Investigation of the Effects of Mutating Iron-Coordinating Residues in Rieske Dioxygenases

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Abstract

Rieske dioxygenases are multi-component enzyme systems, naturally found in many soil bacteria, that have been widely applied in the production of fine chemicals, owing to the unique and valuable oxidative dearomatization reactions they catalyze. The range of practical applications for these enzymes in this context has historically been limited, however, due to their limited substrate scope and strict selectivity. To overcome these limitations, our research group has employed the tools of enzyme engineering to expand the substrate scope or improve the reactivity of these enzyme systems in specific contexts. Traditionally, enzyme engineering campaigns targeting metalloenzymes have avoided mutations to metal-coordinating residues, based on the assumption that these residues are essential for enzyme activity. Inspired by the success of other recent enzyme engineering reports, our research group investigated the potential to alter or improve the reactivity of Rieske dioxygenases by altering or eliminating iron coordination in the active site of these enzymes. Herein, we report the modification of all three iron-coordinating residues in the active site of toluene dioxygenase both to alternate residues capable of coordinating iron, and to a residue that would eliminate iron coordination. The enzyme variants produced in this way were tested for their activity in the *cis*-dihydroxylation of a small library of potential aromatic substrates. The results of these studies demonstrated that all three iron-coordinating residues, in their natural state, are essential for enzyme activity in toluene dioxygenase, as the introduction of any mutations at these sites resulted in a complete loss of *cis*-dihydroxylation activity for all substrates tested.

Keywords: Rieske dioxygenase, toluene dioxygenase, oxidative dearomatization, green chemistry, enzyme engineering, bioremediation

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Introduction

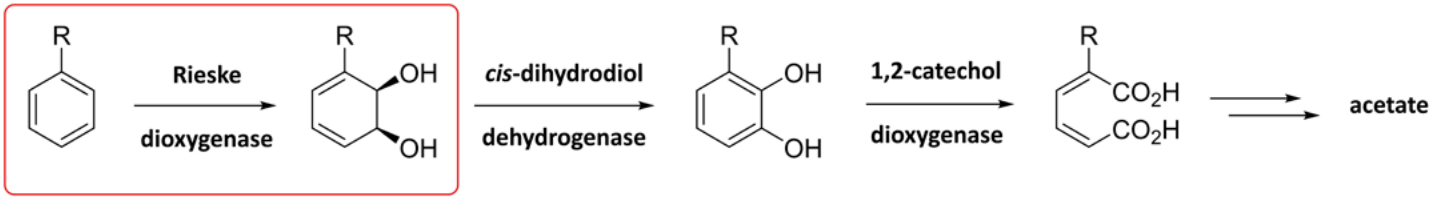
In recent years, the environmentally deleterious effects of the chemical industry have increasingly been recognized, leading to a concerted effort to employ more sustainable methods for the production of fine chemicals (19, 36). These environmentally sustainable chemical methods have collectively been referred to as "green chemistry", which has been defined by a series of principles that serve to guide the application of sustainable chemistry (15, 34). Owing to this increased focus on green chemistry, the application of enzymes as catalysts in the chemical industry has grown in popularity (7). Enzymatic catalysts are entirely biodegradable, they operate in aqueous media as opposed to petroleum-derived solvents, they do not require high-temperature applications, and they are produced from renewable resources, complying with many of the principles of green chemistry (15, 34).

One class of enzymes that have been widely applied as catalysts in the production of fine chemicals are the Rieske dioxygenases (16, 23). Rieske dioxygenases are non-heme iron dioxygenases that are commonly found in soil bacteria (39). These enzymes have frequently evolved as a means for soil bacteria to metabolize aromatic pollutants in their environment and to use these pollutants as carbon and energy sources (**Figure 1**) (12, 42). The ability of these enzymes to catalyze the *cis*-dihydroxylation of aromatics to produce chemically versatile *cis*-diene-diol metabolites with high selectivity has made them very useful catalysts in the production of valuable compounds (16, 23).

Rieske dioxygenases are produced by a wide range of soil bacteria strains, including *Pseudomonas* (42), *Burkholderia* (6), *Nocardioides* (32), and *Comamonas* (22). In fact, metagenomic sequencing studies have revealed that aromatic dioxygenases are ubiquitous, particularly in contaminated environments (17). Owing to their natural role in the remediation of aromatics in the soil and therefore the detoxification of these environments (12,42), these enzymes play crucial roles in soil ecology. Because of this role for Rieske dioxygenases, these enzymes also have tremendous potential for utility in the field of bioremediation, the use of microorganisms to degrade anthropogenic pollutants in an environment. Aromatic pollutants such as benzene and toluene are ubiquitous in the environment and pose significant threats to human health, as these compounds are often

Figure 1

Metabolic pathway by which soil organisms break down aromatic pollutants in their environment (12, 42).



Note. The role of Rieske dioxygenases in this metabolic pathway is highlighted (red).

carcinogenic and endocrine disruptors (4). These aromatic pollutants are frequently introduced into the environment through fossil fuel combustion, oil spills, and the misuse of petroleum products and solvents, creating a need for a means to remove these toxic compounds from the environment (4). To this end, Rieske dioxygenases have been employed as bioremediation catalysts, including applications in the degradation of polychlorinated biphenyls (PCBs), which remain a hazardous presence in many environments (14, 21).

Despite their applications in chemical synthesis and in bioremediation, the utility of Rieske dioxygenases in these contexts has remained limited by their strict selectivity and by their finite substrate scopes. As many Rieske dioxygenases have evolved to metabolize non-polar aromatics, their active sites are organized to effectively bind non-polar substrates and orient them for 2,3-dihydroxylation (9, 18, 28). This results in the substrate scopes and the activities of these enzymes being restricted by the size and by the electronics of any potential substrates (10, 35). To alleviate these restrictions on the utility of Rieske dioxygenases, multiple research groups have applied the tools of enzyme engineering to improve their reactivity or to expand their substrate scopes (5, 3, 11, 20, 26, 33, 37, 38). This has included studies that have engineered Rieske dioxygenases specifically to improve their utility in the bioremediation of aromatic pollutants in the soil (1, 21). Our lab has recently reported the development of improved Rieske dioxygenase variants through the application of rational enzyme engineering (27). These studies have led to the development of Rieske dioxygenases with improved reactivity or expanded substrate scopes, increasing the practical utility of these green chemical tools (1, 3, 5, 11, 20, 21, 26, 27, 33, 37, 38).

When engineering metalloenzymes, studies have traditionally avoided mutations to the residues responsible for coordinating metal ions, based upon the assumption that these residues are essential for enzyme activity. Recently, however, studies have shown this assumption to be incorrect (29, 40). These studies have shown that mutations altering, or even eliminating, iron coordination in heme proteins can alter or improve the reactivity of these proteins in specific contexts (29, 40). Our laboratory was inspired by these studies to investigate whether a similar strategy could result in the development of improved Rieske dioxygenase variants. To our knowledge, no such study has been reported for Rieske dioxygenases, thus this investigation would serve to inform future engineering studies performed with this enzyme class. Based upon reported results with other metalloenzymes (29, 40), it is predicted that mutating the iron-coordinating residues of a Rieske dioxygenase enzyme will result in altered enzyme activity and/or substrate scope. This strategy has the potential to improve the activity of Rieske dioxygenases for specific substrates or expand their substrate scopes.

Materials and Methods

General Experimental

E. coli BL21 (DE3) competent cells were obtained from ThermoFisher. Plasmid isolation/purification was performed using New

Table 1

Sequences of mutagenic primers used.

Primer Name	Primer Sequence
TDO H222A fwd	CGACATGTAC**GCG**GCCGGGACGACCTCGCATCTGTCTGGCATCCTG
TDO H222A rev	GTCGTCCCGGC**CGC**GTACATGTCGCTGCAAAACTGCTCTGCGGCG
TDO H222C fwd	CGACATGTAC**TGC**GCCGGGACGACCTCGCATCTGTCTGGCATCCTG
TDO H222C rev	GTCGTCCCGGC**GCA**GTACATGTCGCTGCAAAACTGCTCTGCGGCG
TDO H222D fwd	CGACATGTAC**GAT**GCCGGGACGACCTCGCATCTGTCTGGCATCCTG
TDO H222D rev	GTCGTCCCGGC**ATC**GTACATGTCGCTGCAAAACTGCTCTGCGGCG
TDO H222E fwd	CGACATGTAC**GAA**GCCGGGACGACCTCGCATCTGTCTGGCATCCTG
TDO H222E rev	GTCGTCCCGGC**TTC**GTACATGTCGCTGCAAAACTGCTCTGCGGCG
TDO H228A fwd	GACGACCTCG**GCG**CTGTCTGGCATCCTGGCAGGCCTGCCAGAAGAC
TDO H228A rev	GATGCCAGACAG**CGC**CGAGGTCGTCCCGGCATGGTACATGTCGCTGC
TDO H228C fwd	GACGACCTCG**TGC**CTGTCTGGCATCCTGGCAGGCCTGCCAGAAGAC
TDO H228C rev	GATGCCAGACAG**GCA**CGAGGTCGTCCCGGCATGGTACATGTCGCTGC
TDO H228D fwd	GACGACCTCG**GAT**CTGTCTGGCATCCTGGCAGGCCTGCCAGAAGAC
TDO H228D rev	GATGCCAGACAG**ATC**CGAGGTCGTCCCGGCATGGTACATGTCGCTGC
TDO H228E fwd	GACGACCTCG**GAA**CTGTCTGGCATCCTGGCAGGCCTGCCAGAAGAC
TDO H228E rev	GATGCCAGACAG**TTC**CGAGGTCGTCCCGGCATGGTACATGTCGCTGC
TDO D376A fwd	CGAGCAGGAC**GCG**GGGGAGAACTGGGTCGAGATCCAGCACATCCTG
TDO D376A rev	CAGTTCTCCCC**CGC**GTCCTGCTCGAACACGCCACCGGCAGAGAAGG
TDO D376C fwd	CGAGCAGGAC**TGC**GGGGAGAACTGGGTCGAGATCCAGCACATCCTG
TDO D376C rev	CAGTTCTCCCC**GCA**GTCCTGCTCGAACACGCCACCGGCAGAGAAGG
TDO D376E fwd	CGAGCAGGAC**GAA**GGGGAGAACTGGGTCGAGATCCAGCACATCCTG
TDO D376E rev	CAGTTCTCCCC**TTC**GTCCTGCTCGAACACGCCACCGGCAGAGAAGG
TDO D376H fwd	CGAGCAGGAC**CAT**GGGGAGAACTGGGTCGAGATCCAGCACATCCTG
TDO D376H rev	CAGTTCTCCCC**ATG**GTCCTGCTCGAACACGCCACCGGCAGAGAAGG

England Biolabs Monarch® miniprep kit. Transformations of electrocompetent cells were performed on an Eppendorf Eporator®. Whole-cell assay cultures were grown in Greiner Bio-One polystyrene clear, round-bottom 96-well plates. All cultures were incubated in a Barnstead MaxQ 4000 Digital Orbital Incubator Shaker equipped with an Enzyscreen universal clamp system unless otherwise stated. Fluorescence analyses were performed using a Biotek® Synergy™ H1 monochromator-based multi-mode plate reader, using Corning® polystyrene black, opaque, flat-bottom 96-well plates. All reagents were obtained from MilliporeSigma unless otherwise stated. Media were made at pH 7.2 and streptomycin was added at 50 μg mL^{-1}. All *E. coli* cultures were maintained at 37 °C unless otherwise stated.

Targeted Mutagenesis Protocol

The pCP-02 expression system was used as the template for toluene dioxygenase mutant generation (30). Saturation mutagenesis was performed following the polymerase chain reaction (PCR)-based procedure of Liu and Naismith (24, 41). Amplification was performed using an ABI GeneAmp® 9700 Thermal Cycler and Q5® DNA polymerase (New England Biolabs). Mutagenic primers were designed according to the procedure of Liu and Naismith (24, 41). Primer sequences are shown below (**Table 1**). Parameters for the relevant PCR reactions are shown below (**Table 2**). Sequencing analyses were performed by Eurofins Genomics© (Louisville, KY).

Whole-cell fermentation 96 well-plate assay protocol

E. coli (BL21 (DE3)) electrocompetent cells were transformed with isolated pCP-02 plasmids expressing toluene dioxygenase (parent and/or mutant libraries), and with isolated pCP-01 plasmids as negative controls (30). The transformation cultures were selected on LB + streptomycin plates overnight. Single colonies were inoculated into 160 μL LB + streptomycin media with 0.3% glucose in a 96-well round bottom seed plate and incubated

Table 2

PCR reaction components and thermocycling protocol utilized for the generation of toluene dioxygenase mutants.

PCR reaction components	Thermocycling protocol
Sterile H2O – 22.5 μL	98 °C – 30 s
Q5® reaction buffer – 10 μL	98 °C – 10 s
Q5® high GC enhancer – 10 μL	72 °C – 4 min } x20
dNTPs (10 mM) – 1 μL	72 °C – 2 min
Forward primer (10 μM)- 2.5 μL	
Reverse primer (10 μM)- 2.5 μL	
Template DNA (pCP-02) – 1 μL	
Q5® DNA polymerase – 0.5 μL	
Total – 50 μL	

with shaking overnight. All plates included 3 or more wells containing *E. coli* (BL21 (DE3)) pCP-02 cells expressing the parent toluene dioxygenase enzyme, and 3 or more wells containing *E. coli* (BL21 (DE3)) pCP-01 (negative control) (30). Seed plates were used to inoculate 5 μL into 155 μL LB media containing streptomycin in a fresh 96-well round bottom assay plate, and the cultures were incubated with shaking for 2.75 h. The assay plates were then pelleted, and the supernatant discarded. Cultures were resuspended in 150 μL minimal media (KH_2PO_4 – 7.5 g L^{-1}; citric acid – 2 g L^{-1}; $MgSO_4 \cdot 7H_2O$ – 5 g L^{-1}; trace metal solution – 2 mL L^{-1} [Na_2SO_4 – 1 g L^{-1}; $MnSO_4$ – 2 g L^{-1}; $ZnCl_2$ – 2 g L^{-1}; $CoCl_2 \cdot 6H_2O$ – 2 g L^{-1}; $CuSO_4 \cdot 5H_2O$ – 0.3 g L^{-1}; $FeSO_4 \cdot 7H_2O$ – 10 g L^{-1}; pH 1.0]; conc. H_2SO_4 – 1.2 mL L^{-1}; ferric ammonium citrate – 0.3 g L^{-1}; glucose – 4 g L^{-1}; thiamine – 0.034 g L^{-1}; pH 7.2) (8) containing streptomycin and incubated for a 1 h recovery period. Following this, the cultures were induced to a final concentration of 0.5 mM IPTG and the incubation temperature was reduced to 30 °C. After a 2 h induction period, aromatic substrates were added as 68 mM stock solutions in DMSO to a final concentration of 2 mM. Cultures were incubated with aromatic substrates for 1.5 h at 30 °C, after which the cultures were pelleted. A 100 μL portion of supernatant from each well was transferred to 96-well black opaque assay plates. The reaction was initiated by adding a 50 μL of $NaIO_4$ stock solution to each well to a final concentration of 10 mM, and the assay plates were incubated with shaking at room temperature for 30 min. Cleaved diols were detected by adding 50 μL of fluoresceinamine solution (prepared with 3 μL conc. HCl (11.65 M)/1 mL fluoresceinamine solution) to each well to a final concentration of 0.1 mM (30). Assay plates were incubated with shaking at room temperature for 5 h. The fluorescence response from each well was analyzed at 485 nm (ex), 520 nm (em), and normalized to the mean fluorescence response of the negative controls ($[I - I_0]/I_0$).

Results

Identification of Iron-Coordinating Residues

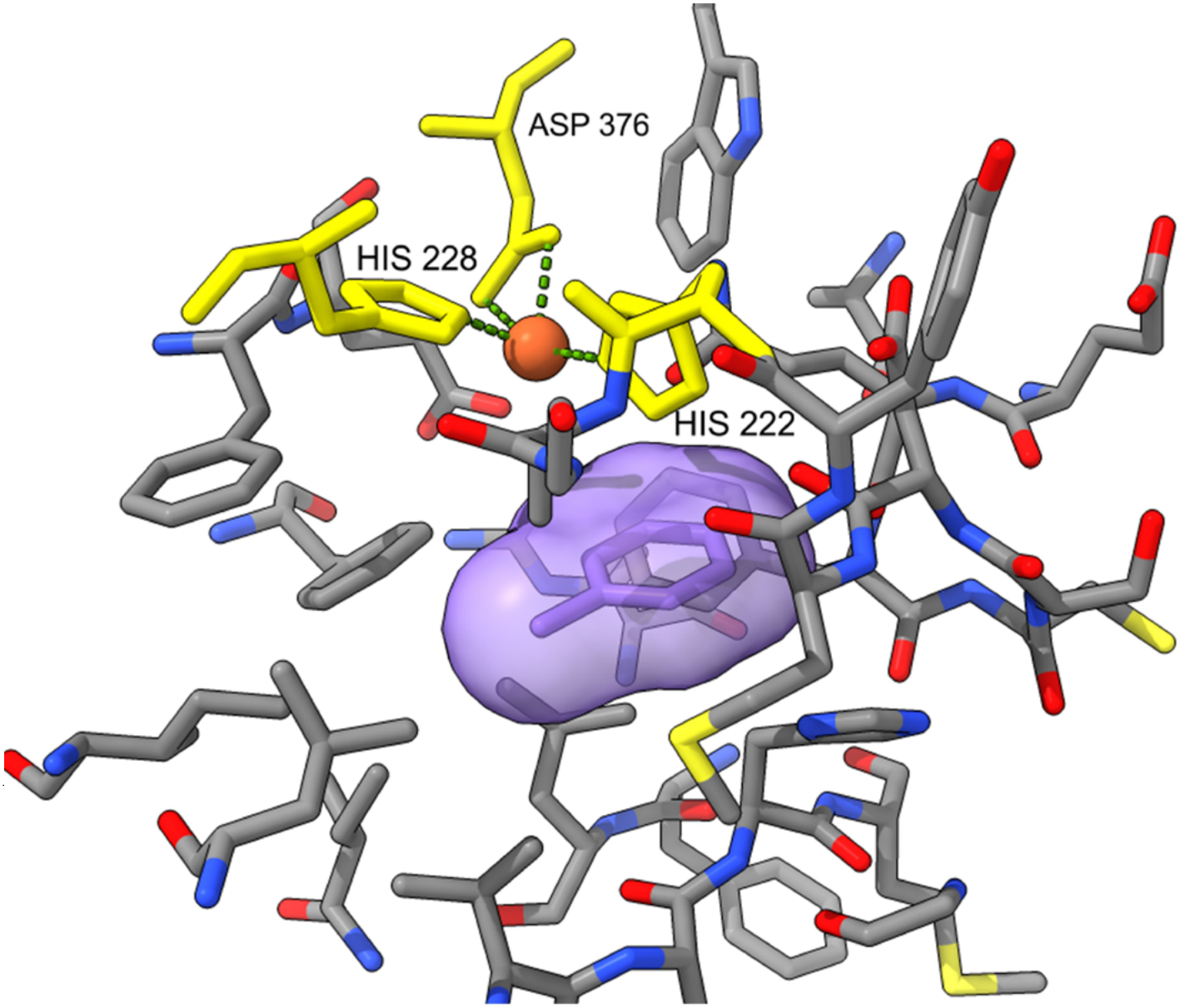
Because our lab has extensive experience working with toluene dioxygenase (TDO) (27, 30), and because TDO has been one of the most widely used Rieske dioxygenases in the field of organic synthesis (16), this enzyme was selected as the template for mutagenesis in this study. To identify the residues that would be targeted for mutagenesis, the reported crystal structure of toluene dioxygenase was analyzed using the molecular visualization software ChimeraX (9, 13). This analysis revealed that the catalytic iron atom of toluene dioxygenase is coordinated by two histidine residues (HIS222 and HIS228) and by one aspartate residue (ASP376) (**Figure 1**). These residues were therefore determined to be the appropriate targets for mutagenesis in this study.

Production of Targeted Toluene Dioxygenase Variants

To test the hypothesis of this study, it was determined that each iron-coordinating residue of TDO would be mutated to three alternate residues capable of coordinating iron (aspartate, glutamate, and cysteine for native histidine residues; glutamate, cysteine, and histidine for the native aspartate residue) and to one residue that would eliminate iron-coordination at that

Figure 2

Visualization of the TDO active site with bound substrate (toluene, purple) (9).



site (alanine). To generate the desired variants of toluene dioxygenase, targeted mutations were introduced through the PCR-based method of Liu and Naismith (24, 41). Mutagenic primers were designed according to this well-established protocol (**Table 1**), and the corresponding mutagenic PCR reactions were carried out as described. The successful introduction of the targeted mutations was confirmed through sequencing analysis performed by a third-party contractor. Representative aligned sequencing data is shown for the H222 mutants in **Figure 3**. Upon completion of this work, expression systems had been developed for twelve novel toluene dioxygenase variants with mutations introduced at the iron-coordinating residues (H222A, H222C, H222D, H222E, H228A, H228C, H228D, H228E, D376A, D376C, D376E, D376H).

Figure 3

Multiple sequence alignment of sequencing data from TDO variants with single active site mutations.

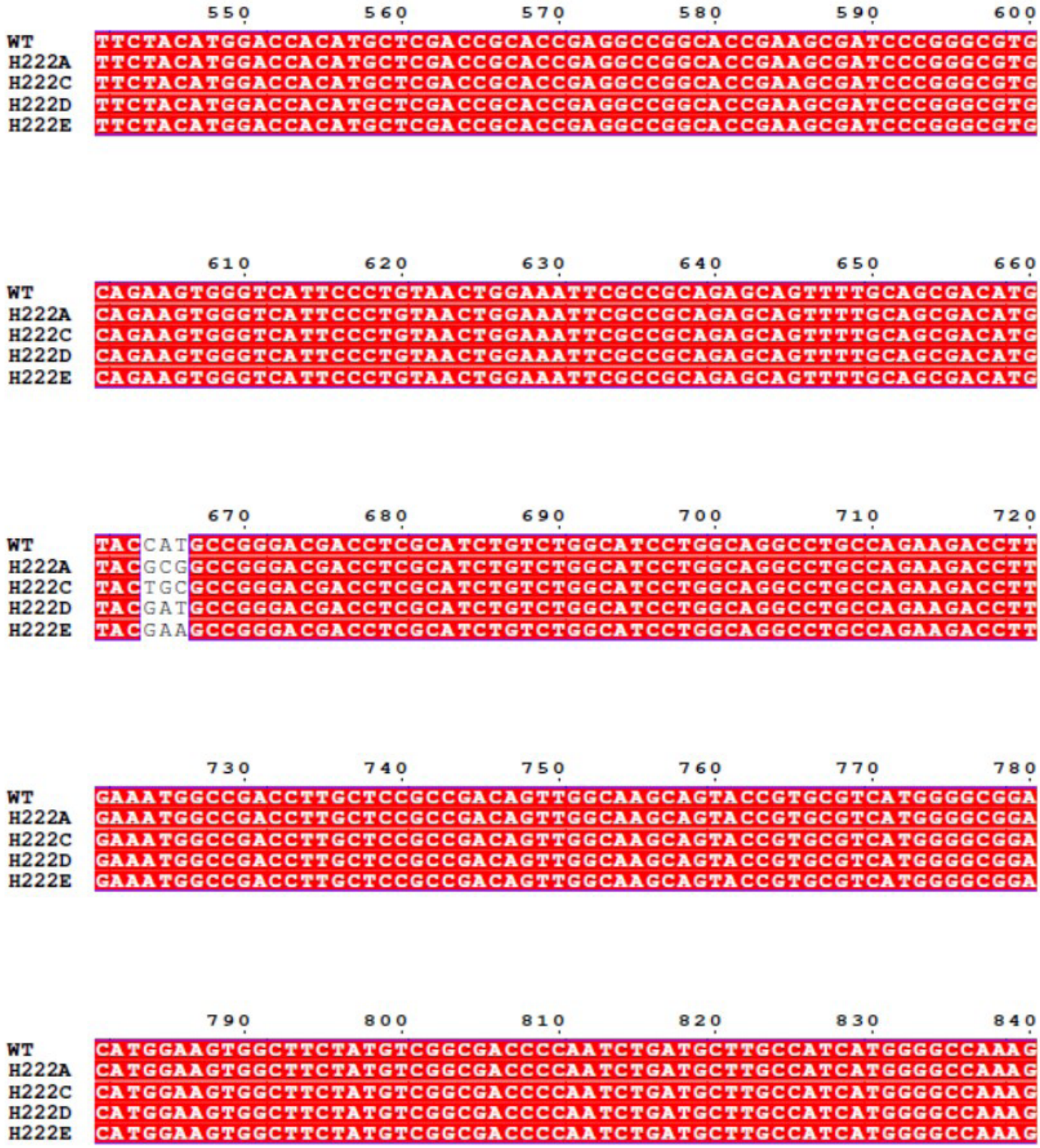
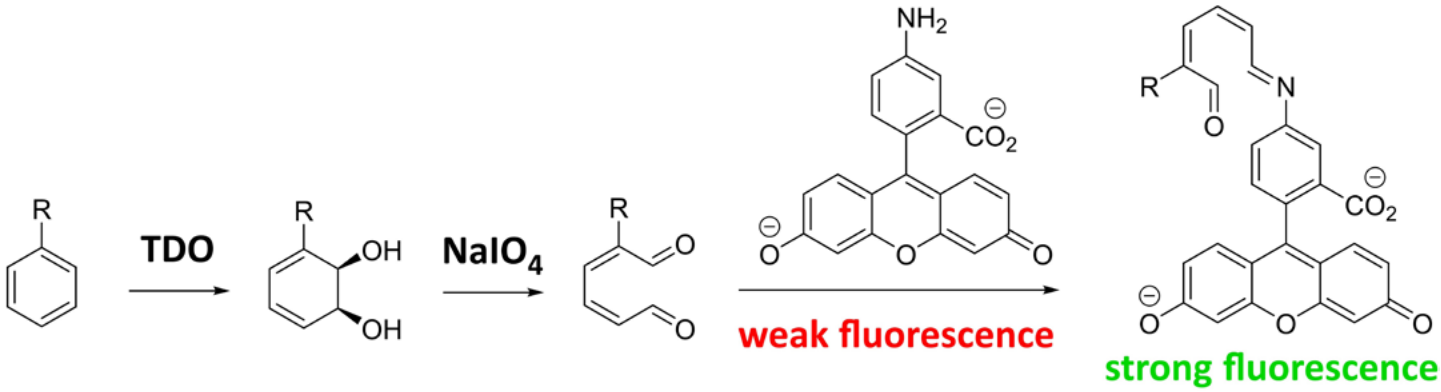


Figure 4

Coupled reactions employed by the fluorescence-based assay to detect and quantify the cis*-diol metabolites produced by active dioxygenases (30).*



Screening of Toluene Dioxygenase Variants

To test the *cis*-dihydroxylation activity of the TDO variants produced in this study, a fluorescence-based assay that had previously been reported by our laboratory was applied (30). This assay, performed in 96-well plates, detects the presence of *cis*-diol metabolites produced by active dioxygenases in the bacterial growth media through the conversion of the *cis*-diol metabolites to a corresponding dialdehyde, and subsequent conjugation of the dialdehyde to a fluorescent probe (30) (**Figure 4**). In this way, the amount of fluorescence detected in each well provides information as to the amount of *cis*-diol metabolite produced by the enzyme variant present in the corresponding well.

As the possibility existed that the introduced mutations would cause the structure of the TDO active site to be altered, while retaining some *cis*-dihydroxylation activity, it was determined that the enzyme variants should be tested on a small library of diverse aromatic substrates (**Figure 5**). This would afford the opportunity to determine the effect of the introduced mutations on the enzyme's activity for a wide range of substrates, including those the native enzyme has high activity for, those the native enzyme has low activity for, and those the native enzyme has no activity for.

To test the *cis*-dihydroxylation activity of the designed TDO variants, vectors expressing each set of variants were separately transformed into *E. coli* (BL21 (DE3)) alongside vectors expressing the parent enzyme (pCP-02) and a negative control (pCP-01) (30). Six colonies expressing each variant were then inoculated into separate wells of a 96-well plate and cultured according to an optimized assay protocol (30), before being treated with the relevant aromatic substrate. Following an incubation period in the presence of the relevant substrate, the cells were removed, and the growth media was carried through the fluorescence-based assay protocol (**Figure 4**). Analysis of the resultant data from these assays revealed that all twelve of the designed TDO variants (H222A, H222C, H222D, H222E, H228A, H228C, H228D, H228E, D376A, D376C, D376E, D376H) lacked any activity for all eight substrates used in the screens (**Figure 6**). This included a

complete loss of activity for the native substrate (toluene), and other substrates for which the native enzyme possesses activity (benzyl alcohol, benzyl acetate, *n*-butyl benzene, *t*-butyl benzene, methyl benzoate) and no gain in activity for substrates that are not metabolized by the parent enzyme (benzyl acetamide and benzylamine) (**Figure 6**).

Discussion

To study the effect of mutating the iron-coordinating residues of Rieske dioxygenases on the activity of these enzymes, the first step was to select a member of this class of enzymes to act as the template for mutagenesis. Toluene dioxygenase (TDO) is a well-characterized member of the Rieske dioxygenase enzyme family, which is derived from the soil bacterium *Pseudomonas putida* F1 (42). Due to the historic importance of TDO in the production of valuable compounds (16), and because of our laboratory's experience working with this Rieske dioxygenase system (27, 30), TDO was chosen as the engineering scaffold for this study. Due to the structural similarity observed with many Rieske dioxygenases (2), the results of this study would be expected to be applicable across many members of this enzyme family.

The goals of this study were to investigate the effects of both altering and eliminating the iron-coordination of key residues in the active site of toluene dioxygenase. As TDO has been well characterized (9), the identification of the iron-coordinating residues of this enzyme (HIS222/HIS228/ASP376) using ChimeraX (13) was trivial (**Figure 2**). To effectively test the hypothesis of this study, it was determined

Figure 5

Aromatic substrates used to screen for cis*-dihydroxylation activity among the TDO mutants produced.*

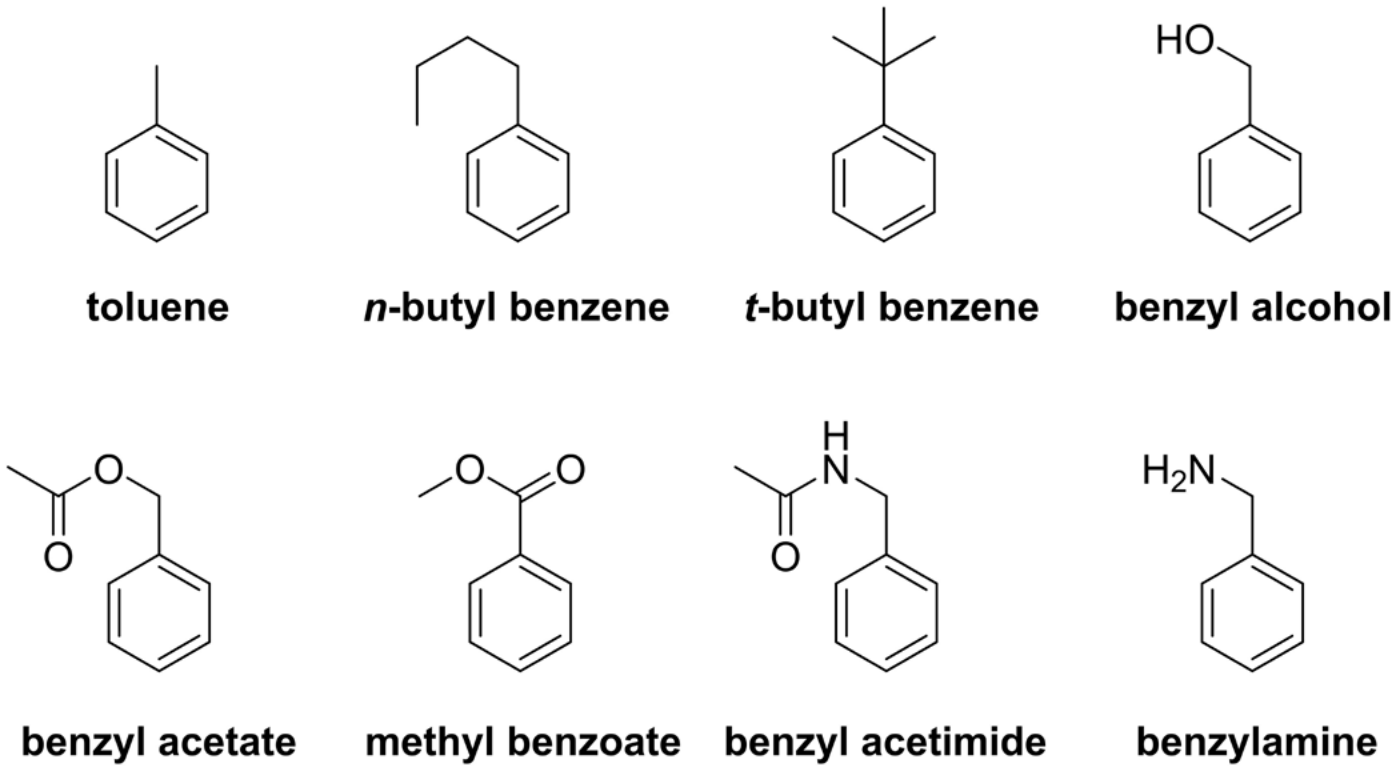
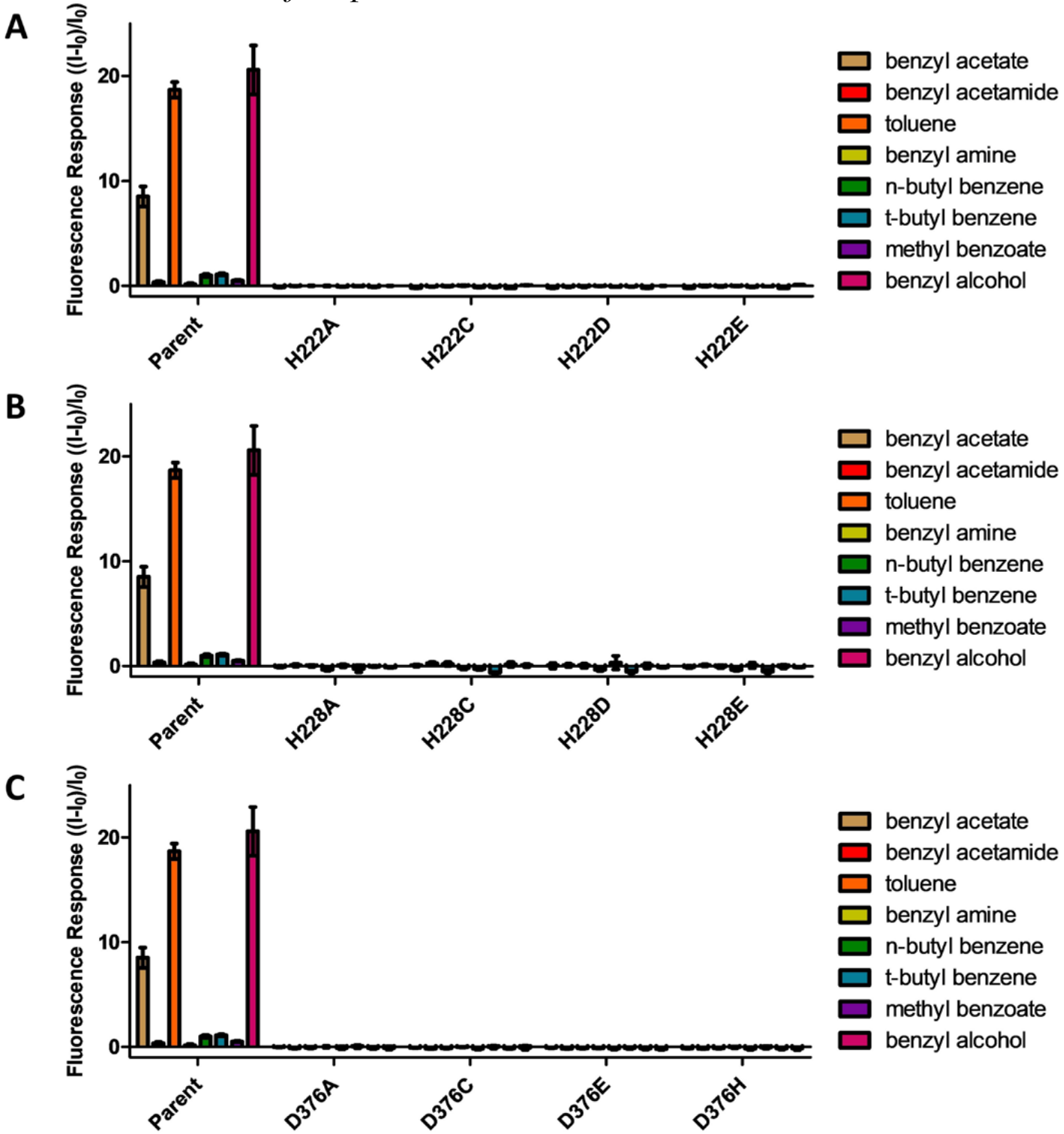


Figure 6

cis-*Dihydroxylation activity of H222 TDO variants (***A***), H228 TDO variants (***B***) and D376 TDO variants (***C***) for representative aromatic substrates* (n = *6*).



Note. Fluorescence response was normalized to the mean fluorescence response of the negative control (*E. coli* BL21 (DE3) pCP-01) ($[I - I_0]/I_0$) (21). Parent activity was determined from positive controls (*E. coli* BL21 (DE3) pCP-02), with the fluorescence response normalized to the negative control ($[I - I_0]/I_0$) (30).

that all three of the residues coordinating the catalytic iron would be mutated to three alternate residues capable of coordinating iron (aspartate, glutamate, and cysteine for native histidine residues; glutamate, cysteine, and histidine for the native aspartate residue) and to one residue that would eliminate iron-coordination at that site (alanine). This afforded the opportunity to evaluate the effects not only of eliminating iron coordination at specific sites, but also the potential beneficial effects of remodeling the active site and/or altering the electronics of the catalytic iron center by changing the iron coordinating residues while maintaining iron coordination.

Once the successful generation of the targeted TDO variants had been confirmed, the next step was to test the *cis*-dihydroxylation activity of these variants. As the possibility existed that the introduced mutations would cause the structure of the TDO active site to be altered, while retaining some *cis*-dihydroxylation activity, it was determined that the enzyme variants should be tested on a small library of diverse aromatic substrates. These aromatic substrates included the native substrate (toluene), one polar substrate for which the native enzyme possesses high activity (benzyl alcohol), one polar substrate for which the native enzyme possesses moderate activity (benzyl acetate), three polar substrates for which the native enzyme possess little or no activity (methyl benzoate, benzyl acetamide, and benzylamine), and two sterically bulky substrates for which the native enzyme possesses low activity (*n*-butyl benzene and *t*-butyl benzene) (**Figure 5**). By testing the activity of the designed mutants across these substrates, it would be possible to determine whether the introduced mutations had advantageous or detrimental effects in the *cis*-dihydroxylation of the native substrate, as well as in the *cis*-dihydroxylation of both sterically bulky and polar classes of substrates.

Upon testing the activity of each designed TDO mutant for all the designated substrates, it was revealed that none of the mutants designed in this study possessed activity for any of the diverse substrates selected (**Figure 6**). These results revealed the critical role played by the iron-coordinating residues of Rieske dioxygenases in their native state, as any alteration of these residues, either to other residues with the potential for iron-coordination or to residues incapable of coordinating iron, resulted in complete ablation of enzyme activity. Although the hypothesis that the mutations introduced in this study would significantly alter the activity and/or substrate scope of the enzyme was proven correct, these changes were shown not to be beneficial for enzyme activity. The loss of activity observed with mutations to non-coordinating alanine likely indicates that coordination by three residues is essential for iron to be bound to the active site of Rieske dioxygenases in a catalytically active state. The loss of activity observed with mutations to alternate iron-coordinating resides may indicate that these alterations result in a significant remodeling of the active site that prevents iron from binding in a catalytically active state. Alternately, these results may indicate that the active site iron of Rieske dioxygenases must specifically be bound by two histidine residues and one aspartate residue to effectively participate in the catalytic mechanism. Further studies, including enzyme structure elucidation/homology modeling,

docking analysis, and molecular dynamics simulations are required to precisely elucidate the cause of the loss of activity observed from these alterations to the iron-coordinating residues. These studies will be performed in due course.

Although this study did not succeed in producing novel TDO variants with improved or expanded activity, the results described will serve to guide future studies targeting the engineering of Rieske dioxygenases. With the increasing recognition of the need to employ more sustainable techniques in the chemical industry, and to remove harmful pollutants in contaminated environments, the field of enzyme engineering will continue to increase in importance. This is reflected in the fact that the engineering of Rieske dioxygenases to improve their utility either as green-chemical catalysts or as tools for the bioremediation of contaminated environments continues to be an active area of research (1, 3, 5, 20, 21, 26, 27, 33, 37, 38). Metagenomics research has shown that Rieske dioxygenases commonly evolve among soil bacteria (17), meaning that many unannotated Rieske dioxygenases remain to be studied in the context of enzyme engineering. As the field of microbiology continues to discover more diverse organisms and the powerful natural catalysts they produce, these new catalysts will provide valuable templates for the field of enzyme engineering. In this way, the natural ecological role of soil organisms can be harnessed and enhanced to create a safer and more sustainable future. This study, by demonstrating the importance of preserving key active site residues in the generation of Rieske dioxygenase mutant libraries in the pursuit of enzyme engineering, will inform and expedite these efforts.

Conclusions

Inspired by recent reports that have shown that the alteration of iron-coordinating residues in metalloenzymes can alter or even improve the activity of these enzymes in certain contexts (29, 40), the iron-coordinating residues of toluene dioxygenase (TDO) were comprehensively mutated in this study. These residues were mutated both to alternate residues that are capable of coordinating iron and to a residue that is incapable of iron coordination. Following the confirmation of successful mutagenesis, these new TDO variants were tested for their ability to catalyze the *cis*-dihydroxylation of a diverse group of potential aromatic substrates through a fluorescence-based assay system (30). The results of this study demonstrated the critical role played by the iron-coordinating residues of Rieske dioxygenases in their native state. These results revealed that any alteration of these residues, either to other residues with the potential for iron-coordination or to a residue incapable of coordinating iron, resulted in a complete loss of *cis*-dihydroxylation activity for any of the classes of substrates tested in this study. Although this study did not produce any Rieske dioxygenase variants with improved or altered *cis*-dihydroxylation activity, the findings of this study will serve to inform future engineering studies targeting these enzymes. Based on these results, it is clear that any attempts to engineer novel variants of Rieske dioxygenases should make every effort to preserve the iron-coordinating residues in their native state.

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