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Structural basis for selectivity in flavin-dependent monooxygenase-catalyzed oxidative dearomatization

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Abstract

Biocatalytic reactions embody many features of ideal chemical transformations, including the potential for impeccable selectivity, high catalytic efficiency, mild reaction conditions and the use of environmentally benign reagents. These advantages have created a demand for biocatalysts that expand the portfolio of complexity-generating reactions available to synthetic chemists. However, the tradeoff that often exists between the substrate scope of a biocatalyst and its selectivity limits the application of enzymes in synthesis. We recently demonstrated that a flavin-dependent monooxygenase, TropB, maintains high levels of site- and stereoselectivity across a range of structurally diverse substrates. Herein, we disclose the structural basis for substrate binding in TropB, which performs a synthetically challenging asymmetric oxidative dearomatization reaction with exquisite site- and stereoselectivity across a range of phenol substrates, providing a foundation for future protein engineering and reaction development efforts. Our hypothesis for substrate binding is informed by a crystal structure of TropB and molecular dynamics simulations with the corresponding computational TropB model and is supported by experimental data. In contrast to canonical class A FAD-dependent monooxygenases in which substrates bind in a protonated form, our data indicate that the phenolate form of the substrate binds in the active site. Furthermore, the substrate position is controlled through twopoint binding of the phenolate oxygen to Arg206 and Tyr239, which are shown to have distinct and essential roles in catalysis. Arg206 is involved in the reduction of the flavin cofactor, suggesting a role in flavin dynamics. Further, QM/MM simulations reveal the interactions that govern the facial selectivity that leads to a highly enantioselective transformation. Thus, the structural origins of the high levels of site-and stereoselectivity observed in reactions of TropB across a range of substrates are elucidated, providing a foundation for future protein engineering and reaction development efforts.

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Supporting Information Available

The Supporting Information is available free of charge on the ACS publications website. Detailed experimental procedures, supporting figures and tables.

Graphical Abstract

Keywords

Biocatalysis; flavin-dependent monooxygenase; oxidative dearomatization; molecular dynamics; enantioselective hydroxylation

INTRODUCTION

The demand for biocatalytic transformations is growing as chemists recognize the advantages of these processes, including high levels of selectivity, low catalyst loadings, environmentally benign reagents, and excellent sustainability profiles.¹ For transformations that pose a challenge to traditional chemical synthesis, there is a greater interest in developing biocatalytic methods. The oxidative dearomatization of phenols to generate quinol products is a reaction that presents a substantial selectivity challenge using traditional chemical methods (see **1** to **2**, Fig. 1).² A suite of I(III), I(V), Cu(I) and Pb(IV) reagents have been developed for oxidative dearomatization.^{2–6} In these reactions, a phenolic substrate is activated as an electrophile, which is intercepted by an oxygen nucleophile in either an intra- or intermolecular fashion.^{7,9} Achieving high levels of selectivity in this reaction is desirable as site-selectivity is often substrate rather than catalyst- controlled. Further, controlling

stereoselectivity under catalytic conditions remains a challenge.^{3,9–13} For example, Maruoka and coworkers recently reported an indane-based organoiodine catalyst that provides *p*-quinol products in up to 84% enantiomeric excess (ee), the highest stereoselectivity for catalytic oxidative dearomatization reported to date.¹³ Anticipating that a biocatalyst could provide exceptional selectivity and a superior sustainability profile, we recently investigated the substrate scope and scalability of a panel of flavin-dependent monooxygenases that mediate oxidative dearomatization reactions in various secondary metabolite pathways.¹⁴ In contrast to traditional chemical transformations in which site-selectivity is governed by the substrate, selectivity in the flavin-dependent monooxygenase reaction is dictated by the orientation of the substrate in the active site relative to the oxidant, C4a-hydroperoxyflavin (**12**).^{15,16} We envisioned that a suite of flavin-dependent monooxygenases could provide complementary selectivity and redefine the state-of-the-art method for mediating this transformation. Our initial study demonstrated that three biocatalysts, TropB, AzaH, and SorbC, perform well in preparative-scale reactions on a range of substrates while maintaining high levels of site- and stereoselectivity, providing a single product in 94% to >99% ee.¹⁴ For example, TropB oxidizes non-native substrates such as **3–11**, which vary significantly in electronic and steric properties compared to the native TropB substrate **1**, yet for each substrate a single product is generated in 98% ee (Fig. 1).¹⁴

We aim to enhance the synthetic utility of these biocatalysts by expanding the substrate scope, developing a suite of catalysts with complementary selectivities, and accessing non-native mechanistic pathways. However, to accomplish these goals and fully realize the synthetic potential of TropB and related enzymes, a structural and mechanistic understanding of this highly selective biocatalyst is required. We solved a crystal structure of TropB, which indicated that the active site is sufficiently spacious to accommodate a range of substrates, but must maintain a conserved binding orientation to achieve high site- and stereoselectivity across an array of substrates. To provide a detailed understanding of the active site architecture, substrate binding pose, and residues required for catalysis, molecular dynamics simulations were carried out with a corresponding computational enzyme model and detailed characterization of wild type TropB alongside several variants revealed distinct roles for two amino acids in the active site.

Structure of TropB.

As a first step toward elucidating the structural basis for selectivity, we solved a 1.7 Å crystal structure of TropB with its flavin adenine dinucleotide (FAD) cofactor bound (Fig. 2A, Table S2). TropB crystallized as a dimer, consistent with a dimeric form observed by size-exclusion chromatography (Figure S3). The large dimer interface (1,244 Å² of buried surface area) includes 14 salt bridges and 6 additional hydrogen bonds. The overall structure comprises two domains: (a) FAD-binding domain 1, a typical type A flavin-dependent monooxygenase with a nucleotide binding site for the adenosine diphosphate (ADP) moiety of the cofactor and (b) catalytic domain 2 with a binding site for the isoalloxazine ring (Figures S7A and S8A).¹⁵ Part of the interdomain loop between α4 (domain 1) and β4 (domain 2) is disordered (residues 81–86, Fig. 2A). Two long helices (α11 and α12) also connect domains 1 and 2. A molecule of FAD sits in the narrow inter-domain cleft, with a planar isoalloxazine ring that is consistent with the observed yellow color of crystals (Figure

S7). FAD contacts the protein through a combination of hydrogen bonds and van der Waals interactions. The adenosine and pyrophosphate groups are bound to TropB as in other class A FAD-dependent monooxygenases.¹⁷ The isoalloxazine ring sits in a binding pocket formed by Pro329, His330 and His331 with hydrogen bonds of O4 and N3 to Ala55, N1 to Ala335 and O2 to the Cys336 amide. A chloride ion sits in a tight pocket between the isoalloxazine ring and Pro329, Gly332 and Gly334, as in other FAD class A monooxygenases (Figure S7A).^{18–20}

In the structure of TropB crystallized in the absence of substrate, a large cavity adjacent to the isoalloxazine ring is anticipated to be the site of substrate binding. This site is defined by several side chains (Arg206, Leu226, Met228, His235, Ile237, Tyr239, Ala250, Phe252, Pro329, His330, His331, and Tyr397), some of which are conserved in other class A flavin monooxygenases.¹⁸ One molecule of hexanediol, a component of the crystallization solution, was bound in the cavity, based on well-defined electron density (Figure S9).

As crystallization with the flavin cofactor in the oxidized state should permit formation of a dead-end substrate complex, significant effort was dedicated to obtaining crystals of a TropB ternary complex with FAD and the native substrate, 3-methylorcinolaldehyde (**1**). Crystal soaking and co-crystallization experiments with **1** or structural analogs of **1** resulted in maps with new electron density in the active site cavity; however, despite extensive experimentation, none of the conditions explored led to well-defined density for a single orientation of the substrate or to the full displacement of hexanediol from the cavity. Although full occupancy of a TropB ternary complex was not captured, crystallization in the presence of **1** induced a change in position of the cofactor isoalloxazine ring, consistent with flavin dynamics of class A flavin-dependent monooxygenases (Figure S10).^{21,22} Substrate binding in class A flavin-dependent monooxygenases triggers flavin reduction by inducing a “flavin-out” conformation, in which the flavin is accessible to the reduced cofactor, nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide (NADH).^{22–24} The TropB crystal structure obtained in the absence of substrate depicts the oxidized cofactor in the “flavin-in” conformation, whereas the structure in the presence of substrate indicated partial occupancy of a flavin-out conformation (Figure S10). This change in flavin position upon substrate binding would allow for reduction of FAD to FADH₂ by an external reductant (NADH or NADPH), consistent with other class A flavin dependent monooxygenases.^{22–24}

Substrate binding and catalysis.

Arg206 and Tyr239 are the only amino acids in the TropB active site that possess relevant functional groups and are near the flavin cofactor and partially occupied substrate site. In the absence of clear density to illustrate the contacts of the native substrate (**1**), we sought to draw an analogy to substrate complexes of other class A flavin-dependent monooxygenases of known structure. TropB has low sequence identity to other class A flavin-dependent monooxygenases, except for SalH (31%);¹⁷ nonetheless, structural features involved in either substrate binding or catalysis remain constant across enzymes in this class (Figure S5–6). In *p*-hydroxyl benzoate hydroxylase (PHBH) a hydrogen bond network composed of Tyr201, Tyr385, His72 and a conserved water molecule is responsible for substrate binding

and further catalysis.¹⁶ This network is absent in TropB; however, Tyr239 and Arg206 are within hydrogen bonding distance of the substrate in the TropB active site. Additionally, the Arg206 guanidinium group is in a position analogous to the Arg220 guanidinium group in PHBH. This is also the case for other class A monooxygenases, PHHY (Tyr289, Arg287),²⁵ MHBH (Tyr271, Arg262)²⁶ and SalH (Arg190).¹⁷ This led us to hypothesize that Tyr239 and Arg206 might be involved in substrate binding and perhaps catalysis.

A set of TropB variants was generated to probe the role of Tyr239 and Arg206 in substrate binding. Arg206 was substituted with either Glu (R206E) or Gln (R206Q) and Tyr239 was replaced with Phe (Y239F) to probe the role of hydrogen bonding in substrate binding (Table S1, Figures S1 and S4). TropB R206E, TropB R206Q, and TropB Y239F produced no detectable product by UPLC-PDA analysis under conditions where the wild type TropB led to complete consumption of the substrate with a specific activity of $0.33 \mu\text{mol min}^{-1} \text{mg}^{-1}$ (Fig. 3 and Figures S13–19, and S28). These results indicate that Arg206 and Tyr239 each have a critical role in achieving productive catalysis.

Having established that TropB R206E, TropB R206Q, and TropB Y239F produce no detectable product with the native substrate, 3-methylorcinolaldehyde (**1**), we sought to identify which step of the catalytic cycle was disrupted. TropB binding of **1** was detected by the change in absorbance at 492 nm, revealing a K_d of $21 \pm 2 \mu\text{M}$ for wild-type TropB and $32 \pm 4 \mu\text{M}$ for TropB R206Q (Fig. 3, Figures S29–32). Substituting Arg206 or Tyr239 with an amino acid that cannot act as a hydrogen bond donor led to a significant increase in K_d values, suggesting that hydrogen bond donors at positions 206 and 239 are critical for substrate binding. Next, we investigated whether NADPH can reduce FAD to FADH₂ in each TropB variant in the presence of substrate by monitoring NADPH absorbance at 362 nm. Complete consumption of NADPH was observed in 800 s with wild type TropB and partial consumption of NADPH was observed with TropB Y239F over 1000 s (Fig. 3, Figure S35). Additionally, the reduction rate of TropB Y239F was significantly slower compared to that of the wild-type but no product formation was detected. (Figures S36–S37) However, no depletion of NADPH was detected for TropB R206Q or R206E. This was an unanticipated result for TropB R206Q because this variant possesses a similar K_d to WT TropB. Thus, we increased the enzyme concentration by 200-fold and under forcing conditions we observed partial consumption of NADPH at extended time periods (Figures S38 – S39).²⁷ Previously Ballou and coworkers demonstrated that the corresponding Arg residue (Arg220) in PHBH, is involved in the flavin dynamics required for FAD to toggle from the “in” conformation to the “out” conformation. for reduction to FADH₂ by stabilizing the “out” conformation.²²

To test the ability of each TropB variant to engage molecular oxygen to form the labile C4a-hydroperoxyflavin intermediate **12**, an intermediate corroborated by stopped-flow analysis (Figure S41), the hydrogen peroxide concentration was measured in samples containing each TropB variant with 2.5 mM substrate and NADPH, generating a readout on the level of peroxide formed through uncoupling (see **12** to **14**, Fig. 3D). In this assay, relatively low levels of H₂O₂ were measured (0.2 mM) with wild type TropB, whereas, significantly higher concentrations of H₂O₂ were present in reactions with TropB Y239F (1.4 mM), indicating a higher level of uncoupling. In reactions with TropB (R206E and R206Q), trace amounts of H₂O₂ were detected (0.03 mM), which did not increase over time, further indicating that

these substitutions hindered flavin reduction (Figure S40). Characterization of the Arg206 and Tyr239 variants supports a role of these two amino acids in substrate binding. These results indicate that Arg206 is also important for FAD reduction to FADH₂ and that Tyr239 stabilizes the substrate in a position suitable for rapid reaction with labile C4a-hydroperoxyflavin. Additionally, TropB R206Q and TropB Y239F were crystallized. In the TropB Y239F structure, Phe239 maintains the same position as Tyr239 in the wild-type protein (Figure S12, Table S2). In the crystal structure of TropB R206Q, Gln206 is ordered and unavailable to stabilize FAD in the “out” conformation, thus, affecting flavin reduction in the catalytic cycle (Figure S11, Table S2).

Next we considered which additional residues could be critical for a productive reaction. Deprotonation is a common step for other class A flavin-dependent monooxygenases,²⁸ and it is generally accepted that this class of monooxygenases requires a catalytic base for deprotonation of phenolic substrates in order to obtain a sufficiently nucleophilic reactant.^{15,29,30} Deprotonation is thought to occur prior to flavin reduction or during substrate hydroxylation.¹⁶ In the case of TropB, His235 has been predicted to act as a catalytic base,²⁸ however, the crystal structure indicates that His235 is too far from the substrate to perform a deprotonation. The apparent lack of a basic residue near the anticipated site of substrate binding led us to hypothesize that the substrate binds as the phenolate, eliminating the requirement a base to initiate catalysis as previously suggested (Fig. 4A).^{15,28,31}

To interrogate this hypothesis, we first investigated the protonation state of the substrate under the reaction conditions, specifically the optimum pH 8.²⁸ The protonation state of the native substrate (**1**) was monitored by UV absorbance, as the phenolate absorbs at 334 nm, whereas, the protonated form is characterized by a maximum absorbance at 292 nm (Figures S33 and S34). This indicates that, under the reaction conditions at pH 8, the predominant form of the substrate is the anionic form (**13**) with an experimentally determined pK_a of 7.1. To experimentally test the proposal that H235 is necessary for catalysis (Fig. 4A),²⁸ TropB H235A was generated (Figure S2). Substitution of His235 with Ala produced a catalytically active TropB variant (10% conversion, Figures S20–S21). To probe the role of a neighboring His residues, His330 and His331 were substituted with Ala, affording an active variant capable of 11% and 71% conversion of substrate to dearomatized product, respectively (Figures S2, S22–S25). Since these residues are close together, one could imagine His235 and His330 having redundant function as the requisite base. Thus, a double substitution was performed to generate H235A/H330A and the variant remained active (10% conversion, Figures S2, S26–S27). These results are consistent with substrate binding in the anionic form, eliminating the need for a basic residue in the active site for substrate deprotonation. Further, the initial rates of reactions with **1** and wild-type TropB, TropB H331A or TropB H235A/H330A follow the reactivity trend with wild-type TropB and TropB H331A having initial velocities of 2.9 and 3.0 $\mu\text{M min}^{-1}$, respectively (Fig. 4B). The TropB H235A/H330A variant has a decreased initial velocity, 0.24 $\mu\text{M min}^{-1}$, that corresponds with the lower conversions observed in reactions with this variant (Figure S42). These data indicate that the His residues are not required for catalysis but they contribute to an ideal active site environment.

Computational approaches to elucidate substrate binding pose.

To provide additional insight on the orientation of the native substrate **1** in the active site, we turned to computational analysis. Several enzyme-substrate models with the flavin cofactor as either FAD (**14**) or C4a-hydroperoxyflavin (**12**) were evaluated with ligand-protein docking calculations that use the CDocker protocol.³² The protocol employs i) flexible active site sidechains and a soft-grid representation of the remaining receptor during multiple rounds of simulated annealing followed by ii) geometry optimizations with the CHARMM molecular mechanical force field and iii) scoring of the complexes with the force field and an implicit solvent model. In addition, molecular dynamics (MD) simulations were performed using the docked complexes in explicit solvent using classical force fields and hybrid quantum mechanical/molecular mechanical (QM/MM) methods. In contrast, flexible docking of **1** in the anionic form (see **13**) to TropB with the flavin cofactor in either the oxidized or hydroperoxo form led to a favorable, low-energy pose consistent with the known site- and stereoselectivity of the reaction. This docking indicated that both Arg206 and Tyr239 hydrogen bond with the phenolate oxygen of **13** (Figs. 2B and 4A). The pose showed a significant change in the location of the Arg206 side chain from its position in the crystal structure, while the movement of Tyr239 was minimal (Fig. 2B and Figure S43). The displacement of Arg206 is reasonable given that the enzyme was crystallized with the flavin in the oxidized “in” state and must go through several catalytic steps before reaching the C4a-hydroperoxyflavin state (**12**, see Fig. 3). The charge distribution in the molecular mechanics (MM) force field delocalized the negative charge on the substrate in accordance with gas phase and solvated QM calculations and was not concentrated on the phenolate oxygen. This indicates that the interaction with Arg206 and Tyr239 was not driven by an unphysical electrostatic interaction that could arise if the charges were overly localized by standard charge distribution methods.

To characterize enzyme-substrate contacts, several MD simulations of the identified docking pose were performed. Specifically, models with different tautomeric states of His235, His330 and His331 were constructed since the tautomeric states of His residues are not directly observed in crystal structures and these states can be challenging to accurately predict. During each of the 25 nanosecond (ns) simulations, the substrate exhibited only minimal fluctuations (0.24 to 0.65 Å root mean square deviation [rmsd] from average atomic position), remaining in a bound position (averaging 5.2 to 6.8 Å for the O_{distal}-C3 distance, Figures S47, S49, S50 and Table S3). A reactive configuration was observed in 1.3% of the snapshots as strictly defined by (1) an O_{distal}-C3 distance less than 4 Å, and (2) an O_{distal}-C3-C3_{methyl} angle between 60° and 115° (Figure S48). All of these snapshots showed a hydrogen bond between the anionic substrate **13** and Arg206, and approximately half of the snapshots also showed a hydrogen bond with Tyr239, supporting the relevance of this interaction.^{33,34} One of the Arg206 terminal amine groups hydrogen bonded to the phenolate oxygen throughout the simulation (average O-H distance 2.0 Å, 88% of frames < 2.4 Å). Interestingly, the phenolic proton of Tyr239 formed a tighter hydrogen bond as the simulation progressed. The average distance for this hydrogen bond was 2.7 Å over 24 ns but averaged 2.0 Å in the final 10 ns with 90% of the snapshots showing a distance less than 2.4 Å (Figure S51). Thus, MD simulations with a classical force field suggest that both Arg206 and Tyr239 form hydrogen bonding interactions with the substrate. To investigate

the interactions between the substrate and the active site environment with a more accurate description, MD simulations were also performed using a QM/MM representation.³⁵ In DFTB3 simulations, water molecules adorn the polar region of the active site, separating His235 from the substrate. Thus, neither a direct nor Grotthaus-like mechanism for substrate activation by His235 would be feasible. The substrate maintained hydrogen bonding interactions with both Tyr239 and Arg206 throughout these simulations.

The combined knowledge from the TropB crystal structure and subsequent MD suggests that the anionic form of the substrate, phenolate **13**, is the predominant form under the reaction conditions and that the negative charge is stabilized through interactions with both Arg206 and Tyr239, thus positioning the substrate for a productive reaction with the C4a-hydroperoxyflavin cofactor (**12**, Fig. 4A) and leading to a highly site- and stereoselective transformation.

Computational insights on facial selectivity.

To identify the factors controlling facial selectivity we turned to computation. While QM/MM simulations indicated that hydrogen bonding of Arg206 and Tyr239 with the phenolate form of the substrate is the dominant intermolecular interaction (Figure S52), these interactions alone are insufficient to be the basis of for the facial selectivity observed experimentally. Additional interactions between substrate and TropB were observed in MD simulations. Both hydrophobic C3 and C6 methyl groups of substrate **1** interacted with hydrophobic residues (Figures S53 – S54). The C6 methyl group was near Ala55 and the phenyl group of Phe119. In contrast, the C2 hydroxyl group was positioned in a solvated environment, hydrogen bonding exclusively with the aldehyde moiety (Figures. S55 – S56). If the opposite face of the substrate were presented to the C4a-hydroperoxyflavin factor (**12**) by rotating along the C1–C4 axis, the C2 hydroxyl group would occupy an unsuitable hydrophobic region and the C6 methyl group would be in a hydrophilic space, placing these two groups in uncomplimentary environments (Fig. 5A and Figure S58). Similarly, the C3 methyl group makes hydrophobic contacts with Phe252, Ala250 and Ile237. If the substrate rotated about the C1–C4 axis, however, this methyl group would sit near the backbone carbonyl of Gly53 and the hydroperoxy group of the flavin (Figure S57). Thus, this region would be an energetically unfavorable position for the hydrophobic C3 methyl group if the substrate presented the opposite face to the cofactor. The bulkier methyl group would also clash with Tyr239, disrupting the hydrogen bond with the substrate. Thus, we conclude that the facial selectivity in the hydroxylation of substrate **1** in TropB arises from the amphipathic active site environment as illustrated in Fig. 5A. Docking of non-native substrates revealed binding poses analogous to native substrate **1** (Fig. 5B–D and Figures S44 – S46). For example, substrates **3** and **8** adopt a similar pose to native substrate **1**, occupying the same space in the TropB active site and maintaining hydrogen bond interactions with Arg206 and Tyr239 (Fig. 5B and 5C, respectively). Substrates with significantly more sterically demanding substituents such as **7**, provide docking solutions with the substrate slightly shifted in the active site to accommodate bulky substituents but maintain the relevant hydrogen bonding contacts and facial selectivity as observed for the native substrate (Fig. 5D). These docking results are consistent with the site- and stereoselectivity observed in reactions with non-native substrates.¹

Through structural insights gained from both the crystal structure and molecular dynamics simulations, we have uncovered the origins of the exquisite site- and stereoselectivity exhibited in reactions of TropB with a breadth of substrates. A crystal structure of TropB has enabled computational studies that support binding of the anionic form of the substrate, which is further supported by experimental data. Molecular dynamics simulations and characterization of TropB variants demonstrated that the phenolate substrate has significant interactions with both Arg206 and Tyr239, controlling the pose of the substrate within the active site and the selectivity observed in subsequent reactions. The excellent stereoselectivity observed in reactions with TropB can be attributed to pronounced facial selectivity in substrate binding. These findings clearly illustrate the structural features that control the site- and stereoselectivity in the complexity-generating reaction mediated by TropB, critical for future protein engineering and reaction development efforts with TropB and related enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Chemistry of flavin-dependent monooxygenase TropB.¹⁴ Oxidative phenol dearomatization mediated by TropB in stipitatic acid biosynthesis (see **1** to **2**) and substrate scope. Non-native substrates with perturbation of a single substituent compared to the native substrate (**1**, blue) were evaluated for reactivity with TropB, measured in total turnover numbers (TTNs), maximum TTN = 1000 in this assay.

Figure 2.

(A) TropB dimer, with monomer 1 colored in indigo with disordered residues 81–86 shown as a dashed line and monomer 2 shown in gray; (B) TropB crystal structure overlaid with the TropB QM/MM model with substrate **1**.

Variant	Product Formation	Specific Activity (umol min ⁻¹ mg ⁻¹)	Kd (uM)	NADPH Consumed	H ₂ O ₂ formed in the reaction
WT	Yes	0.33	21 ± 2	Yes	0.2 mM
Y239F	No	0	> 300	Yes	1.4 mM
R206Q	No	0	32 ± 4	No	-
R206E	No	0	> 100	No	-

Figure 3.

Activity of wild type TropB and TropB variants. (A) Substrate (**1**) binding to wild type TropB; (B) NADPH depletion upon **1** binding; (C) Product (**2**) formation; (D) Formation of peroxide in shunt pathway. *Reaction conditions: 2.5 mM substrate **1**, 2.5 μM TropB variant, 1 mM NADP⁺, 5 mM glucose-6-phosphate (G6P), 1 u mL⁻¹ glucose-6-phosphate dehydrogenase (G6PDH), 50 mM potassium phosphate buffer pH 8.0, 30 °C, 1h. 0% conversion indicates that no substrate consumption or product formation was detected.

Reaction Conditions: 100 nM TropB variant, 250 μM NADPH, 125 μM **1 monitored at 362 nm over 1,000 sec. ***Concentration of hydrogen peroxide was measured using an established assay.²⁷

Figure 4.

Hypotheses for substrate activation and positioning. A. The mechanism on the right requires deprotonation of the substrate in the active site, which has been proposed to be performed by His235.²⁸ The mechanism proposed on the left relies on binding of the anionic form of the substrate, including interactions with Arg206 and Tyr239 consistent with our experimental findings. The C4a-hydroperoxoflavin O_{distal} is highlighted in red. B. Initial velocity rates for TropB wild-type and the histidine variants.

Figure 5.

QM/MM simulation of the native substrate (1) in the active site to illustrate facial selectivity.

A. Left: Substrate (1) (orange) bound in the active-site, showing favorable contacts with Ala250 and Ile237; Right: 180° degree rotation along the C1-C4 axis of 1 (green), illustrates unfavorable substrate orientation. **B-D.** Substrate docking of **3**, **8**, **7**, respectively with the correct face presented to the flavin.