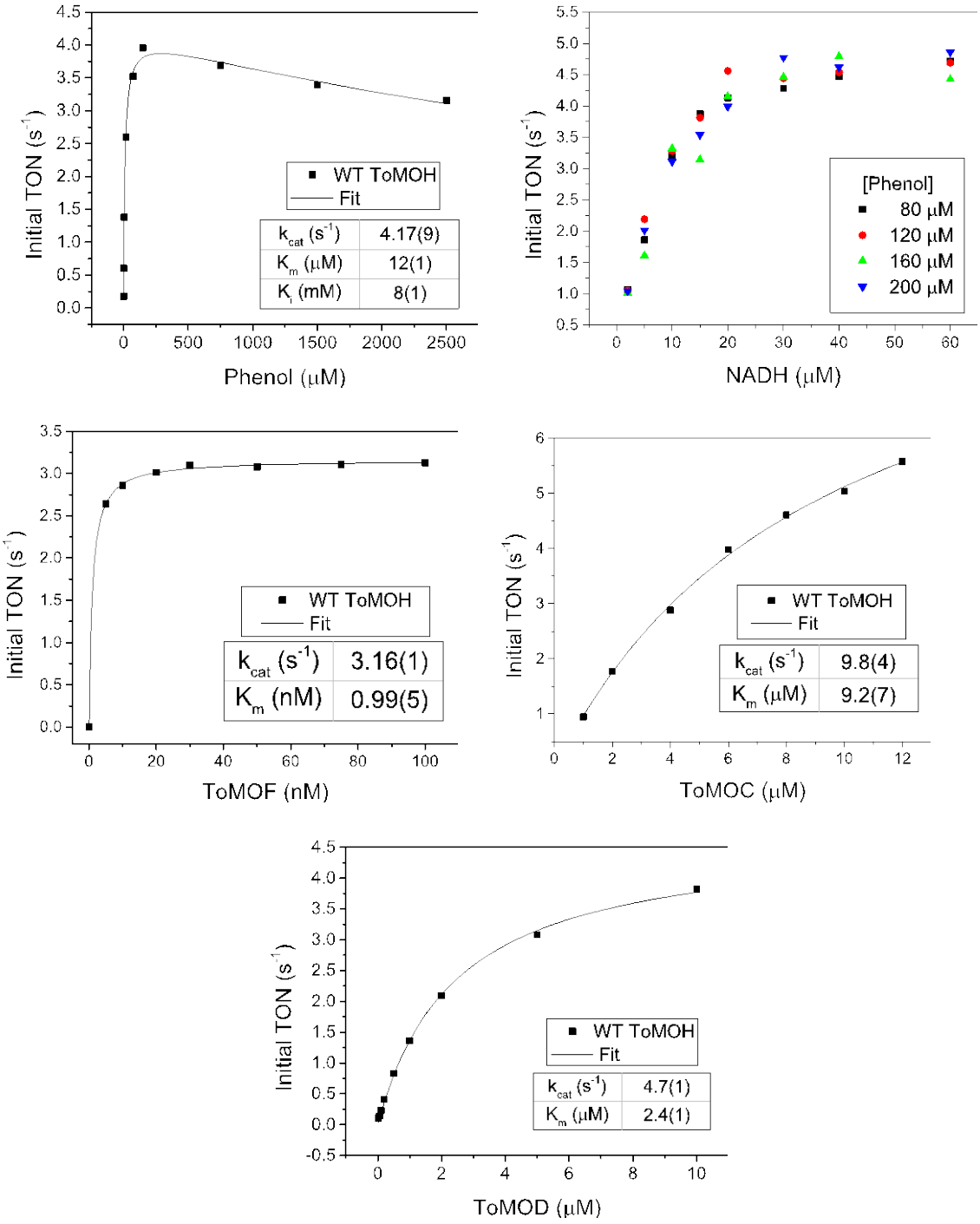


Figure S3. The component proteins used in the ToMO reactivity studies were systematically varied to determine their contributions to catalysis and the concentrations necessary to reach optimal catalytic activity. In all of these studies, the base concentrations of each component were as follows, except for the analyte varied in the given assay: 0.15 μM ToMOH, 6 μM ToMOD, 6 μM ToMOC, 0.06 μM ToMOF, 400 μM phenol, 200 μM NADH, and C23O. The reactions were studied by the catechol formation assay.

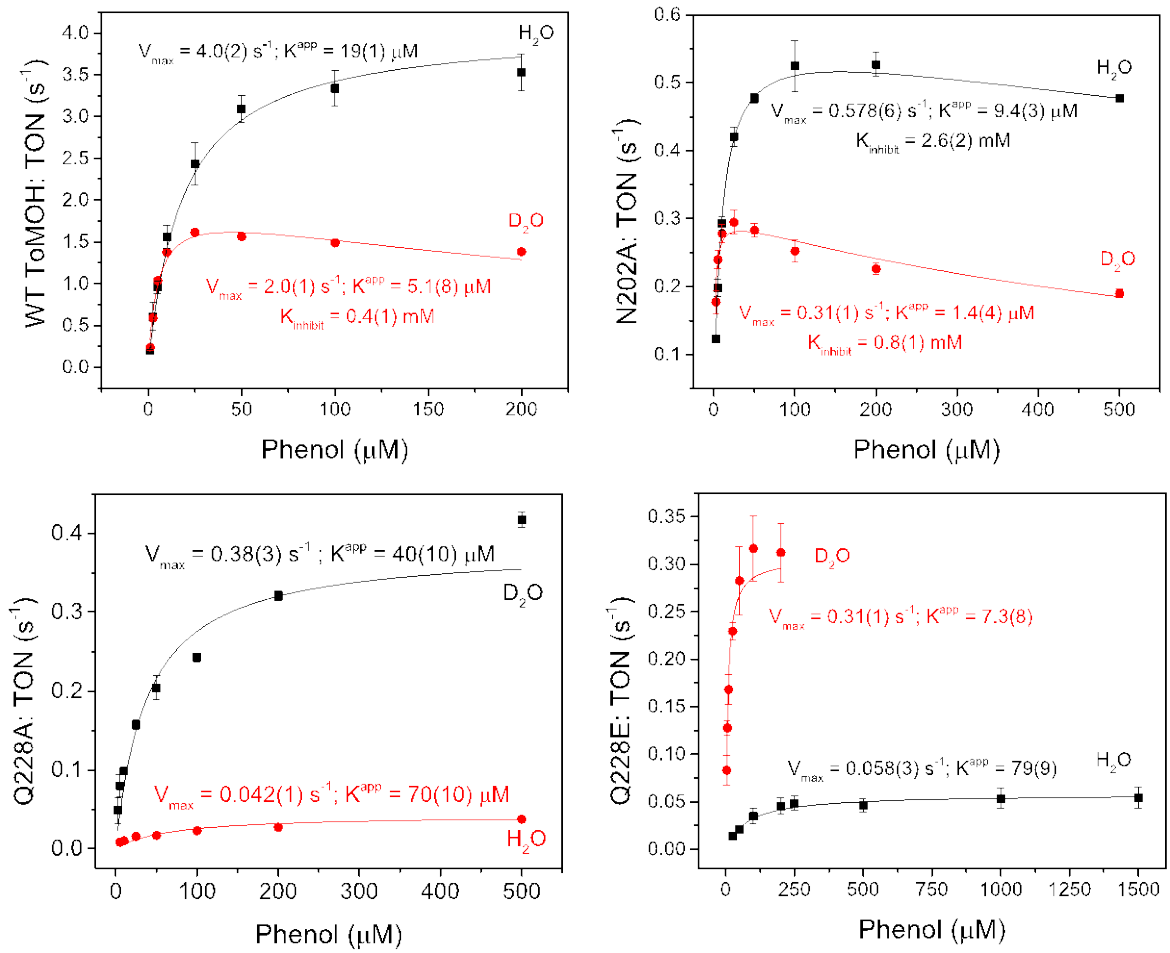


Figure S4. The reactions of each of the hydroxylase isoforms were studied in H_2O (black) and D_2O (red) by the catechol formation assay. In all of these studies, the component concentrations were as follows: 0.15 μM ToMOH, 6 μM ToMOD, 6 μM ToMOC, 0.06 μM ToMOF, 0-1500 μM phenol, 200 μM NADH, and C23O. The results are summarized in Table 1 of the main text.

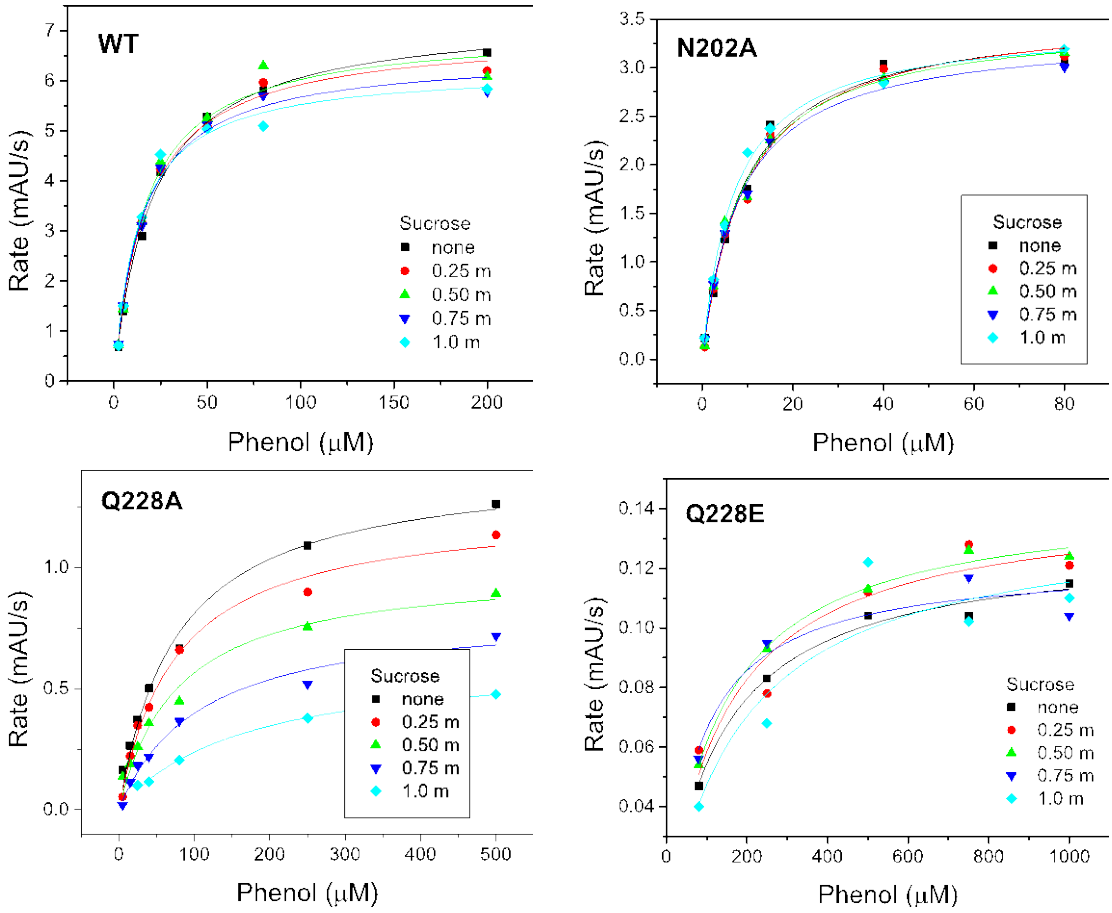


Figure S5. The un-normalized data of the hydroxylase isoforms in varying concentrations of sucrose. In all of these studies, the component concentrations were as follows: 0.15 μM ToMOH, 6 μM ToMOD, 6 μM ToMOC, 0.06 μM ToMOF, 0-1000 μM phenol, 200 μM NADH, and C23O. The reactions were studied by the catechol formation assay. The data are normalized for clarity in Figure 2 of the main text.

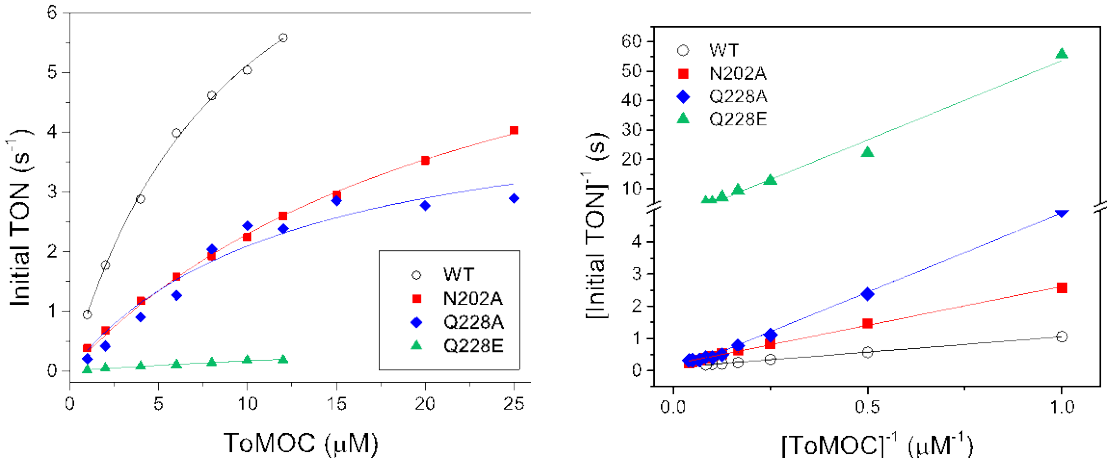


Figure S6. The reactions of each of the hydroxylase isoforms were studied with varying concentration of ToMOC by the catechol formation assay. In all of these studies, the component concentrations were as follows: 0.15 μM ToMOH, 6 μM ToMOD, 1-25 μM ToMOC, 0.06 μM ToMOF, 400 μM phenol, 200 μM NADH, and C23O. The reactions were studied by the catechol formation assay. The fitted parameters are summarized in Table 2 of the main text.

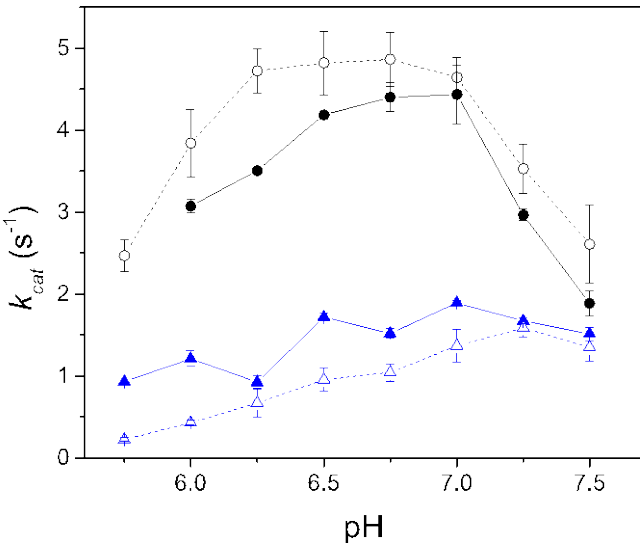


Figure S7. The results from the quenched-activity (filled shapes) and the NADH (open shapes) assays. The unnormalized data of the hydroxylase isoforms in varying concentrations of sucrose. In all of these studies, the component concentrations were as follows: 0.15 μM ToMOH, 6 μM ToMOD, 6 μM ToMOC, 0.06 μM ToMOF, 200-500 μM phenol, and 200 μM NADH. The data are normalized and quantitatively analyzed in Figure 3 of the main text.

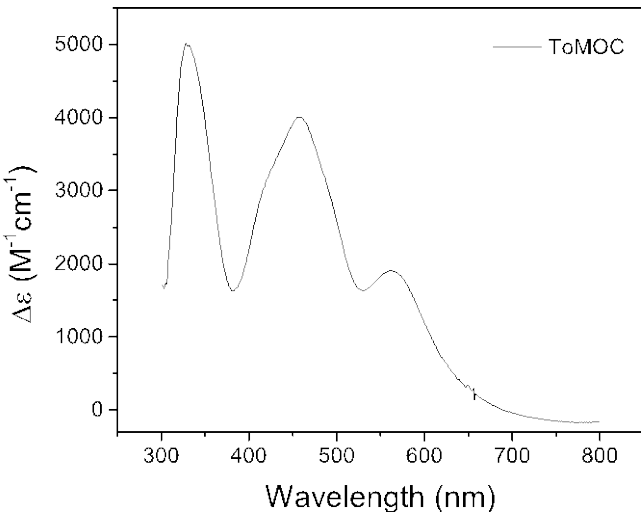


Figure S8. The change of extinction coefficient of ToMOC upon reduction with phosphate buffered dithionite, pH 7.0. Peaks corresponding to 458 nm and 565 nm are within the visible range and were observed to determine the rate of re-oxidation by the hydroxylase.

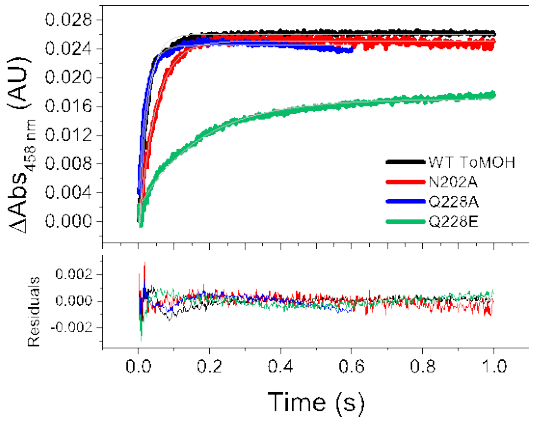


Figure S9. The change of absorbance at 458 nm upon mixing $ToMOC_{red}$ with an oxidized hydroxylase isoform. The final protein concentrations were 10 µM ToMOC and 100 µM ToMOH. The fit parameters are shown in Table 1 of the main text.

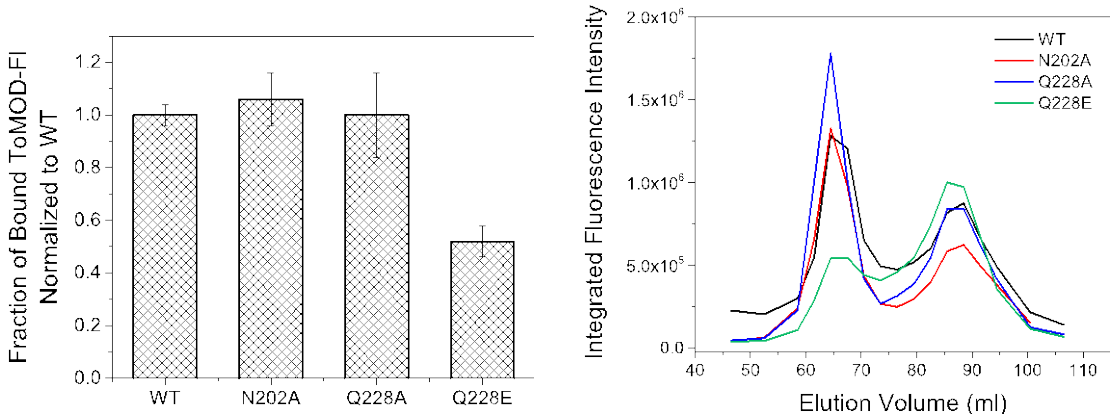
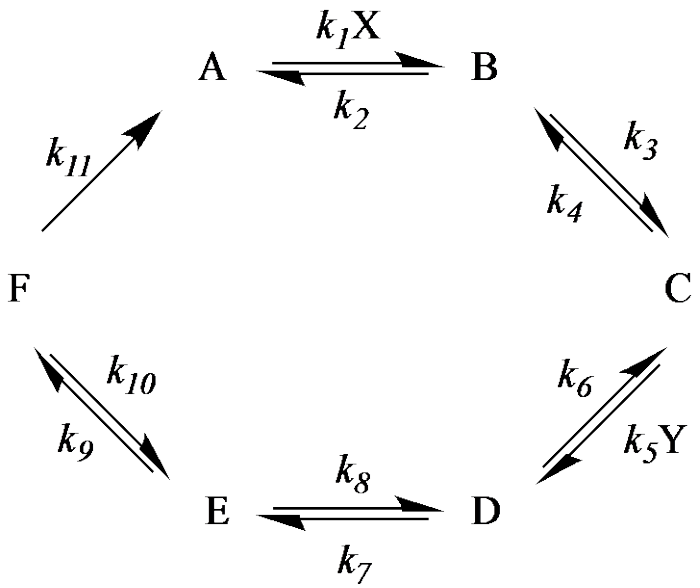


Figure S10. Size-exclusion chromatograms for binding between ToMOD-Fl and hydroxylase variants. Left: Fraction of ToMOD-Fl bound to each hydroxylase variant relative to WT ToMOH. Right: Representative fluorescence chromatograms for size exclusion chromatography of the pre-mixed solution of ToMOD-Fl and each hydroxylase.



Scheme S2. A hypothetical diagram depicting catalytic turnover of A of a bisubstrate reaction. A is the resting state of the catalyst; X and Y are substrates; B, C, D, E, and F are catalytic intermediates. A mathematical interpretation is given in Scheme 1 of the Supporting Information (next page).

(A)

$$\frac{1}{v} = \frac{\begin{array}{c}[k_2k_4k_6k_8(k_{10}+k_{11})]+[k_4k_9k_{11}(k_6+k_7)(k_1+k_2)]+[k_1k_6k_8(k_3+k_4)(k_{10}+k_{11})+k_1k_3k_9k_{11}(k_6+k_7)]\cdot[X] \\ +[k_5k_7k_9k_{11}(k_2+k_3)]\cdot[Y]+[k_1k_3k_5(k_7+k_8)(k_{10}+k_{11})+k_1k_5k_9(k_3k_7+k_3k_{11}+k_7k_{11})]\cdot[X][Y]\end{array}}{k_1k_3k_5k_7k_9k_{11}\cdot[X][Y][E_t]}$$

(B)

if $k_4 = 0$

$$\frac{1}{v} = \left(\frac{1}{[X]}\right)\cdot\left(\frac{(k_2+k_3)}{k_1k_3[E_t]}\right)+\frac{\begin{array}{c}[k_3k_6k_8(k_{10}+k_{11})+k_3k_9k_{11}(k_6+k_7)]+ \\ [k_3k_5(k_7+k_8)(k_{10}+k_{11})+k_5k_9(k_3k_7+k_3k_{11}+k_7k_{11})]\cdot[Y]\end{array}}{k_3k_5k_7k_9k_{11}[Y][E_t]}$$

$$\frac{1}{v} = \left(\frac{1}{[Y]}\right)\cdot\left(\frac{[k_6k_8(k_{10}+k_{11})+k_9k_{11}(k_6+k_7)]}{k_5k_7k_9k_{11}[E_t]}\right)+\frac{\begin{array}{c}[k_5k_7k_9k_{11}(k_2+k_3)]+ \\ [k_1k_3k_5(k_7+k_8)(k_{10}+k_{11})+k_1k_5k_9(k_3k_7+k_3k_{11}+k_7k_{11})]\cdot[X]\end{array}}{k_1k_3k_5k_7k_9k_{11}[X][E_t]}$$

(C)

if $k_8 = 0$

$$\frac{1}{v} = \left(\frac{1}{[X]}\right)\cdot\left(\begin{array}{c}[k_4(k_6+k_7)(k_1+k_2)] \\ +[k_5k_7(k_2+k_3)]\cdot[Y] \\ \hline k_1k_3k_5k_7[Y][E_t]\end{array}\right)+\frac{[k_1k_3k_9k_{11}(k_6+k_7)]+[k_1k_3k_5k_7(k_{10}+k_{11})+k_1k_5k_9(k_3k_7+k_3k_{11}+k_7k_{11})]\cdot[Y]}{k_1k_3k_5k_7k_9k_{11}[Y][E_t]}$$

$$\frac{1}{v} = \left(\frac{1}{[Y]}\right)\cdot\left(\begin{array}{c}[k_4(k_6+k_7)(k_1+k_2)] \\ +[k_1k_3(k_6+k_7)]\cdot[X] \\ \hline k_1k_3k_5k_7[X][E_t]\end{array}\right)+\frac{[k_7k_9k_{11}(k_2+k_3)]+[k_1k_3k_7(k_{10}+k_{11})+k_1k_9(k_3k_7+k_3k_{11}+k_7k_{11})]\cdot[X]}{k_1k_3k_5k_7k_9k_{11}[X][E_t]}$$

Scheme S3. A mathematical representation of the bisubstrate system shown in Diagram 1. (A) Mathematical representation of the initial rate. (b) Two representations of the initial rate if $k_4 = 0$, causing the first irreversible step to occur after X binding and before Y binding. (c) Two representations of the initial rate if $k_8 = 0$, causing the first irreversible step to occur after both X and Y bind.

Literature Cited

(1) Kassebaum, J. W., and Silverman, D. N. (1989) Hydrogen/Deuterium Fractionation Factors of the Aqueous Ligand of Cobalt in $Co(H_2O)_6^{2+}$ and Co(II)-Substituted Carbonic Anhydrase. *J. Am. Chem. Soc. 111*, 2691-2696.

(2) Hangasky, J. A., Saban, E., and Knapp, M. J. (2013) Inverse Solvent Isotope Effects Arising from Substrate Triggering in the Factor Inhibiting Hypoxia Inducible Factor. *Biochemistry 52*, 1594-1602.

(3) Cook, P. F. (1991) *Enzyme Mechanism from Isotope Effects. p40-42, 83-116, 160,* CRC Press, Boca Raton.