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Review

Choose Your Own Adventure: A Comprehensive Database of Reactions Catalyzed by Cytochrome P450 BM3 Variants

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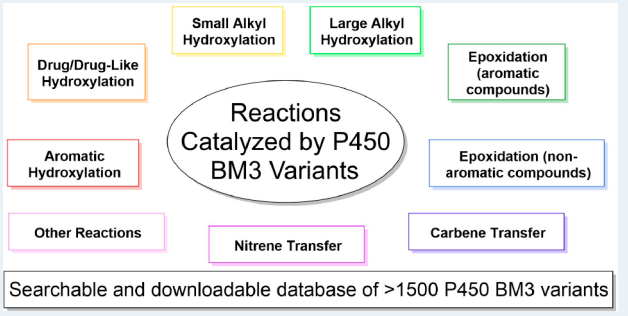
ABSTRACT: Cytochrome P450 BM3 monooxygenase is the topic of extensive research as many researchers have evolved this enzyme to generate a variety of products. However, the abundance of information on increasingly diversified variants of P450 BM3 that catalyze a broad array of chemistry is not in a format that enables easy extraction and interpretation. We present a database that categorizes variants by their catalyzed reactions and includes details about substrates to provide reaction context. This database of >1500 P450 BM3 variants is downloadable and machine-readable and includes instructions to maximize ease of gathering information. The database allows rapid identification of commonly reported substitutions, aiding researchers who are unfamiliar with the enzyme in identifying starting points for enzyme engineering. For those actively engaged in engineering P450 BM3, the database, along with this review, provides a powerful and user-friendly platform to understand, predict, and identify the attributes of P450 BM3 variants, encouraging the further engineering of this enzyme.



KEYWORDS: *CYP450 BM3, directed evolution, biotransformation, database, C−H functionalization*

INTRODUCTION

CYP102A1, also known as P450 BM3, is a cytochrome P450 monooxygenase that natively catalyzes the hydroxylation of long-chain fatty acids. It is highly active and self-sufficient, enabling catalysis to occur from a single polypeptide that contains the heme-and reductase domains. It has been the topic of extensive research over the past three decades and numerous laboratories have worked on evolving this enzyme to generate novel enzymatic properties and features. As a result, P450 BM3 and its variants have been shown to catalyze a wide variety of reactions, including hydroxylation,[1−16] epoxidation,[17−29] carbene transfer,[30−50] and nitrene transfer,[51−65] with a diverse range of substrates. The advances reported by the group of Frances Arnold on the evolution of P450 BM3 toward novel biocatalytic reactions, which were prominently featured in her 2018 Nobel-winning research, are among the most transformative.

In 2012, the review article "P450 BM3 (CYP102A1): Connecting the Dots" presented a thorough account of the reaction outcomes for the variants of P450 BM3 available at the time.[66] Its utility and interest are seen in its high citation index (cited over 450 times to date). In the intervening decade, directed evolution of P450 BM3 has become increasingly prevalent in academia and industry. The rate of discovery has been amplified by new technologies in variant library construction and in screening methods. As a result, there is an abundance of information on increasingly diversified variants of P450 BM3 that catalyze a broader array of chemistry. This information, however, is not structured in a way that currently allows computational mining.

We recognized the challenge of organizing information regarding the various new reactions and mutations that enable them. We determined that a database would provide the optimal means to categorize this knowledge and, most importantly, to enable extraction of information. The Turner lab recently presented the RetroBioCat Database, which serves as a platform for synthetic biotransformations.[67] The database is highly recommended for researchers who are unfamiliar with enzymes but are considering using them for synthetic transformations. Moreover, this database facilitates comprehension of enzyme activity as well as the limitations. The RetroBioCat Database's initial data came predominantly from their own group, which facilitated establishment of common reaction conditions and metrics for evaluating enzyme variants. However, P450 monooxygenases were excluded from the RetroBioCat Database.[67]

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Here, we present a complete and current overview of P450 BM3 variants and their catalyzed reactions. This review conveniently associates each P450 BM3 variant with its corresponding catalyzed reaction, including hydroxylation, epoxidation, carbene transfer, and more, in the form of a database, available as Supplementary Table 1. Additionally, we provide substrate information to facilitate navigation. Compiling the outcomes of over 1500 variants into this database offers an effective means of identifying and comprehending the properties of P450 BM3 variants and facilitating further evolution of this enzyme toward new reactions and improved reaction properties.

The database is downloadable, increasing its value relative to the static format of published tables. It focuses solely on P450 BM3 and has the potential to be incorporated into existing databases or serve as a framework for adding other P450s to the database. Ultimately, this extensive machine-readable database will provide the community with the means to develop machine learning methods to further accelerate reaction discovery.

P450 BM3: PROPERTIES AND LIMITATIONS

Efforts to functionalize C−H bonds in late-stage processes are highly sought-after for the synthesis of small molecules and pharmaceuticals production. However, achieving high yield and desired stereoselectivity remains a challenge with chemical synthesis methods.[68] Biocatalysts offer an environmentally sustainable alternative for generating various hydroxylated products and use atmospheric O_2 as an oxidant.[69] The demand for cost-effective and eco-friendly solutions for small-molecule oxidation continues to rise, leading to the constant development of new biocatalytic techniques. Enzymatic synthesis has become a standard industrial practice, providing high catalytic efficiency and yield, while limiting the formation of undesired side-products.[70] Promising multienzyme cascades have also been demonstrated.[69,71−74] In recent years, Merck & Co. has enhanced the original 12−18 step chemical pathway for producing islatravir, a drug used in the treatment of HIV infection, by developing a three-step manufacturing process that utilizes five evolved enzymes.[75]

Cytochrome P450 monooxygenases (P450s) are heme-containing enzymes that catalyze oxidation reactions on organic compounds, including those on nonactivated C−H bonds. The bacterial P450 CYP102A1 from *Bacillus megaterium* has gained attention as a biocatalyst for numerous synthetic applications due to its high stability and catalytic activity. However, further research is needed to fully understand its potential.[76,77] CYP102A1, also known as P450 BM3, is a self-sufficient cytochrome P450 enzyme, meaning that a single polypeptide chain encodes the oxygenase and reductase domains needed to complete the catalytic cycle.[78,79] Including both domains in one protein gives it unique properties, such as rapid electron transfer and high catalytic efficiency. This enables the enzyme to carry out various reactions efficiently while utilizing low substrate concentrations, thus rendering it an attractive option for an array of synthetic applications. NADPH serves as the electron donor, transferring electrons to the FAD domain of ferredoxin reductase, which then transport the electrons to the catalytic heme via FMN.[80] An appealing feature of P450 BM3, as a biocatalyst, is that it is not membrane-bound but is expressed in a soluble form. As a result, it can be functionally expressed in large quantities in *Escherichia coli* and can be stabilized by immobilization,[81] making it suitable for large-scale bioproduction applications.[82−85] This further highlights the potential of P450 BM3 as a valuable tool for synthesizing a diverse range of compounds through sustainable and eco-friendly means.

P450 BM3 naturally hydroxylates long-chain fatty acids, such as lauric acid. However, enzyme engineering has expanded the range of relevant substrates and reactions.[86−88] Selective hydroxylation is a desirable feature for synthesizing fine chemicals, including chiral compounds used in flavorings, fragrances, and pharmaceuticals, as well as for developing new antibiotics and drug precursors.[89,90] In recent years, enzyme engineering has broadened its reactivity to include various oxidation reactions, as well as carbene,[30,31] and nitrene transfer reactions, which are new-to-nature reactions.[52,53,58]

Given the attractive features of P450 BM3, the enzyme has garnered significant attention and has been the subject of numerous reviews highlighting its exceptional properties and characteristics. Reviews have been conducted on various aspects of P450 BM3, including structural and mechanistic details,[90−100] hydroxylation,[76,93,101−131] pharmaceutical compound hydroxylation,[127,131−133] epoxidation,[115,118,126,134] and carbene/nitrene transfer.[68,101,112,116,125,128,135−144] Additionally, studies have explored the use of decoy molecules to improve enzymatic activity,[92,105,117,118,134,141,145−149] as well as P450 BM3-inorganic complexes.[150−152] Reviews of P450 BM3 frequently place it within a broader context of other P450 enzymes, resulting in a lack of focus. Though some reviews do describe reactions catalyzed by P450 BM3 variants, they are typically limited to discussion of the "best" variants for a given reaction and do not offer a comprehensive review of reported variants. Due to the significant number of P450 BM3 variants and reactions reported, identifying all properties of a given variant often requires an extensive literature search. Therefore, selecting an appropriate starting variant for enzyme engineering can be difficult. To address this challenge, we developed a database that catalogues P450 BM3 variants, their corresponding reaction type, and substrate usage.

Our objective was to categorize reactions into meaningful classes based on their respective chemical processes, substrate properties, or general application. We first grouped the chemical reactions catalyzed by P450 BM3 into five categories: hydroxylation, epoxidation, carbene transfer, nitrene transfer, and other reactions. Hydroxylation encompasses aromatic hydroxylation, drug/drug-like hydroxylation, small alkyl hydroxylation, and large alkyl hydroxylation. It is worth noting that compounds in the drug/drug-like hydroxylation class are classified according to their application rather than similarities in structure. Epoxidation is classified into alkyl substrates, which resemble the native substrates, and aromatic substrates, which are typically styrene derivatives. The expression "aromatic epoxidation," used for brevity, is meant to encompass epoxidation of a molecule containing an aromatic group; it does not mean epoxidation on an aromatic ring, which would be a dearomatization reaction. A representative substrate for this class is styrene which forms styrene oxide after epoxidation by BM3 variants. Similarly, the expression "alkyl epoxidation" pertains to epoxidation of alkenes not containing an aromatic ring. Last, some compounds do not obviously fit within the outlined categories and were then added to the category based on where they are frequently screened together. As an example, valencene could be placed within the small or large alkyl hydroxylation class but is typically screened with other terpenoids that are grouped

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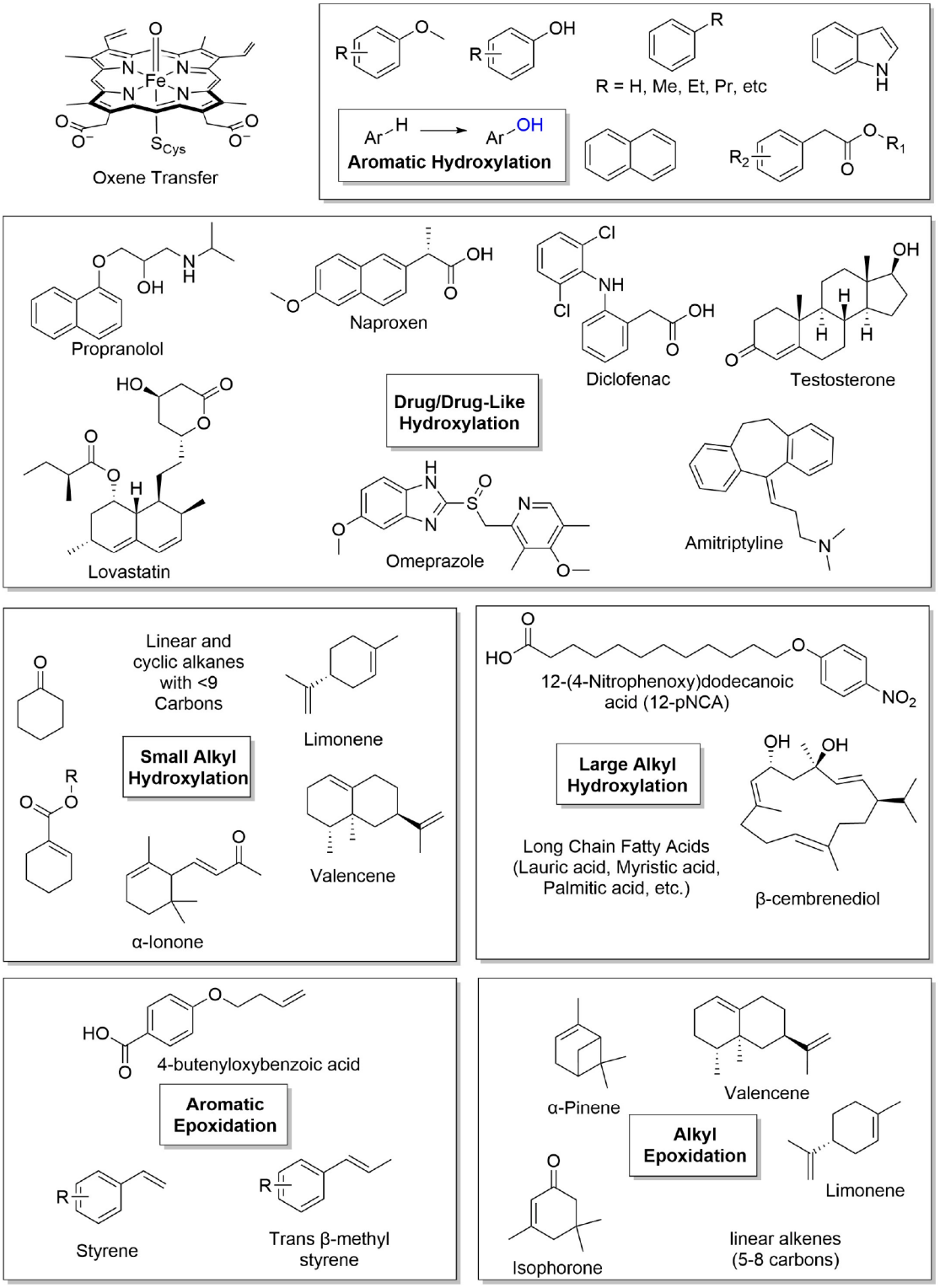
Scheme 1. Representative Substrates of the Hydroxylation and Epoxidation Reaction Classes Catalyzed by P450 BM3[a]



[a]The classes are further subdivided into aromatic hydroxylation, drug or drug-like hydroxylation, small alkyl hydroxylation, large alkyl hydroxylation, aromatic epoxidation, and alkyl epoxidation.

within the small-alkyl hydroxylation class. Consequently, we have divided P450 BM3 variants into nine categories based on this classification. Representative substrates from the hydroxylation and epoxidation categories are presented in Scheme 1. Corresponding substrates and products from the carbene transfer, nitrene transfer, and other reaction categories are displayed in Scheme 2.

DATASET STRUCTURE

We collected the publications discussing P450 BM3 that had been published up until July 2023. Over 600 publications

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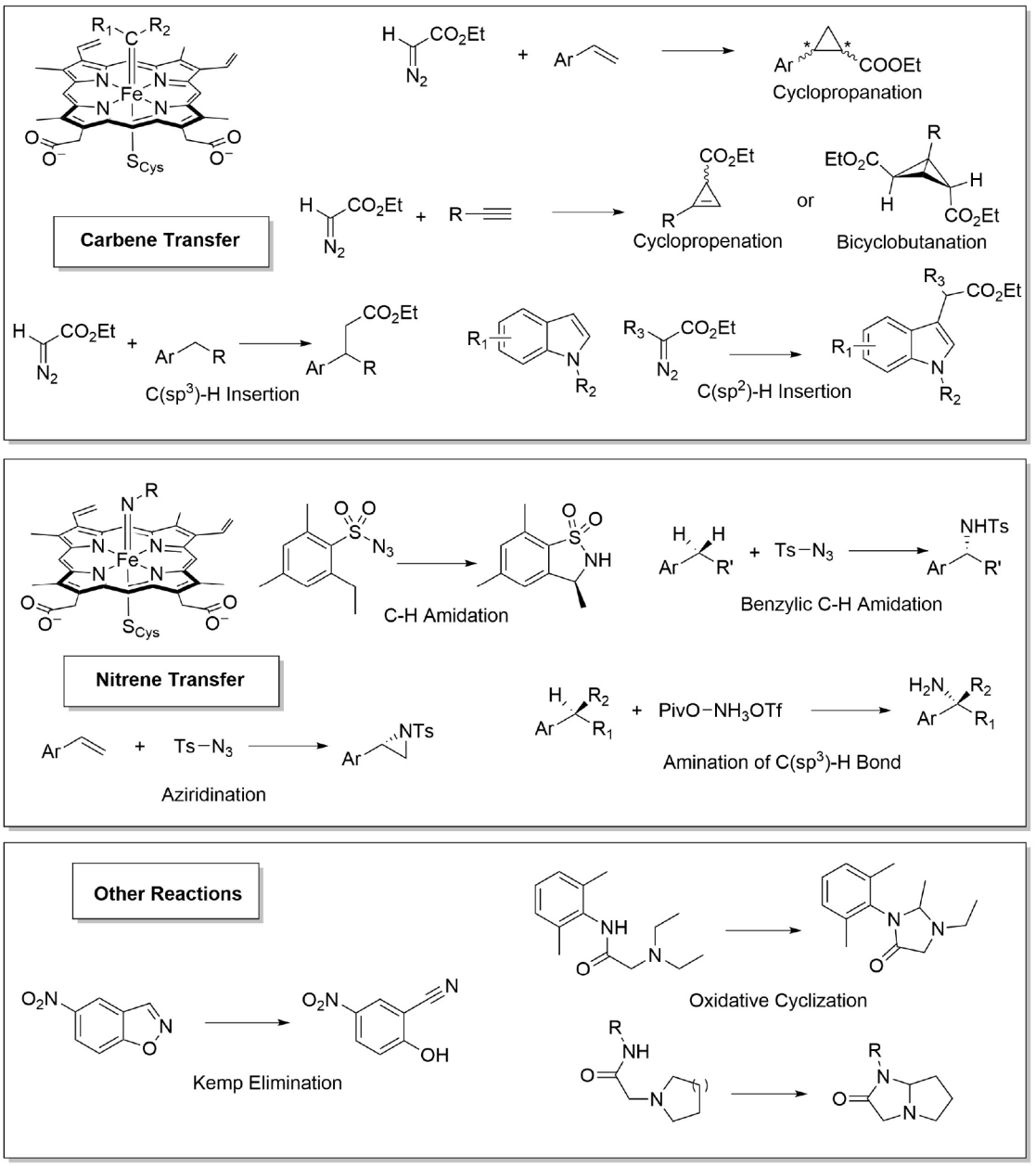
Scheme 2. Representative Substrates and Corresponding Products of the Carbene and Nitrene Transfer Reaction Classes Catalyzed by P450 BM3



referring to P450 BM3 were identified which collectively contain thousands of variants, a testimony to the enzyme's popularity. To facilitate navigation of the database, we opted to only include P450 BM3 variants that show improvement relative to the parent enzyme. Given the frequent concurrence of amino acid substitutions, the parent enzyme is often the native P450 BM3 but can also be any variant to which further mutations are introduced. Potential improvements of the modified P450 BM3 enzymes include increased product yield, altered selectivity toward certain product isomers or enantiomers, or enhanced enzyme stability. The variant names and their corresponding parent enzymes as reported in each cited publication, along with the mutations introduced to generate each variant are included (Supplementary Table 1). The substrate utilized by each P450 BM3 variant is also provided to facilitate the interpretation of the catalyzed reactions (Supplementary Table 1). Supplementary Tables 2−10 provide a comprehensive list of substrates tested for each reaction class, namely aromatic hydroxylation, drug/drug-like hydroxylation, small and large alkyl hydroxylation, aromatic and alkyl epoxidation, carbene transfer, nitrene transfer, and other reactions. These tables are an easy reference to check whether a specific substrate of interest has been tested, as they list all substrates with duplicates removed.

To maintain focus, this review will not delve into mechanistic details or provide explanations for how specific mutations affect reaction outcomes; such information, where available, can be found in the cited publications. Additionally, quantifying values are not catalogued here because the diverse metrics used to evaluate enzyme activity (i.e., k_{cat}, K_M, k_{cat}/K_M, TTN, % conversion, % yield, rate of NADPH consumption, rate of product formation, etc.) preclude direct comparison.

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Indeed, until recently there was no set protocol for presenting biocatalytic data. STRENDA (Standards for Reporting Enzymology Data) has addressed this with its guidelines on enzymology data reporting.[153] Upon wider adoption by the community, it will enable reporting data in a machine-readable format that will significantly boost the speed of data collection for database creation. However, research articles without a consensus still require manual curation.[154] Additionally, lab-to-lab variation in reaction conditions can affect activity, even when utilizing the same metric (i.e., % conversion after 1 h vs 16 h).

Variants possessing properties less favorable than the parent molecule will not be included, unless a specific property has been exchanged to gain another property (for example, a decrease in product formation is traded for improved enantioselectivity), which will be mentioned explicitly. However, because this review does not specify the metrics used to assess the degree of improvement of one variant over another, the reader is encouraged to refer to the cited publications for further details about variant properties.

The discussed requirements yielded approximately 400 publications reporting over 1500 distinctive variants with improved features (Supplementary Table 1). These encompassed more than 220 singular variants, over 250 double variants, and more than 140 triple variants with the corresponding mutations for each variant listed in Supplementary Tables 11−13. Each of these variations has exhibited an enhancement above the parent enzyme and implies that, in many cases, only a handful of modifications are necessary to enhance the catalytic abilities of P450 BM3. Variants containing four mutations are listed in Supplementary Table 14, while those with more than four mutations are listed in Supplementary Table 15 in ascending order based on the number of mutations.

Together, these variants consist of over 900 unique mutations, with some containing up to 34 mutations. The mutations are listed in Supplementary Table 16 based on amino acid position. Researchers can refer to these tables to quickly determine if a variant of interest is present in the database. If the variant is present, readers should consult Supplementary Table 1 for substrate information and corresponding references that provide further characteristics on the P450 BM3 variant. If a variant of interest is not listed in the database, mutations included in the variant may be identified in a similar manner.

Wild-type P450 BM3 possesses the innate ability to natively catalyze diverse reaction types with varying degrees of efficiency and a range of substrate types (Table 1). Numerous alkyl hydroxylation and alkyl epoxidation reactions are catalyzed by wild-type BM3 because they closely resemble the natural substrates, medium-to-long-chain fatty acids, such as lauric acid. The database also reports wild-type hydroxylation of aromatic, drug/drug-like, and small alkyl substrates, generally with lower reaction conversions than for large alkyl hydroxylation. Interestingly, wild-type BM3 has exhibited detectable activity for carbene[30−36] and nitrene transfer reactions[51−56] and other reactions[155,156] with certain substrates. However, the conversion, regioselectivity, and enantioselectivities are often inadequate and impractical. Nonetheless, the wild-type BM3's native promiscuity showcases its potential as a platform for enzyme engineering, explaining why many researchers use it as a starting point.

Table 1. Reactions Catalyzed by Wild-Type BM3[a]

Reaction Class	Code	References
Aromatic Hydroxylation	1a	4, 5, 8, 10, 13, 16, 21, 23, 25, 157-203
Drug/ Drug-Like Hydroxylation	1b	21, 79, 82, 172, 204-217
Small Alkyl Hydroxylation	1c	4, 21, 22, 28, 84, 157, 167, 217-246
Large Alkyl Hydroxylation	1d	2, 15, 21, 79, 85, 157, 166-168, 192, 203, 207, 218-220, 228, 229, 238, 244, 245, 247-325
Aromatic Epoxidation	2a	20, 21, 23, 25, 29, 174, 184, 326-329
Alkyl Epoxidation	2b	21, 22, 26, 28, 29, 221, 223, 226-228, 255, 282, 302, 323, 324, 330-333
Carbene Transfer	3	30-36
Nitrene Transfer	4	51-56
Other Reactions/Stabilizing Mutations	5	155, 156, 290, 318, 334, 335

[a]Citations pertain to instances where wild-type P450 BM3 performed the corresponding reaction in each reaction class category.

Our objective was to examine amino acid positions and mutations which enable a broad range of reactions, to provide a unique perspective attainable only by gathering hundreds of variants into a database. We will discuss recurring positions or mutations in each reaction class that enhance the performance for commonly encountered substrates. This will assist researchers not currently utilizing P450 BM3 in identifying appropriate variants or substrates to begin their investigations. It will also facilitate targeting residues for producing site-saturation mutagenesis (SSM) libraries to screen various reaction classes and identify starting points for further protein engineering.

REPRESENTATION OF P450 BM3 MUTATIONS IN RELATION TO THE REACTIONS THEY CATALYZE

Our primary goal was to examine positions where mutations occurred, regardless of the resulting mutation. By identifying regions that undergo frequent mutation across various reaction classes, we can pinpoint positions that, when mutated, enable reaction promiscuity. These positions serve as possible starting points for creating SSM libraries capable of catalyzing more than one reaction.

A crystal structure of the heme domain of P450 BM3 (residues 1−471, PDB: 1FAG) highlights key secondary structure elements: the B′-helix (residues 72−83), the E-helix (142−159), the F-helix (171−190), the largely solvent exposed G-helix (197−226), and the I-helix (Figure 1). These elements cluster above the plane of the heme. The cyan-colored I-helix and the red heme will serve as orientation reference points for the active site in all figures. While numerous mutations made beyond these regions have been

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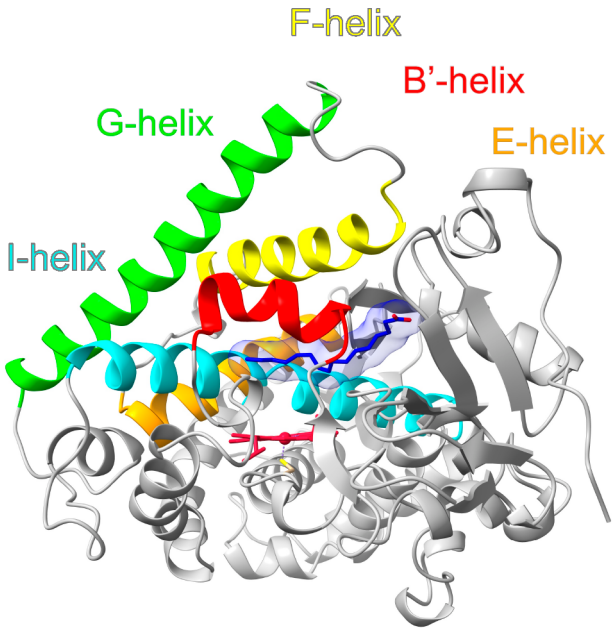

Figure 1. Crystal structure of the P450 BM3 heme domain (PDB: 1FAG) representing key secondary structure elements. These are the B′-helix (red; residues 72−83), E-helix (orange; 142−159), F-helix (yellow; 171−190), G-helix (green; 197−226), and I-helix (cyan; 250−282). The heme is red, and the cocrystallized lauric acid is in navy sticks surrounded by its van der Waals radius.[66]

reported, the highlighted elements encompass several of the most commonly mutated positions that will be discussed in more detail in this review. Figure 1 depicts the interaction between the heme-iron and the axial cysteine (Cys400). The substrate access tunnel, containing the cocrystallized substrate lauric acid, is highlighted by dark blue and is adjacent to the clustered key elements presented above. The B′-helix, F-helix, and I-helix feature many residues within the substrate access channel that interact with the substrate (Figure 1). For this reason, most of the commonly mutated positions on P450 BM3 are in these regions.

P450 BM3 VARIANTS CATALYZING AROMATIC HYDROXYLATION

We have identified 140 positions where at least one mutation improved reactivity toward aromatic hydroxylation (Figure 2). The relevant citations for the mutated positions in both heme and reductase domains are listed in the caption of Figure 2 (as for each of the following figures); "chimeras" refer to variants created using gene shuffling-type approaches, where identifying which gene is the "parent" and corresponding number of mutations can be difficult. These mutations span the entire heme domain, with many of them located in or near the active site. Figure 2 also shows some regions where no beneficial mutation has been reported, but it is uncertain whether mutations have been carried out and were not beneficial or whether those positions have not been investigated.

Mutations at positions 47, 51, 74, 78, 82, 87, 188, 263, 328, 330, and 353 have been frequently associated with improved hydroxylation of aromatic substrates, as evidenced by multiple references to each position. A complete list of substrates can be found in Supplementary Table 2. The majority of these positions are adjacent to the substrate access channel, including the B′-helix, F-helix and I-helix, and in the active

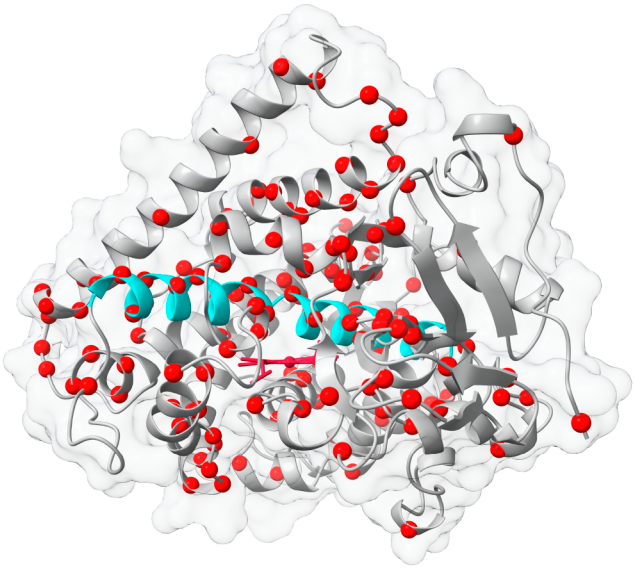

Figure 2. All positions of the P450 BM3 heme domain that have been mutated and show improvement toward aromatic hydroxylation are shown (red spheres). Mutated positions in the heme domain (residues 1−471) and in the reductase domain (472−1049) are listed below, with refs: **1** (338), **11** (338−340), **23** (338), **42** (338), **47** (5, 23, 25, 158, 163−165,167, 170, 173, 175−177, 182−184, 186, 187, 189, 193−195, 200, 202, 338−347, 384, 349−354), **51** (5, 23, 25, 158, 165, 167, 173, 175−177, 182, 184, 186, 187, 189, 193, 194, 202, 342−344,346, 355, 356), **58** (357−359), **64** (173, 183, 200, 340, 342, 349−351, 354), **68** (338, 339), **72** (343, 345), **73** (195), **74** (19, 157, 159, 165, 169, 173, 179, 187, 195, 202, 342, 343, 360−365), **75** (13, 19, 166, 201), **78** (163, 164, 181, 187, 198, 337, 343, 348, 364, 366), **81** (166, 173, 187, 195, 200, 338−340, 342, 349−351, 354), **82** (1, 5, 163, 187, 198, 201, 319, 337, 343, 345, 351, 367, 368), **86** (173, 183, 200, 347, 349, 350), **87** (5, 6, 9, 13, 16, 21, 157−160, 162, 164, 165, 168−171, 173−175, 179, 181−183, 187, 189, 190, 194, 195, 200−202, 327, 337−343, 345−365, 368−379), **88** (181), **94** (163, 164, 348), **96** (9, 358), **100** (357−359), **103** (338, 340), **106** (338), **107** (338, 357−359), **108** (195), **109** (338, 339), **110** (340), **112** (340), **113** (338, 339), **115** (9), **122** (196), **135** (357−359), **136** (338, 340), **138** (163, 366), **142** (163, 164, 348), **143** (173, 183, 195, 200, 338−340, 342, 349−351, 354), **145** (357−359), **146** (338), **148** (206), **149** (338), **150** (338, 339), **152** (339), **159** (338, 340), **160** (9), **162** (206, 340−342), **167** (368), **168** (361), **171** (23, 25, 165, 175, 184, 187, 343, 363), **175** (163, 164, 348, 366), **178** (163, 366), **180** (1, 319), **181** (1, 5, 16, 181, 319), **184** (1, 16, 163, 164, 187, 319, 343, 348, 366), **185** (206), **187** (206, 340, 341), **188** (1, 5, 157, 159, 165, 169, 170,173, 179, 182, 183, 187, 195, 200, 202, 206, 319, 338−343, 345, 347, 349−351, 354, 360−365, 370), **189** (166), **190** (340), **191** (25, 165, 168, 175, 184, 185, 187, 343), **192** (341), **198** (9, 349, 350, 354), **199** (338, 339), **205** (163, 164, 348), **213** (338, 339), **220** (195), **222** (364), **223** (163), **224** (368), **225** (361), **226** (163, 164, 348), **227** (163), **228** (206), **236** (163, 164, 206, 342, 348, 366), **237** (9, 206, 340, 341, 358), **238** (353), **239** (25, 165, 168, 175, 184, 185, 187, 343, 357−359), **248** (341), **252** (163,164, 348, 366), **254** (341), **255** (163, 164, 196, 348, 352, 366), **256** (9), **259** (25, 165,168, 175, 184, 185, 187, 343), **262** (201), **263** (19, 166, 187, 201, 343, 363), **264** (158, 173, 201, 337, 342, 343), **267** (173, 183, 187, 195, 200, 338−340, 342, 343, 345, 349−351, 354), **268** (23, 161, 166, 181, 373), **270** (338), **274** (357−359), **276** (25, 165, 168, 175, 184, 185, 187, 343), **278** (9), **281** (341, 353), **285** (349, 350, 354), **286** (166), **290** (163, 164, 348, 366), **295** (163, 366), **305** (9, 166), **307** (23, 25, 165, 175, 184, 187,343, 363), **312** (338), **319** (23, 25, 165, 175, 184, 187, 338, 339, 343, 349, 350, 363), **328** (5, 13, 16, 163, 180, 181, 187, 189, 194, 198, 202, 319, 343, 346, 363, 364, 367), **329** (19, 196), **330** (19, 21, 25, 165, 167, 175, 186, 187, 193, 319, 343, 379), **331** (196), **335** (341), **338** (338), **345** (9), **347** (338, 339), **351**

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Figure 2. continued

(341), **353** (25, 163−165, 168, 175, 184, 185, 187, 343, 348, 366), **354** (19, 352, 353), **359** (341), **363** (352, 353), **366** (166), **370** (338), **377** (165, 175, 379), **383** (338, 339, 368), **390** (189), **392** (189), **393** (189, 358), **399** (203), **401** (5, 167, 176, 177, 184, 186, 187, 193, 341, 343, 344, 380), **403** (187, 203, 339), **405** (9, 358), **407** (368, 381, 382), **408** (338−340), **410** (25), **413** (339), **415** (345, 349−351, 354), **417** (340), **425** (165, 175, 379), **434** (357−359, 361, 368), **435** (361), **437** (19, 187, 189, 194, 201, 345, 350), **438** (201), **440** (361), **445** (362), **446** (357−359), **471** (349, 352, 353), **474** (338−341), **490** (189), **494** (349), **543** (352), **558** (338−341), **575** (353), **584** (367), **595** (353), **664** (338−341), **675** (338−341), **678** (338−341), **685** (367), **687** (338−341), **741** (338−341), **760** (367, **813** (338−341), **814** (343), **825** (338−341, 367), **836** (338−341), **853** (367), **870** (338−341), **881** (338−341), **887** (338−341), **894** (338−341), **915** (367), **954** (338−341), **964** (349, 350), **967** (338−341), **981** (338−341), **1008** (338−341), **1011** (353), **1016** (353), **1021** (338−341), **1022** (338−341, 353), **1024** (349), **1049** (349), **Chimeras** (383, 384).

site; this trend is evident throughout this review. SSM libraries of these positions can be invaluable for screening different substrate classes. Indeed, the aromatic hydroxylation reactions comprise more than 400 variants, with half containing only one or two mutations (Supplementary Figure 1). Since variants with more mutations have seldom been reported for this class of reactions, this implies that few mutations are sufficient to create variants that are operationally effective.

Position 87, located within the active site, has been frequently mutated to smaller residues, enabling accommodation of larger substrates.[174,336] SSM at positions 78, 82, 87, 264, and 268 has produced P450 BM3 variants with mutagenesis coverage ranging between 50 and 70% at each position.[337] Over 25% of the library of 53 variants screened were effective at hydroxylating indole. This highlights the efficiency of utilizing SSM libraries at specific P450 BM3 positions.[337]

P450 BM3 Variants Catalyzing Drug/Drug-Like Hydroxylation. Aromatic hydroxylation and drug/drug-like hydroxylation reaction categories exhibit similar patterns of mutations, which is unsurprising due to the presence of an aromatic moiety in most drugs.[385−388] Among the drug/drug-like hydroxylation classification, 129 mutated positions have been reported that display improvement compared to the parent enzyme (Figure 3). These positions are spread throughout the heme domain and beyond, with numerous frequently mutated positions within the active site, indicating a similar trend to aromatic hydroxylation.

Mutation at positions 47, 51, 64, 72, 74, 78, 81, 82, 86, 87, 143, 188, 267, 319, and 415 occurs frequently and is linked to the metabolism of various drug and drug-like substrates. A complete list of substrates can be found in Supplementary Table 3. Similar to aromatic hydroxylation, the majority of frequently mutated positions are situated on the B′-helix, F-helix, or I-helix. Caution is necessary when interpreting common mutation positions. As an example, the mutation of glycine to serine at position 415 does not lead to any activity enhancement but was acquired during random mutagenesis along with E267V.[389] This variant, named M01, includes the mutations R47L/F87V/L188Q/E267V/G415S. In some cases, researchers use the M01 variant without the G415S mutation yet still name it M01; for this reason, we specify this variant's name and identity in the database.

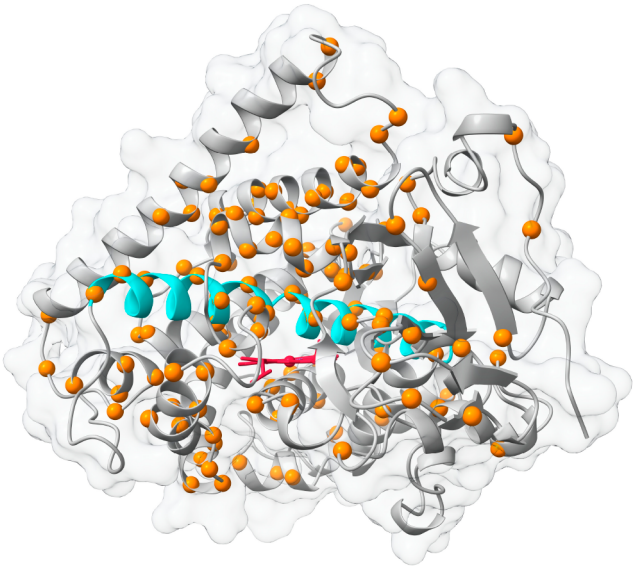

Figure 3. All positions of the P450 BM3 heme domain that have been mutated and show improvement toward drug/drug-like hydroxylation are shown (orange spheres). Mutated positions in the heme domain (residues 1−471) and in the reductase domain (472−1049) are listed, with refs: **8** (390), **11** (391, 392), **23** (391, 392), **24** (393, 394), **39** (391), **47** (3, 11, 14, 164, 182, 200, 204, 205, 209−211, 213, 214, 217, 334, 348, 350, 351, 354, 389−419), **49** (11, 396, 397, 405, 408, 420), **51** (11, 182, 204, 205, 209, 211, 395−397, 400, 403, 405, 406, 408), **52** (217, 348, 420), **58** (359, 393, 394, 420), **64** (200, 204, 205, 209, 210, 350, 354, 389, 396, 400, 401, 404, 407, 409−413, 415−420), **72** (3, 14, 211, 216, 350, 391, 396, 403, 404, 409, 410, 413, 415), **74** (182, 204, 205, 209, 211, 350, 393, 394, 400−404, 406, 407, 421, 422), **75** (334, 393, 394, 420), **76** (14), **77** (3, 14), **78** (3, 14, 164, 211, 216, 217, 334, 348, 393, 394, 396, 397, 403, 405, 408, 410, 423, 424), **79** (14), **80** (14), **81** (3, 14, 200, 204, 205, 209−211, 217, 350, 354, 389, 391, 392, 396, 399−401, 403, 404, 406, 407, 409−413, 415−419), **82** (3, 14, 79, 207, 211, 216, 217, 348, 350, 393, 394, 396, 397, 403−406, 408, 410, 413), **86** (200, 204, 205, 209, 350, 389, 390, 399−402, 404, 407, 409, 411−415, 420), **87** (3, 11, 14, 21, 79, 164, 182, 200, 204, 205, 207, 209−211, 213−215, 217, 334, 348, 350, 351, 354, 359, 377, 389−422), **88** (3, 14, 216), **94** (164, 217, 334, 348, 393), **96** (391), **100** (359, 393, 394, 420), **102** (393, 394), **103** (391), **106** (391, 420), **107** (359, 391−394, 420), **108** (391), **109** (391, 420), **110** (391, 392, 420 **112** (391), **113** (391), **122** (420), **135** (359, 393, 394, 420), **136** (391, 392), **138** (217, 348, 423, 424), **140** (420), **142** (164, 217, 334, 348, 393), **143** (200, 204, 205, 209, 210, 350, 354, 389, 391, 392, 396, 400−402, 404, 409, 410, 413, 415−420), **145** (359, 393, 394), **148** (206), **149** (391), **152** (391), **158** (391), **159** (391), **162** (206, 209, 400−402, 420), **165** (206, 401), **170** (420), **171** (211, 403, 406), **173** (402), **175** (164, 217, 334, 348, 393, 423, 424), **177** (3, 14, 206, 334, 401), **178** (217, 348, 423, 424), **180** (217), **181** (334, 396), **182** (420), **184** (164, 211, 217, 334, 348, 393, 403, 406, 420, 423, 424), **185** (3, 14, 206, 209, 397, 405, 420), **186** (402), **187** (206, 209, 391, 400−402), **188** (3, 14, 182, 200, 204−206, 209−211, 213, 214, 217, 350, 351, 354, 389−392, 396−406, 409−419, 421, 422), **190** (391, 392), **191** (211, 403), **197** (217), **198** (350, 354, 404, 409, 411, 413, 415, 417−419), **205** (14, 164, 217, 334, 348, 393) **209** (3, 14), **211** (391), **212** (391), **213** (391, 392), **226** (164, 217, 334, 348, 393), **228** (206, 209, 401), **231** (420), **233** (420), **236** (164, 206, 209, 217, 334, 348, 393, 401, 423, 424), **237** (206, 209, 400−402), **239** (211, 359, 393, 394, 403, 420), **251** (212), **252** (164, 217, 334, 348, 393, 423, 424), **255** (164, 217, 334, 348, 393, 423, 424), **259** (211, 391, 403), **260** (334, 393, 406), **261** (391, 406), **262** (391, 406), **263** (211, 403, 406), **264** (395, 400−402, 406), **267** (200, 204, 205, 209−211, 214, 350, 351, 354, 389−392, 396, 399−404, 407, 409−413, 415−419), **268** (420), **270** (391, 406), **274** (359, 391, 393, 394, 420), **276** (211, 403), **285**

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Figure 3. continued

(350, 354, 404, 409, 411, 413, 415, 417−419), **290** (164, 217, 334, 348, 393, 423, 424), **294** (420), **295** (217, 348, 423, 424), **305**, 420), **307** (211, 212, 403, 406), **309** (391), **319** (211, 350, 389, 390, 399, 403, 404, 406, 409, 411−415), **324** (393, 394, 420 **328** (3, 14, 211, 216, 217, 348, 393, 403, 406), **329** (211, 406), **330** (3, 14, 21, 211, 216, 396, 403, 405, 406, 408), **338** (391), **340** (420), **349** (348), **353** (164, 211, 217, 334, 348, 393, 403, 423, 424), **366** (217, 334, 348, 393, 394, 420), **372** (393), **398** (420), **401** (211, 391, 403, 406), **403** (211, 391, 403, 406), **408** (391, 392), **409** (391, 420), **413** (391, 420), **415** (210, 214, 350, 354, 389, 390, 396, 399, 404, 409−413, 415, 417−419), **417** (391), **422** (334), **434** (359, 393, 394, 420), **437** (210, 334, 350, 396, 404, 407, 409, 413, 415), **438** (3), **440** (404), **442** (393, 394, 420), **446** (359, 393, 394, 420), **464** (217, 348), **474** (391, 392), **558** (391, 392), **664** (391, 392), **675** (391, 392), **678** (391, 392), **687** (391, 392), **710** (217, 348), **741** (391, 392), **813** (391, 392), **825** (391, 392), **836** (391, 392), **870** (391, 392), **881** (391, 392), **887** (391, 392), **894** (391, 392), **954** (391, 392), **964** (350, 390, 404, 409, 411, 413, 415), **967** (391, 392), **981** (391, 392), **1008** (391, 392), **1021** (391, 392), **1022** (391, 392), **1049** (404, 411, 413), **Chimeras** (383, 384, 425).

For the drug/drug-like hydroxylation class, there are over 400 variants in the database. Of these, 9% are single variants, 17% are double variants, 11% are triple variants, and other variants contain up to 29 mutations (Supplementary Table 1 and Supplementary Figure 2). It is plausible that multiple mutations may be necessary to achieve a desirable enhancement in activity, given that these substrates do not resemble the natural substrates. In some cases, variants that exhibit promiscuous activity toward drug/drug-like substrates can be applied to substrates from different reaction classes without the need for additional mutations and will be expanded upon further in this article.

P450 BM3 VARIANTS CATALYZING SMALL ALKYL HYDROXYLATION

Alkyl hydroxylation has been classified into two subclasses: small alkyl hydroxylation and large alkyl hydroxylation. Small alkyl hydroxylation involves substrates with a linear chain of <8 carbons or monocyclic alkyl alkanes such as cyclohexane. The aim is to distinguish these smaller substrates from those that are more similar to lauric acid. A complete list of substrates can be found in Supplementary Table 4. There are 81 positions within the heme domain that exhibit improved small alkyl hydroxylation ability compared to the parent enzyme as a result of mutations (Figure 4).

The most frequently mutated positions include 47, 74, 78, 82, 87, 188, and position 328. There are a total of 375 unique variants identified in the small alkyl hydroxylation group, which is comparable to the number found in aromatic and drug/drug-like hydroxylation. The small alkyl hydroxylation category includes a significant proportion of single and double substitutions, accounting for 25% and 24%, respectively (Supplementary Figure 3). Since these small alkyl substrates have nonaromatic and hydrophobic properties similar to those of the original substrates, observing a gain of function often requires few mutations. However, additional mutations may be necessary when using very small alkyl substrates (such as butane, propane, or ethane)[244,426] or when screening for improvements in regioselectivity or enantioselectivity.[217,231,240]

Screening SSM libraries at popular positions (74, 78, 82, 87, 188) within this class may yield significant benefits. These top

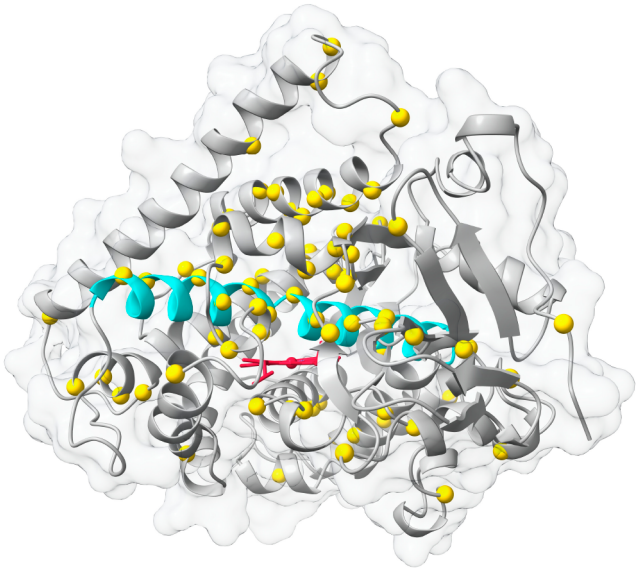

Figure 4. All positions of the P450 BM3 heme domain that have been mutated and show improvement toward small alkyl hydroxylation are shown (yellow spheres). Mutated positions in the heme domain (residues 1−471) and in the reductase domain (472−1049) are listed, with refs: **4** (428), **26** (230, 287, 288, 429, 430), **47** (28, 83, 167, 217, 219, 221, 222, 224, 226, 230, 231, 233, 237, 238, 242, 244, 245, 287, 288, 334, 426, 429−438), **51** (28, 167, 219, 221, 222, 237, 238, 242, 245, 431, 436), **52** (217, 233, 244, 433−435), **64** (224, 231), **70** (432), **72** (226), **74** (19, 28, 157, 222, 223, 231, 237, 238, 244, 287, 288, 306, 428−431, 434, 439−442), **75** (19, 219, 226, 334, 428, 437, 438), **78** (217, 220, 226, 230−233, 244−246, 246, 334, 348, 366, 424, 426−428, 432−438, 442), **81** (217, 224, 230, 231, 319, 348, 442), **82** (217, 226, 230−233, 244−246, 319, 348, 367, 426−428, 432−435, 437, 438, 442), **86** (231), **87** (7, 16, 19, 21, 22, 28, 83, 157, 217, 222−227, 229−232, 234, 235, 237, 238, 240, 245, 287, 288, 306, 334, 348, 376, 428−433, 437, 438, 440−445), **88** (437, 438), **94** (217, 226, 233, 244, 245, 334, 426, 432−435, 437, 438), **111** (432), **138** (217, 220, 244, 245, 366, 424, 426, 436), **141** (432), **142** (217, 226, 233, 244, 245, 334, 348, 426, 432−435, 437, 438, 442), **143** (224, 231), **147** (432), **153** (428), **159** (432), **162** (428), **171** (237, 238), **175** (217, 220, 226, 233, 244, 245, 334, 348, 366, 424, 426, 432−438, 442), **178** (217, 220, 244, 245, 366, 424, 426, 436), **180** (217, 348, 432, 442), **181** (16, 219, 230, 243, 246, 334, 428), **182** (243), **184** (16, 217, 220, 226, 233, 244, 245, 319, 334, 348, 366, 424, 426, 428, 432−438, 442), **185** (232), **188** (28, 83, 157, 217, 223, 224, 230−233, 237, 238, 244, 287, 288, 306, 319, 428−431, 433−435, 439−441), **191** (185, 237, 238), **197** (217, 348, 442), **198** (224, 231), **205** (217, 226, 233, 244, 245, 334, 348, 426, 432−435, 437, 438, 442), **226** (217, 226, 233, 244, 245, 334, 348, 426, 432−435, 437, 438, 442), **232** (428), **235** (242, 428), **236** (217, 220, 226, 233, 244, 245, 334, 348, 366, 424, 426, 432−438, 442), **239** (185, 237, 238, 242), **241** (432), **252** (217, 220, 226, 233, 244, 245, 334, 348, 366, 424, 426, 432−438, 442), **255** (217, 220, 226, 233, 244, 245, 334, 348, 366, 424, 426, 432−438, 442), **259** (185, 237, 238), **260** (19, 230, 334, 428, 437, 438), **263** (19, 226, 230, 246, 319, 432), **264** (445), **266** (432), **267** (224, 231), **268** (235, 443), **276** (185, 237, 238), **285** (224, 231), **290** (217, 220, 226, 233, 244, 245, 334, 348, 366, 424, 426, 432−438, 442), **295** (217, 220, 244, 245, 366, 424, 426, 436), **307** (237, 238), **315** (432), **319** (231, 237, 238, 243), **328** (7, 16, 22, 217, 226, 227, 229, 230, 232−435, 236, 240, 244−246, 319, 367, 426−428, 432−435, 437−440, 440, 444, 445), **329** (19, 230, 233, 435), **330** (19, 21, 167, 317, 319, 428, 432), **345** (432), **353** (185, 217, 220, 226, 233, 237, 238, 244, 245, 334, 348, 366, 424, 426, 432−438, 442), **359** (428), **366** (217, 233, 244, 334, 433−435), **386** (222), **390** (203), **393** (203), **399** (203 **401**), (4, 167, 237, 238, 242, 380), **403** (203), **407** (381), **411** (243), **415** (224, 231), **422** (334), **436** (243), **437** (19, 230, 231, 334, 445), **438** (246, 427), **443** (244,

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Figure 4. continued

434), **464** (217, 244, 434, 437), **471** (441), **584** (367), **654** (434), **685** (367), **698** (434), **710** (217, 244, 434, 437), **760** (367), **825** (367), **853** (367), **915** (367), **964** (231), **966** (233, 245, 435, 436, 444), **1037** (434), **1046** (233, 245, 435, 436, 444), **1049** (231).

single variants can subsequently be recombined to create higher-order variants with enhanced activity compared to their point-mutated counterparts.[240] Using pooled SSM libraries has also been successful, although most researchers prefer to use individual libraries and recombine top variants.[427]

P450 BM3 VARIANTS CATALYZING LARGE ALKYL HYDROXYLATION

Large alkyl hydroxylation is the substrate class that most closely resembles and includes the native substrates, such as lauric acid. Within the heme domain, there are 123 positions that are mutated and display an enhancement relative to the parent (Figure 5). Commonly mutated positions in this reaction class include 47, 51, 74, 78, 82, 87, 175, 184, 188, 236, 252, 255, 328, and 353. Approximately 300 unique variants are present in this reaction class, with the largest portion (19%) consisting of single variants. Over 70% of the variants within this reaction category have ≤6 mutations, as observed in Supplementary Figure 4.

A majority of the variants in this reaction category have high conversions with native-like substrates.[168] A complete list of substrates can be found in Supplementary Table 5. Mutations are made to alter regioselectivity or enantioselectivity, since attempting to improve a reaction conversion that is already near-quantitative leads to diminishing returns.[296,325] Position 47 is situated near the entrance of the substrate access channel where the native Lys47 interacts with the carboxylate group of long-chain fatty acids such as lauric acid.[66] In the case of a mutation to neutral residues at this position, P450 BM3 can accept substrates with various ionic characteristics.[293]

P450 BM3 VARIANTS CATALYZING AROMATIC EPOXIDATION

The epoxidation reactions can be categorized based on whether the substrate resembles the native substrates or not. A complete list of substrates can be found in Supplementary Table 6. Within the database, the smallest category is aromatic epoxidation, which includes only 34 unique variants with mutations across 49 positions on the heme domain (Figure 6). Variants with either one or five mutations account for approximately 40% of all variants within the aromatic epoxidation subclass (Supplementary Figure 5).

Wild-type P450 BM3 catalyzes the formation of styrene oxide from styrene through epoxidation with a preference for (*R*)-styrene oxide formation, exhibiting a conversion of only 14% and an enantiomeric excess of 20%.[326] Introduction of the F87G substitution enhanced (*R*)-styrene oxide formation to an enantiomeric excess of up to 90%.[17,18] Variant 139-3 is the product of engineering toward improved small alkyl hydroxylation: its reaction conversion was improved to 40% but with an enantiomeric excess of only 6%. Despite having 11 substitutions, variant 139-3 is outperformed by the F87G single variant in terms of enantioselectivity; substitution at position 87 frequently enhances or even inverts enantiose-

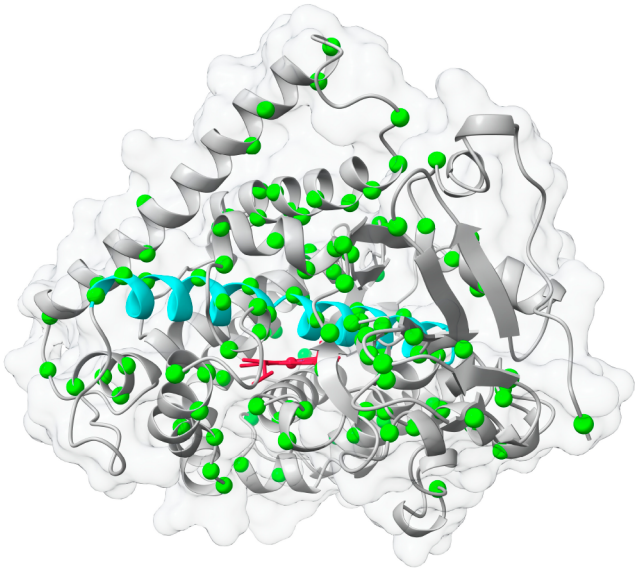

Figure 5. All positions of the P450 BM3 heme domain that have been mutated and show improvement toward large alkyl hydroxylation are shown (light green spheres). Mutated positions in the heme domain (residues 1−471) and in the reductase domain (472−1049) are listed, with refs: **1** (320), **21** (325), **25** (446), **26** (2, 287, 320, 429, 446, 447), **27** (325), **28** (320), **30** (325), **47** (2, 195, 217, 238, 241, 244, 245, 287, 288, 293, 348, 426, 429, 433, 438, 446−454), **51** (2, 238, 241, 245, 270, 325, 446, 451), **52** (217, 244, 290, 348, 433), **55** (325), **58** (290, 359, 455, 456), **62** (381), **67** (325), **69** (24), **70** (325), **72** (2, 24, 296), **73** (195, 313), **74** (2, 157, 238, 241, 244, 287, 288, 306, 429, 431, 441, 442, 446, 447, 453, 457, 458), **75** (2, 12, 24, 166, 219, 309, 319, 452, 458−461), **78** (2, 12, 24, 217, 220, 244, 245, 296, 309, 319, 348, 424, 426, 433, 438, 442, 452−454, 458−462), **81** (2, 166, 195, 217, 442, 453, 454, 462), **82** (2, 12, 79, 207, 217, 244, 245, 252, 314, 319, 348, 367, 433, 438, 442, 452−454, 462), **86** (281), **87** (2, 7, 12, 21, 24, 79, 157, 195, 207, 217, 229, 238, 241, 245, 276, 285, 287, 288, 290, 296, 304, 306, 309, 325, 335, 336, 348, 359, 369, 376, 429, 431, 441, 442, 446, 447, 451−468), **88** (458), **94** (217, 244, 245, 348, 426, 433, 438, 452, 454), **96** (278), **97** (381, 469, 470), **100** (290, 359, 455, 456), **106** (290), **107** (290, 359, 455, 456), **108** (195), **109** (325, 470), **119** (325), **127** (320), **135** (290, 320, 359, 455, 456), **138** (220, 244, 245, 424), **142** (12, 217, 244, 245, 348, 426, 433, 438, 442, 452−454, 461, 462), **143** (195), **145** (290, 325, 359, 455, 456), **148** (325), **156** (381), **171** (2, 238), **175** (12, 217, 220, 244, 245, 348, 424, 426, 433, 438, 442, 452−454, 461, 462), **177** (12, 452, 461), **178** (220, 244, 245, 424), **180** (217, 442, 453, 454, 462), **181** (12, 219, 442, 461, 462), **184** (2, 12, 217, 220, 244, 245, 290, 348, 424, 426, 433, 438, 442, 452−454, 461, 462), **185** (325), **188** (2, 157, 195, 217, 238, 241, 244, 287, 288, 306, 312, 429, 431, 433, 441, 446, 447, 451, 457), **189** (166), **191** (2, 168, 238), **197** (217, 325, 442, 453, 454, 462), **198** (325), **202** (325), **205** (12, 217, 244, 245, 348, 426, 433, 438, 442, 452−454, 462), **214** (325), **216** (325), **220** (195), **226** (12, 217, 244, 245, 348, 426, 433, 438, 442, 452−454, 461, 462), **228** (325), **235** (471, 472), **236** (12, 217, 220, 244, 245, 348, 424, 426, 433, 438, 442, 452−454, 461, 462), **237** (325), **239** (2, 168, 238, 290, 325, 359, 455, 456), **251** (310), **252** (12, 217, 220, 244, 245, 348, 424, 426, 433, 438, 442, 452−454, 461, 462), **255** (12, 217, 220, 244, 245, 325, 348, 424, 426, 433, 438, 442, 452−454, 462), **259** (2, 168, 238), **260** (2), **263** (2, 12, 166, 296, 309, 325), **264** (2, 309, 325, 446), **267** (2, 195, 473), **268** (24, 166, 254, 291, 294), **274** (290, 359, 455, 456), **276** (2, 168, 238), **278** (325), **286** (166), **290** (12, 217, 220, 244, 245, 348, 424, 426, 433, 438, 442, 452, 454, 461, 462), **295** (220, 244, 245, 424), **301** (325), **305** (166), **307** (2, 238, 310), **315** (325), **319** (2, 238), **324** (290), **327** (325), **328** (2, 7, 12, 24, 217, 229, 244, 245, 319, 325, 348, 367, 426, 433, 438, 452, 458), **330** (2, 21, 319, 458), **331** (458), **332** (2), **334** (325), **335** (325), **340** (290), **345** (325), **349** (348), **353** (2, 12, 168, 217, 220, 238,

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Figure 5. continued

244, 245, 348, 424, 426, 433, 438, 442, 452−454, 461, 462), **357** (325), **363** (325), **366** (166, 217, 244, 290, 348, 433, 461), **371** (325), **385** (325), **390** (325), **393** (474), **397** (469, 470), **399** (203), **401** (2, 238, 241, 380), **403** (2, 203, 475), **407** (381, 382, 470, 475−477), **408** (325), **422** (325), **434** (290, 359, 455, 456), **437** (2, 12), **442** (290, 461), **443** (244), **446** (290, 359, 455, 456), **452** (320), **463** (320), **464** (217, 244, 348), **470** (320), **471** (441, 471, 472), **473** (320), **474** (320), **494** (471, 472), **546** (320), **584** (367), **589** (320), **599** (320), **624** (320), **637** (320), **639** (320), **660** (320), **664** (320), **674** (320), **685** (367), **710** (217, 244, 348), **715** (320), **716** (320), **741** (320), **760** (367), **769** (472), **782** (320), **796** (472), **813** (320), **824** (320), **825** (367), **847** (472), **850** (472), **852** (472), **853** (367), **870** (320), **881** (320), **889** (320), **895** (320), **915** (367), **947** (320), **954** (320), **966** (245, 295, 318, 431, 446, 471, 472, 478, 479), **967** (320), **978** (472), **1008** (320), **1019** (320), **1024** (471, 472), **1046** (245, 295, 297, 318, 431, 446, 471, 472, 478, 479), **Chimeras** (305, 323, 324, 383).

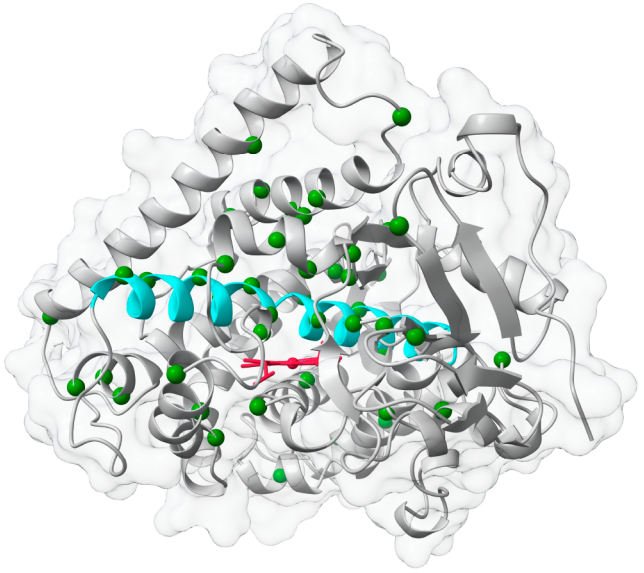

Figure 6. All positions of the P450 BM3 heme domain that have been mutated and show improvement toward aromatic epoxidation are shown (dark green spheres). Mutated positions in the heme domain (residues 1−471) and in the reductase domain (472−1049) are listed, with refs: **47** (23, 25, 27, 184, 226), **51** (23, 25, 27, 184), **58** (456), **75** (20), **78** (20, 27, 226, 326, 366, 480), **82** (27, 226, 298), **87** (17, 18, 20, 21, 174, 226, 326, 327, 371, 456), **94** (27, 226), **100** (456), **107** (456), **135** (456), **138** (326, 366, 480), **142** (27, 226), **145** (456), **171** (23, 25, 184), **175** (27, 226, 326, 366, 480), **178** (326, 366, 480), **181** (20), **184** (27, 226, 326, 366, 480), **191** (25, 27, 184), **205** (27, 226), **226** (27, 226), **235** (326), **236** (27, 226, 326, 366, 480), **239** (25, 27, 184, 456), **252** (27, 226, 326, 366, 480), **255** (27, 226, 326, 366, 480), **259** (25, 27, 184), **262** (20), **263** (20, 27, 226), **264** (20), **268** (23), **274** (456), **276** (25, 27, 184), **290** (27, 226, 326, 366, 480), **295** (326, 366, 480), **307** (23, 25, 184), **319** (23, 25, 184), **328** (20, 27, 226), **330** (21, 25), **353** (25, 27, 184, 226, 326, 366, 480), **372** (27), **401** (184), **410** (25), **434** (456), **437** (20), **438** (20, 298), **446** (456), **471** (326), **494** (326), **1024** (326).

lectivity[17,18,174] and is regularly present in this reaction class (Supplementary Table 1). These examples illustrate that both single point substitution and elaborate engineering can yield operational improvements depending on the desired application, highlighting the complexity of engineering P450 BM3.

P450 BM3 VARIANTS CATALYZING ALKYL EPOXIDATION

The second group of epoxidation involves substrates that closely resemble the fatty acid substrates natively favored by BM3 (Figure 7). A complete list of substrates can be found in

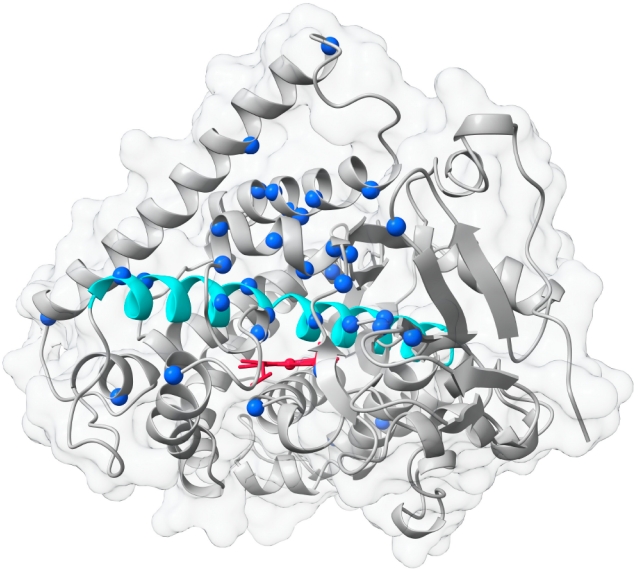

Figure 7. All positions of the P450 BM3 heme domain that have been mutated and show improvement toward alkyl epoxidation are shown (light blue spheres). Mutated positions in the heme domain (residues 1−471) and in the reductase domain (472−1049) are listed, with refs: **26** (429, 430), **47** (28, 221, 226, 331, 429, 430, 448, 449, 481), **51** (28, 221, 331, 449, 481), **69** (24), **72** (24, 226), **74** (19, 28, 223, 331, 429, 430), **75** (19, 226, 309), **78** (24, 226, 309, 366, 424, 462), **81** (462), **82** (226, 462, 482), **87** (19, 21, 22, 24, 28, 221, 223, 226, 227, 309, 331, 332, 429, 430, 445, 448, 449, 462, 481), **94** (226), **138** (366, 424), **142** (226, 462), **175** (226, 366, 424, 462), **178** (366, 424), **180** (462), **181** (462), **184** (226, 366, 424, 462), **188** (28, 223, 331, 429, 430), **197** (462), **205** (226, 462), **226** (226, 462), **236** (226, 366, 424, 462), **252** (226, 366, 424, 462), **255** (226, 366, 424, 462), **260** (19), **263** (19, 226, 309), **264** (309), **268** (24), **290** (226, 366, 424, 462), **295** (366, 424), **328** (22, 24, 226, 227, 445, 481), **329** (19), **330** (19, 21), **353** (226, 366, 424, 462), **401** (482), **437** (19, 445), **Chimeras** (323, 324).

Supplementary Table 7. There are 112 variants reported in this class, but only 38 positions within the heme domain have been reported to provide improved activity upon mutation. This is the fewest number of positions mutated for any of the reaction classes. Almost 50% of the variations in this category consist of point substitutions, which shows that few mutations at a limited number of positions result in improved function (Supplementary Figure 6).

The wild-type enzyme frequently exhibits substantial activity toward numerous substrates in this type of reaction.[302] As the point substituted variants are highly efficient, there is little impetus to introduce further mutations. Therefore, mutations usually enhance the regio- or enantioselectivity of epoxidation rather than the quantity of the resulting product.[226,227] Moreover, there is a common occurrence of competition between substrate epoxidation and hydroxylation, where the utilization of mutations in P450 BM3 can facilitate an increase

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in the proportion of epoxidation to hydroxylation.[28,226,227] Position 87 remains one of the most frequently mutated positions, consistent with previous reaction classes, as its location in the active site can cause significant changes in accepted substrates based on the substitution at that position.

P450 BM3 VARIANTS CATALYZING CARBENE TRANSFER

The carbene transfer reaction, catalyzed by P450 BM3 variants, represents a novel-to-nature reaction and illustrates how enzyme engineering can generate innovative functions. Although mutations at positions 268 and 400 are largely responsible for unlocking this reactivity, it differs considerably from the native reaction and often requires numerous mutations to achieve a modest level of activity.[30−31,135,141] For more information on carbene and nitrene transferase reactions performed by various P450 BM3 variants, including their initial discovery, development, and most promising candidates, please refer to the recent article published by Yang and Arnold.[135]

There are 159 distinct variants across 45 positions in the carbene transfer reaction classes (Figure 8). Variants engineered to perform carbene transfer reactions with operational utility typically include more mutations than those selected for hydroxylation or epoxidation reactions. Indeed, the carbene transfer reaction class is characterized by a prevalence of variants with 18 mutations, with more than 85% of the variants containing at least 13 mutations (Supplementary Figure 7). This can be attributed to the requirement for more extensive modification to achieve appropriate active site architecture and productive substrate binding as the chemistry and substrates used differ importantly from the native reaction. A complete list of substrates can be found in Supplementary Table 8.

For instance, variants 139-3, J, and 9-10A were all initially engineered toward enhanced small alkane hydroxylation.[220,426] These variants feature either 11 mutations (139-3) or 13 mutations (J and 9-10A) and serve as frequent parent enzymes for further engineering of carbene transfer variants.[135] Carbene transferases engineered to be efficient with a particular substrate then often serve as starting variants to alter regio- or enantioselectivity.[135] Because there is no immediate benefit to discern mutations within a variant that do not contribute to the desired function, this reaction class holds the highest proportion of variants that include 25 or more substitutions (compare Supplementary Figure 7 to Supplementary Figures 1−6 and 8−9).

P450 BM3 VARIANTS CATALYZING NITRENE TRANSFER

Similarly to carbene transfer reactions, the nitrene transfer reactions catalyzed by P450 BM3 variants are also a novel-to-nature reaction. As mentioned above, more information on carbene and nitrene transferase reactions by P450 BM3 variants can be found in this recent publication.[135] This reaction class encompasses a total of 94 variants distributed across 46 positions in the heme domain (Figure 9). In a further similarity with carbene transferase variants, approximately 90% of nitrene transferase variants comprise 13 or more mutations and only about 7% have fewer than 10 mutations, illustrating that greater deviations from the native reaction typically require more mutations (Supplementary Figure 8). A complete list of substrates can be found in Supplementary Table 9. In addition, variants with fewer than 10 mutations suffer from low reaction conversions and this renders them operationally unusable.[51−56] Variants exhibiting enhanced nitrene transferase properties are utilized as the starting point for advancing nitrene transfer reactions with diverse substrates, thereby increasing the number of substitutions.[135]

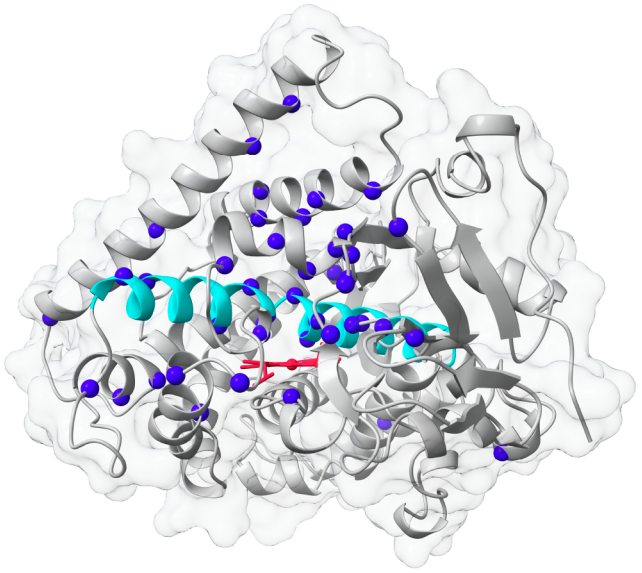

Figure 8. All positions of the P450 BM3 heme domain that have been mutated and show improvement toward carbene transfer are shown (dark blue spheres). Mutated positions in the heme domain (residues 1−471) and in the reductase domain (472−1049) are listed, with refs: **47** (30, 43), **70** (44, 46, 47, 49, 50), **72** (42, 44), **74** (44, 46, 47, 49, 50), **75** (30, 33, 35, 45, 48), **78** (30, 31, 33−36, 38, 40−50), **82** (30, 34, 42, 44, 46, 47, 49, 50), **87** (30, 31, 33−37, 42−50), **92** (34, 40), **94** (30), **100** (34, 40), **118** (43, 44, 47, 49, 50), **142** (30, 31, 33−36, 42−50), **149** (43), **162** (44, 47, 50), **175** (30, 31, 33−36, 42−50), **177** (30, 33, 43, 44, 46, 47, 49, 50), **181** (30, 33−35, 38, 40, 41, 45, 48), **184** (30, 31, 33−36, 42−50), **188** (42), **201** (34), **205** (30), **215** (34), **226** (30, 31, 33−36, 42−50), **233** (43), **236** (30, 31, 33−36, 42−50), **248** (43), **252** (30, 31, 33−36, 42−50), **255** (30), **261** (42), **263** (30, 33−35, 42−44, 46−50), **264** (44, 47, 50), **266** (43, 44, 46, 47, 49, 50), **267** (35, 44, 47, 49), **268** (30, 31, 33−50), **269** (42), **281** (34), **290** (30, 31, 33−36, 42−50), **327** (34, 42−44, 46, 47, 49, 50), **328** (30, 34, 35, 42, 44, 46, 47, 49, 50), **330** (42−44, 46, 47, 49, 50), **332** (44, 47, 50), **353** (30, 31, 33−36, 42−50), **366** (30, 31, 33−36, 42−50), **400** (31, 32, 34, 35, 38, 40−50), **401** (44, 47, 49, 50), **436** (42−44, 46, 47, 49, 50), **437** (30, 33−35, 38, 40−50), **438** (30, 34−36, 38, 42, 45, 46, 48, 49), **442** (30, 31, 33−36, 42−50), **472** (34), **573** (34), **646** (34), **674** (34, 44, 46, 47, 50).

P450 BM3 VARIANTS CATALYZING OTHER REACTIONS

The last reaction class comprises variants that do not adhere to the previously discussed reaction classes. These variants execute a reaction that differs from all previously discussed reaction classes, or their mutations foster enhanced stability during operational conditions. There are 162 variants in this final reaction group, and they include substitutions located over 102 positions in the heme domain (Figure 10). On average, the variants contain six or fewer mutations. This is higher than the observed average for hydroxylation or epoxidation classes but significantly lower than the carbene or nitrene transfer reaction class (Supplementary Figure 9).

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Figure 9. All positions of the P450 BM3 heme domain that have been mutated and show improvement toward nitrene transfer are shown (purple spheres). Mutated positions in the heme domain (residues 1−471) and in the reductase domain (472−1049) are listed, with refs: **70** (64), **72** (65), **74** (57, 64, 65), **75** (56, 58), **78** (52−65), **81** (51, 53, 54, 60), **82** (51, 53−55, 57, 59−61, 63−65), **87** (51−65), **92** (55), **100** (55), **138** (53), **142** (51−65), **175** (51−65), **177** (56−57, 58), **178** (53), **180** (51, 53, 54, 60), **181** (55−58, 64), **184** (51−65), **197** (51, 53, 54, 60), **201** (55), **205** (51, 53, 54, 60), **215** (55), **226** (51−65), **236** (51−65), **252** (51−65), **255** (51, 53, 54, 60), **263** (55−59, 61−65), **266** (60, 65), **267** (60−61, 63, 65), **268** (52, 53, 55−64), **269** (65), **281** (55), **290** (51−65), **295** (53), **327** (57, 63), **328** (54, 55, 57, 59, 61−65), **330** (57), **353** (51−65), **366** (52, 55−58, 61−65), **392** (60), **393** (60), **394** (60), **395** (63, 65), **400** (55−59, 61−65), **401** (64), **402** (60), **436** (57), **437** (55−58, 62−65), **438** (52, 55, 56, 58−65), **442** (52, 55−59, 61−65), **472** (55), **573** (55), **646** (55), **674** (55, 57), **1046** (55).

Figure 10. All positions of the P450 BM3 heme domain that have been mutated and show improvement toward other reactions are shown (light pink spheres). Mutated positions in the heme domain (residues 1−471) and in the reductase domain (472−1049) are listed, with refs: **26** (483), **35** (483), **42** (211), **47** (156, 211, 334, 349, 434), **51** (156, 211), **52** (290, 420, 434, 483), **58** (290, 358, 420, 483), **61** (483), **64** (349), **74** (156, 211, 434, 483, 484), **75** (166, 420, 483), **78** (156, 334, 434, 484), **81** (156, 166, 211, 349), **82** (155, 156, 211, 367, 434, 483, 484), **86** (349, 420), **87** (155, 156, 211, 290, 334, 335, 349, 358, 420, 483, 484), **88** (211), **90** (483), **92** (483), **94** (334, 434), **96** (358), **98** (483), **100** (290, 358, 420, 483), **106** (290, 420, 483), **107** (290, 358, 420, 483), **108** (483), **113** (483), **118** (483), **134** (483), **135** (290, 358, 420, 483), **142** (334, 434, 484), **143** (349), **145** (290, 358, 483), **151** (483), **162** (206, 420), **171** (156, 211), **172** (483), **173** (483), **175** (334, 434, 484), **177** (484), **179** (156), **181** (484), **184** (156, 211, 290, 334, 420, 434, 483, 484), **188** (156, 211, 349, 434, 483), **189** (166), **191** (156, 211), **198** (349), **199** (483), **205** (334, 434, 483), **214** (483), **217** (483), **226** (334, 434, 484), **228** (206), **231** (483), **233** (156), **235** (335, 472), **236** (334, 434, 484), **237** (206, 358, 483), **239** (156, 211, 290, 358, 420, 483), **252** (334, 434, 484), **255** (334, 434), **259** (156, 211), **263** (156, 166, 484), **266** (484), **267** (156, 211, 349, 483, 484), **268** (166, 484), **270** (156), **273** (483), **274** (290, 358, 420, 483), **275** (483), **276** (156, 211), **285** (349), **286** (166), **290** (334, 434, 484), **295** (483), **302** (156), **305** (166), **306** (483), **307** (156, 211), **319** (156, 211, 349), **324** (290, 420, 483), **327** (484), **328** (156, 211, 367, 434, 484), **329** (156), **330** (156, 211, 484), **338** (483), **340** (290, 420, 483), **353** (156, 211, 334, 434, 484), **357** (483), **366** (166, 290, 334, 420, 434, 483, 484), **393** (358), **400** (155, 484), **401** (156, 211), **403** (156, 211), **405** (358), **415** (349), **422** (334), **430** (483), **433** (483), **434** (290, 358, 420, 483), **436** (484), **437** (483, 484), **438** (484), **442** (290, 420, 483, 484), **443** (434), **444** (483), **446** (290, 358, 420, 483), **451** (483), **461** (483), **464** (434), **468** (483), **471** (335, 349, 472), **494** (335, 349, 472), **584** (367), **654** (434), **674** (484), **685** (367), **698** (434), **710** (434), **760** (367), **769** (472), **796** (472), **825** (367), **847** (472), **850** (472), **852** (472), **853** (367), **915** (367), **964** (349), **966** (472), **978** (472), **1024** (335, 349, 472), **1037** (434), **1046** (472), **1049** (349), **Chimeras** (305).

Reactions in this reaction class primarily involve Kemp eliminations or oxidative cyclization of lidocaine-type substrates. A complete list of substrates can be found in Supplementary Table 10. Wild-type P450 BM3 performs a Kemp elimination on 5-nitrobenzisoxazole, although with poor kinetic parameters.[155] Introduction of the A82F mutation results in over a 100-fold increase in the second-order rate constant (k_{cat}/K_m), demonstrating that a single mutation can significantly enhance P450 BM3's inherent promiscuity. With regard to the oxidative cyclization of lidocaine derivatives, wild-type P450 BM3 carries out this reaction with low efficiency. In the initial discovery of this reaction, 45 variants of P450 BM3 with up to 10 mutations were screened for reaction conversion with lidocaine; 24 variants showed a lidocaine conversion rate of over 50%, an improvement over the wild-type P450 BM3.[211] In subsequent screening of 12 different lidocaine derivatives for oxidative cyclization, wild-type P450 BM3 conversion was less than 5%, with no activity detected for many of the compounds.[156] However, some variants with up to 10 mutations showed near-quantitative conversions, demonstrating that when wild-type activity is low, accumulation of mutations can lead to a significant increase in activity. A trend comparable to that observed for the carbene and nitrene transfer reaction classes is seen, in which the innate activity was minimal or absent, warranting numerous mutations to improve the variants' effectiveness and specificity.

IMPORTANT OR FREQUENTLY MADE MUTATIONS TO P450 BM3

As seen in the main database, Supplementary Table 1, and illustrated in Figures 2−10, there is broad variation in the positions selected for mutation across all reaction classes that resulted in improved P450 BM3 variants. On the basis of the database, it is evident that most mutations have been made

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within or adjacent to the substrate access channel or active site, yet it is also clear that mutations to P450 BM3 can enhance function even when distant from the heme. Indeed, error prone PCR methods commonly reveal positions far from the active site. We note that regions near the heme-reductase domain linker or those that are solvent exposed are seldom reported in variants having improved activity. Furthermore, the absence of beneficial mutations reported at positions near the interface between heme-reductase dimers[425] may reflect their critical role in function but such speculation requires further investigation.

The 25 positions with the highest frequency of substitution are 87, 47, 353, 78, 188, 184, 328, 236, 252, 175, 290, 82, 51, 226, 142, 267, 263, 366, 268, 74, 442, 330, 255, 400, and 401, given here in descending order based on their frequency of occurrence in the database. Notably, the most common specific substitutions are concentrated in these positions. The 25 most frequently occurring substitutions, ranked in descending order of frequency as found in the database are R47L, F87V, A184V, F87A, H236Q, E252G, T175I, A290V, L353V, S226R, L188Q, P142S, Y51F, V78A, E267V, I366V, E442 K, R255S, C400S, H171L, A74G, F205C, A328V, N239H, and Q307H.

It is interesting to note that some mutations are often reported as single variants, as seen with F87V and F87A, or exclusively found in higher-order variants, such as S226R. It is crucial to evaluate the frequency of both the position and the identity of the substitution when making conclusions about their significance. Furthermore, it is important to consider the mutational coverage of a position (how many of the 19 other amino acids have been reported as beneficial), in addition to the frequency at which a specific substitution occurs. For instance, while high mutational coverage suggests adaptability, observing preferred mutations among the most active variants can highlight selection toward a desired activity. With increased data, one can delve deeper into the relationship between mutations and functional outcomes. Analyzing the relative frequency and prominence of certain mutations within every position allows for a more nuanced understanding of which changes might be more favorable in specific reactions or environments. This data-driven approach helps in identifying patterns or trends that indicate the most effective substitutions for optimizing a particular process or outcome.

Overall, the 27 positions with mutational coverage of 7 or higher are positions 47, 49, 51, 72, 74, 75, 78, 81, 82, 87, 88, 181, 184, 185, 188, 260, 263, 267, 268, 327, 328, 329, 330, 354, 436, 437, and 438. Among these, the most frequently found positions within the database that have a mutational coverage of 7 or higher are 47, 51, 74, 78, 81, 82, 87, 184, 188, 263, 267, 268, 328, and 330. Frequently reported positions having high mutational coverage are highly significant since they provide abundant data and demonstrate that a variety of substitutions are advantageous over the native amino acid. Indeed, beneficial single variants at these positions are frequently found within the database. These positions are also frequently targeted in the creation of SSM libraries, with the most successful variants then being combined to generate highly active and/or selective enzymes. The creation of this database helps facilitate these types of analyses to determine potentially important positions or substitution patterns and provide a more complete understanding of P450 BM3.

AMINO ACID VARIABILITY IN NATURAL EVOLUTION VS PROTEIN ENGINEERING

Having determined the positions of P450 BM3 where experimental methods have led to beneficial high mutational coverage or frequent mutation, we investigated how amino acid variability in protein engineering compares with natural evolution. To this end, we identified conserved residues or regions between P450 BM3 and related homologues with ConSurf.[485,486] This software creates an amino acid multiple sequence alignment (MSA) and sequence similarity tree utilizing the input structure P450 BM3 (PDB: 1FAG) in our case. The program generates a heatmap incorporated onto the crystal structure input to gauge the degree of conservation based on the amino acid sequences of P450 BM3 homologues. We identified 150 P450 BM3 homologues that had a sequence identity between 95% and 35% and applied the default ConSurf parameters.

It was observed that many of the conserved regions in both P450 BM3 and its naturally evolved homologues are situated within the substrate access channel and active site (Supplementary Figure 10). The significant conservation in these areas is due to the fact that most P450s innately carry out oxidation reactions through C−H activation, such that the region near the heme is generally hydrophobic. T268 and C400, essential residues for the native catalytic function, are also highly conserved in nature. Nonetheless, there is considerable variability in the amino acid identity of surface-exposed residues including those surrounding the entry point to the substrate access channel, presumably reflecting variability in native substrates of the P450 homologues.

To compare trends in mutational patterns between natural evolution and protein engineering, we focused on the 27 positions that had a mutational coverage of 7 or more, described above and highlighted below according to their degree of natural conservation (Figure 11). Mutations to these positions result in variants that are more active than the parent enzyme. Analyzing the mutational patterns between natural evolution and protein engineering immediately reveals an important insight. Specifically, researchers should not necessarily avoid positions with highly conserved amino acids when performing mutagenesis, since our database reveals that locations with a mutational coverage of 7 or higher are often conserved in nature (red spheres). In naturally evolved P450 BM3 homologues, highly conserved amino acids are likely to be catalytically crucial or contribute to substrate binding stabilization. However, if the substrate or reaction of interest differs from the native reaction, it may be necessary to mutate these conserved regions to create a more suitable substrate binding environment. As a result, amino acids that are highly conserved in nature are often mutated in protein engineering to catalyze non-native reactions, proving to be highly beneficial mutations.

Solvent-exposed amino acids typically exhibit high variability (Supplementary Figure 10) but are rarely found in the database with significant mutational coverage. Positions near the entry to the substrate access channel show high amino acid variability in nature and in the database, presumably due to the use of varied substrates by P450 BM3 homologues in nature and in the laboratory. An average level of amino acid variability in nature is seen in the B′-helix and F-helix, located adjacent to the substrate access channel (yellow space-fill), where several residues with high mutational coverage are found. This analysis

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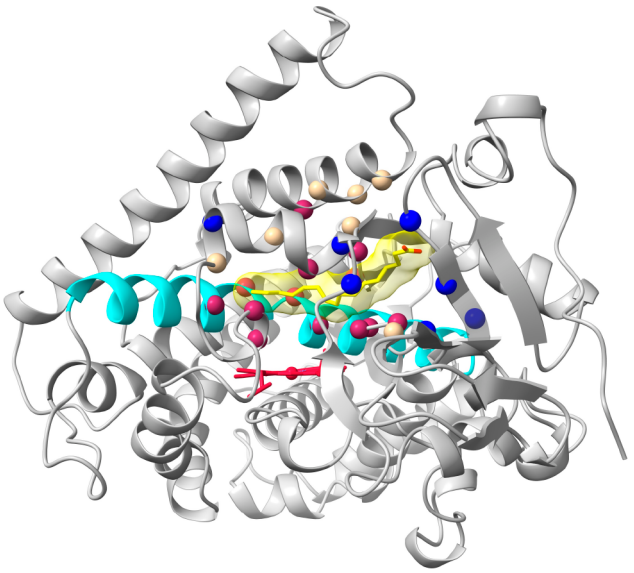

Figure 11. Crystal structure of the P450 BM3 heme domain (PDB: 1FAG) displaying the 27 amino acid positions, as colored spheres, with mutational coverage of 7 or greater. The spheres are colored according to the natural amino acid conservation as determined using ConSurf (Supplementary Figure 10). High (red), medium (beige), and low (dark blue) natural sequence conservation among 150 P450 BM3 variants. The I-helix is in cyan, the heme is in red sticks and the substrate lauric acid is in yellow sticks surrounded by its van der Waals radius.

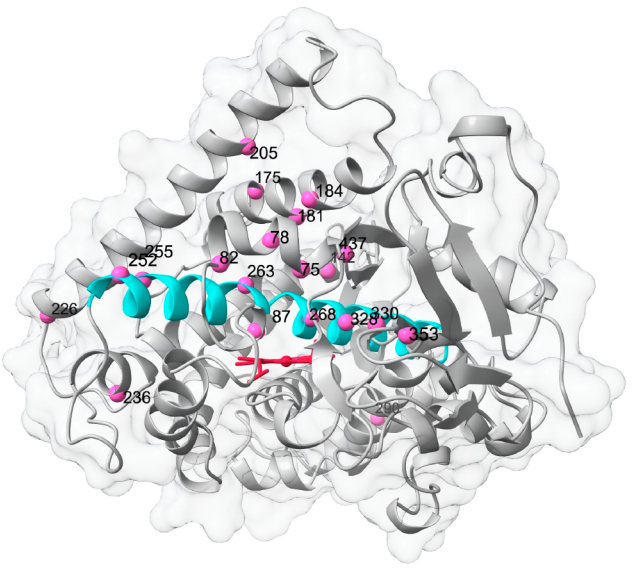

Figure 12. Crystal structure of the P450 BM3 heme domain (PDB: 1FAG) representing positions that are substituted in all reaction classes and belong to improved variants. These positions are represented with dark pink spheres and are labeled in black.

highlights the value of the database in comparing natural evolution and protein engineering, as well as identifying mutational "hot spots".

P450 BM3 MUTATIONS REPORTED IN ALL REACTION CLASSES

Some positions in P450 BM3 have been reported as being substituted in each of the reaction classes. They thus appear to be significant, or at the very least, popular among researchers, for enabling the various chemical reactions investigated. The 20 positions where substitution is included in beneficial variants found across all the reaction classes in the database are positions 75, 78, 82, 87, 142, 175, 181, 184, 205, 226, 236, 252, 255, 263, 268, 290, 328, 330, 353, and 437 (Supplementary Table 1). These positions are highlighted in Figure 12 and are mainly located on the B′-helix, F-helix, or I-helix previously discussed in Figure 1. The remaining positions are localized either adjacent to the substrate access channel or active site or more distantly on the E-helix and G-helix.

It is important to also consider the mutational coverage at positions within the database, in addition to the amino acid position itself. When there is a variety of substitutions reported at a particular position across multiple reaction classes, it suggests that the position promotes reaction and/or substrate promiscuity. On the other hand, if there are only a few different substitutions reported at a position, it may indicate that other substitutions at that position have not yet been engineered or that they are not beneficial and would not be included in this database unless included with others. Indeed, interpreting combinations of substitutions that frequently appear may have significance, or they may have been carried forward through rounds of evolution without having a beneficial impact on function.

To assess the significance of specific substitutions, it is important to consider the frequency at which a position and/or substitution is found in the database and to refer to the cited literature. To facilitate reader navigation and evaluate significance, we generated Supplementary Table 16 that cites the positions and substitutions at those positions that have been reported by researchers. Supplementary Table 16 allows readers to identify the location of mutations, the number of substitutions at each position, and whether they are specific to certain reaction classes or present across all classes. A sample of Supplementary Table 16 is provided below to illustrate its information content (Table 2).

Table 2, which illustrates a simplified sample of Supplementary Table 16, provides details on positions with high mutational coverage, such as positions 78 and 87. These positions have shown favorable outcomes as a result of various substitutions. At position 78, the V78A substitution is cited more frequently than V78F and other substitutions. However, both V78A and V78F are found in the majority of the 9 reaction classes outlined in this review. F87A and F87V are also commonly cited in the database and are present in all reaction classes (Supplementary Tables 1 and 16). F87G is absent in variants that catalyze carbene or nitrene transfer reactions but present in variants catalyzing hydroxylation or epoxidation reactions (Table 2 and Supplementary Table 16). Indeed, wild-type P450 BM3 is responsible for the epoxidation of 3-chlorostyrene to produce (*S*)-styrene oxide; however, F87G is sufficient to provide the inversion of enantioselectivity, favoring the formation of (*R*)-epoxide.[174]

Just because a mutation is less frequently found in the database does not necessarily indicate lack of impact. Certain positions, such as 64, 175, or 226, have limited mutational coverage in the database with only 1 or 2 different substitutions reported (Table 2). Mutations at position 64 predominantly occur in variants that catalyze aromatic and drug/drug-like hydroxylation reactions. The E64G mutation, specifically, is solely reported in variants containing over 5 mutations. The lack of mutational coverage at position 64 and

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Table 2. Occurrence of Substitutions Segregated by Reaction Class[a]

		Reaction Class Code								
Position	Mutation	1a	1b	1c	1d	2a	2b	3	4	5
64	E64G	✓(9)	✓(25)	✓(2)						✓(1)
	E64V		✓(1)							
78	V78A	✓(6)	✓(8)	✓(15)	✓(20)	✓(5)	✓(6)	✓(12)	✓(11)	✓(2)
	V78C		✓(2)	✓(3)	✓(1)				✓(1)	
	V78D				✓(1)					
	V78E		✓(2)							
	V78F	✓(2)	✓(3)	✓(11)	✓(5)	✓(1)		✓(1)	✓(1)	✓(2)
	V78G	✓(1)			✓(4)		✓(1)			
	V78H	✓(1)			✓(1)					
	V78I	✓(2)	✓(5)	✓(4)	✓(2)					✓(1)
	V78K	✓(1)								
	V78L		✓(6)	✓(4)	✓(2)			✓(5)	✓(1)	✓(1)
	V78M		✓(1)	✓(2)	✓(2)			✓(4)	✓(2)	
	V78N			✓(3)	✓(1)					
	V78S	✓(1)		✓(2)	✓(1)				✓(1)	
	V78T		✓(2)	✓(2)	✓(1)					
	V78V		✓(1)							
	V78W	✓(1)		✓(1)	✓(1)					
	V78Y			✓(1)				✓(1)	✓(1)	
87	F87A	✓(36)	✓(22)	✓(17)	✓(34)	✓(7)	✓(8)	✓(8)	✓(12)	✓(9)
	F87D			✓(1)						
	F87G	✓(8)	✓(2)	✓(8)	✓(6)	✓(4)	✓(3)			✓(1)
	F87I	✓(2)	✓(4)	✓(10)	✓(4)	✓(1)	✓(3)			✓(2)
	F87K				✓(1)					
	F87L	✓(7)	✓(1)	✓(4)	✓(1)	✓(1)	✓(2)			
	F87N	✓(1)								
	F87P	✓(1)		✓(4)			✓(1)	✓(3)		
	F87R	✓(2)								
	F87S	✓(2)								
	F87T	✓(2)		✓(3)	✓(1)		✓(2)		✓(1)	
	F87V	✓(47)	✓(47)	✓(32)	✓(24)	✓(5)	✓(12)	✓(11)	✓(8)	✓(6)
	F87W			✓(1)				✓(1)		
175	T175I	✓(4)	✓(7)	✓(19)	✓(16)	✓(5)	✓(4)	✓(15)	✓(15)	✓(3)
226	S226R	✓(3)	✓(5)	✓(15)	✓(14)	✓(2)	✓(2)	✓(15)	✓(15)	✓(3)
	R226T							✓(1)		

[a]This table highlights the positions, number of mutations at each position, and number of references in parentheses, which are grouped according to the reaction class where they were reported. A check mark means that the substitution was reported in that reaction class and the number in parentheses indicates the number of references. Blank cells indicate that the reaction class does not contain variants with that substitution. This is a simplified sample of Supplementary Table 16 which contains all relevant references.

the clustering of references within one reaction class suggest that an SSM library at position 64 may not yield the most fruitful outcomes when screening against multiple substrates. Combining mutations at position 64 with other frequently mutated positions results in systematically advantageous variant properties, as demonstrated within this database. Whether this position and mutation combination is selective for drug/drug-like substrates is not yet clear.

Importantly, the database offers a centralized location for easily accessing this information, eliminating the need for lengthy literature searches. This can prove time-saving where the impact of specific substitutions is unclear. For instance, the only substitution reported at position 175 is T175I, and it occurs only in variants with more than 9 substitutions (Supplementary Tables 1 and 2). A similar pattern is seen for position 226, where substitution only to arginine or threonine are in the database, only in variants with more than 10 mutations. These substitutions at positions 175 or 226 are reported to be beneficial across all reaction classes, but their significance warrants consideration. These two substitutions arose from random mutagenesis and accumulated through multiple rounds of evolution.[426] Their high frequency is largely due to their inclusion in 9−10A and related variants, which are commonly found within this database.[135] Although the roles of T175I and S226R in their respective variants may be important, their significance in higher-order variants is difficult to assess due to potential synergistic effects from multiple mutations. This illustrates the significance of determining the coverage of mutations at the site of interest. Referring to cited literature is necessary to determine the significance of a specific substitution/variant.

IMPACT OF KEY SUBSTITUTIONS ON REACTION SCOPE

This database promises to be of great utility for researchers using or interested in using P450 BM3. It provides instances of individual variants capable of catalyzing previously unexplored reactions or enhancing existing functionality as illustrated earlier. To simplify access to variant and reaction information, we urge researchers producing P450 BM3 variants to consult this database (Supplementary Table 1). Understanding reaction outcome possibilities at a particular position also informs researchers on what they are likely to observe when screening SSM libraries with a given substrate (Supplementary Table 16).

Among the 20 positions present in variants across all reaction classes (Figure 12), certain positions are exclusively substituted in high-order variants. This makes it difficult to interpret the beneficial impact of any given substitution on the different reactions. To determine the impact of specific substitutions on the reactions, we zoomed in on those variants containing only one substitution at any of those 20 positions, with a particular focus on positions where several single

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substitutions are included in the database. Positions 75, 78, 82, 87, 188, 263, 268, and 328 satisfy these criteria.

Positions 75 and 78 (B′-Helix). L75X single variants showed improvement in aromatic hydroxylation, small alkyl hydroxylation, and alkyl epoxidation reactions, as shown in Supplementary Table 17. Moderate mutational coverage is observed, with 11 out of 19 amino acids found in single variants. Additionally, higher-order variants include 15 out of 19 possible substitutions at position 75. Similarly, single variants at position 78 enhance hydroxylation reactions, as demonstrated in Supplementary Table 18. Numerous variants contain a substitution at position 78, consistent with their incorporation into multiple lineages (Supplementary Table 16). Variants with fewer than five substitutions including position 78 typically enhance the activity already present, but they are uncommon in epoxidation, carbene, or nitrene transfer reactions. It is likely that substitutions at position 78 alone do not enable reaction promiscuity toward these other reaction classes but improve existing native function.

Position 82 (B′-Helix). Single variants at position 82 improve hydroxylation and epoxidation (Supplementary Table 19). A82F leads to a marked enhancement in Kemp elimination activity.[155] Ten out of 19 possible substitutions are reported at position 82 for individual variants, and full mutation coverage of all 19 substitutions are reported when higher-order variants are taken into account (Supplementary Tables 16 and 19).

Position 87 (Active Site Residue). Position 87 (active site residue), located within the active site, is often substituted to enhance performance across all reaction classes (Supplementary Table 20). This is likely due to increased active site volume resulting from most F87 mutations, accommodating larger substrates; in particular, substitution of F87 to the smaller alanine, glycine, isoleucine, leucine, or valine is commonly reported.[174,336] The mutational coverage of single variants at position 87 is 13/19, while 15 of the 19 amino acid possibilities are present in higher-order variants. Mutational libraries are frequently created at position 87 and typically produce favorable results.[22] However, there are examples where F87 mutations do not result in a significant gain in function.[232] The oxidation of 1-cyclohexene carboxylic acid methyl ester is inefficiently executed by the wild-type P450 BM3, resulting in a conversion rate of a mere 4%. With the F87A substitution, this conversion rate improves to only 10%.[232] Achieving reaction conversions of more than 50% requires creating a triple variant, which entails introducing substitutions V78L and A82F in addition to F87A.[232] Mutations at positions 78, 82, and 87 are commonly observed in the database, indicating that SSM libraries at these and other frequently mutated positions are suitable for screening a range of substrates. A similar example emphasizing the significance of position 87 is that the wild-type P450 BM3 cannot metabolize testosterone,[398] whereas the F87A variant provides conversion that exceeds 20%.[405] Additionally, while the wild type undergoes hydroxylation of *m*-methyl phenol with only ∼5% conversion, the introduction of F87V boosts conversion to 79%.[5]

There is a considerable degree of variation in the effect of a substitution. Thus, screening SSM libraries could enhance the probability of discovering a useful mutation, as opposed to screening individual variants. Over 700 variants in the database carry either the F87A or F87V mutation, providing additional evidence for the impact of position 87 in broadening the reaction scope. Paired with another substitution, they give rise to variants that show substantial enhancements compared to the parent enzyme;[5,405] double variants containing either the F87A or F87V mutation are detailed in Supplementary Tables 21 and 22. For example, testosterone hydroxylation is enhanced 4-fold by F87A/A330W[405] or A82M/F87A[396] in comparison to F87A alone, emphasizing the potential activity benefits of combining F87A with other mutations. These and other variants resulted from combining different SSM libraries with F87A or F87V.[396,405] These variants do not perform chemistry different from that of F87A or F87V but enhance pre-existing function, which is typical for most substitutions. Later, we will discuss examples of substitutions that promote unique chemistry.

Position 188 (F-Helix). Single variants at position 188 are mainly found within the alkyl hydroxylation and alkyl epoxidation reaction classes (Supplementary Table 23). All 19 substitutions at position 188 are reported and indicate good tolerance of substitution at this position, depending on the substrates used. The hydroxylation patterns of C10−C16 fatty acids by L188X single variants are similar, but their rates vary depending on the mutation at position 188 and the length of the fatty acid being tested.[312] Additionally, allylic hydroxylation of ethyl 6-heptenoate following substitution at position 188 was enhanced while maintaining a >90% *ee*.[28] L188X mutations occur in numerous higher-order variants, including GVQ (A74G/F87V/L188Q), which is widely utilized to enhance hydroxylation reactions and will be discussed below in more detail (Supplementary Tables 1 and 16).

Position 263 (I-Helix). Single variants at position 263 deliver improvements toward hydroxylation of aromatic and small alkyl substrates, as well as epoxidation of alkyl substrates (Supplementary Table 24). Across all variants, regardless of mutation count, 17 out of 19 possible substitutions offer benefits. Among these, I263A and I263G are the most frequent in the database. I263A is reported across all reaction classes and influences the selectivity of reactions such as cyclopropanation reactions[30] or nitrene transfer reactions which form sulfimide products.[56] This substitution affects the stereochemistry of the reaction but alone is not sufficient to enable these new-to-nature reactions.

Position 268 (I-Helix). Single variants T268A, T268E, and T268K have a significantly different impact on reaction outcomes than other previously discussed positions, with alanine being the most common amino acid substitution at this site (Supplementary Table 25). Although wild-type P450 BM3 demonstrates poor performance in carbene transfer reactions, the T268A mutation leads to a >60-fold increase in the total turnover number for cyclopropane formation when using styrene and ethyl diazoacetate.[30] Although this improvement in activity comes with a cost, as the less desirable trans isomer is formed, it can be mitigated by introducing further mutations, such as F87V.[30] The T268A mutation also improves nitrene transfer reactions, albeit to a lesser extent.[52,53,56] The native threonine at this position is important due to its role in heterolytic O−O cleavage necessary for native hydroxylation reactions.[60,125,138] Mutations at this position prove highly beneficial in variants performing carbene and nitrene transfer reactions which do not use molecular oxygen (Supplementary Tables 1 and 16).

Position 328 (Active Site Residue). Single variants at position 328 lead to notable enhancement in hydroxylation reactions (Supplementary Table 26). More than half of the

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possible substitutions at position 328 are found as singular variants. Additionally, the database includes 16 out of 19 amino acid possibilities when considering higher-order variants, with phenylalanine, isoleucine, leucine, and valine being the most frequently observed. A328F alone increased *p*-hydroxylation of *m*-alkylphenols from 5 to 60% conversion while maintaining excellent selectivity.[5] The F87V mutation shows a comparable level of improvement, indicating that various mutations can achieve similar outcomes, and researchers should not concentrate mutagenesis efforts on a single position.[5] Single variants, A328N and A328L, favor epoxidation of alpha-isophorone over hydroxylation, the latter being preferred by the wild-type enzyme.[22]

Position 400 (Axially Coordinated to Heme-Iron). The only amino acid conserved in all functional P450s is the cysteine that serves as axial ligand to the heme. Its substitution leads to the loss of the native activity.[135] In P450 BM3, substitutions at Cys400, similar to those at position 268, significantly enhance carbene and nitrene transferase activity as well as Kemp elimination and oxidative cyclization activity (Supplementary Table 1).[31,32,34,35,38,42−50,52,55−59,61−65,155,484] The Cys400Ser substitution is the most commonly reported, although substitution to histidine is also observed. Multiple studies have shown successful promotion of new-to-nature reactions as a result of these mutations.[135,141] The key factor contributing to this improvement is their impact on the heme cofactor's reduction potential. This alteration enables NAD(P)H-driven activity.[31]

Additionally, there are numerous other noteworthy positions, such as 47, 51, 72, 74, 86, 267, 330, 353, 401, and 437, that were not addressed. The impact of their modification is not widespread across reaction classes, and they offer lower mutational coverage compared to more popular alternatives. Nonetheless, it is important not to overlook the significance of these, and other, positions.

COMMONLY USED P450 BM3 VARIANTS WITH WELL-DEFINED PROPERTIES

The significance and ultimate utility of this database lies in its ability to facilitate identifying the properties of existing variants, or those variants researchers may want to create, and enable identifying trends in substitutions that lead to specific reaction outcomes. Researchers interested in applying existing variants with well-defined functionalities for specific reactions should first locate the relevant reaction class for their desired substrate (Supplementary Table 1). Selection of the "best" variant will depend on the desired regio- or enantioselectivity, and consulting the database and cited literature will offer insights into which variant may be preferable.

Below, we present some commonly employed P450 BM3 variants utilized in a specific reaction or set of reactions. These variants may not necessarily be optimal for a given reaction, but their widespread usage affords greater availability of data on the metrics utilized to measure reaction efficacy with varied substrates. Broader adoption of a variant typically results in the evaluation of more substrates and the gathering of more information on substrate preferences, which is often lacking for less common variants.

GVQ. The A74G/F87V/L188Q variant, also known as GVQ, was produced through random mutagenesis of positions 74, 87, and 188 in order to enhance indole hydroxylation.[365] This variant has been found to exhibit a significant rate improvement over the wild-type enzyme when hydroxylating a diverse range of substrates[157] and is primarily utilized for drug/drug-like and aromatic hydroxylation. GVQ-derived variants demonstrate similar substrate preferences to wild-type BM3 but generally exhibit enhanced rates or conversions (Supplementary Figure 27). Notable variants utilized for catalyzing aromatic hydroxylation reactions are RT2, KT2, and R19, all of which harbor 5 to 7 mutations. Supplementary Tables 28−30 provide details on the substitutions of these variants, as well as the reactions they facilitate.

These and related variants are commonly combined and tested against a substrate panel, which has proven to be a successful approach.[2,25,156,184,187,211,343,403] For example, the screening of RT2, KT2, and R19 variants for lidocaine metabolism produced diverse substrate conversions. The literature reports that wild-type P450 BM3 does not employ lidocaine as a substrate, while GVQ leads to just 14% conversion.[211] However, the variants F87A and KT2 enhance the conversion of this cyclic product to 40% and 43%, respectively. Impressively, RT2 conversion of lidocaine is 87%, and R19 produces the desired cyclic product with nearly quantitative conversion (95%). The major isomer formed in all cases is the oxidative cyclization product.[211] The use of focused libraries necessitates screening additional variants, but it substantially enhances the probability of obtaining a variant that transforms a substrate of interest with high conversion. Such focused libraries can be exceedingly beneficial to researchers who lack the resources or the expertise to generate many variants themselves and would rather screen an existing library with a reasonably well-defined set of properties. These variants can oxidize various drugs, as shown in Supplementary Tables 28−30.

M01 and M16. Variants M01 and M16 are the most utilized for drug and drug-like substrate oxidation (Supplementary Tables 31 and 32). M01 consists of the substitutions R47L/F87 V/L188Q/E267V and is derived from the variant R47L/F87 V/L188Q, which also exhibits high activity toward the oxidation of drug/drug-like substrates. M16 carries additional mutations of F81I and E143G compared to M01. A version of M16, named M16 V2, features 20 mutations in the reductase domain, but it retains the substrate preference for drug or drug-like substrates (Supplementary Table 1).

Screening a small number of variants with well-defined properties frequently results in the detection of variants with altered product oxygenation profiles.[391,396,402,412,413] Hydroxylation of testosterone by the R47L/F87V/L188Q variant generates three primary products, specifically 2β-, 15β-, and 16β-testosterone, whereas the wild-type is incapable of catalyzing this reaction.[398] M01 and M11 are both active toward testosterone and show comparable product distributions, albeit M11 exhibits nearly 10 times higher activity than M01.[410] To enhance or modify regio- or enantioselectivity, combinatorial mutations at one or more positions can be applied to top-performing variants. By introducing mutations at positions 72 and 82 into M01 or M11, these new variants generate a previously unidentified metabolite, known as 16α-testosterone.[410]

139-3. Another commonly used variant is 139-3, which was developed for hydroxylation of small alkyl substrates,[220] but it also accepts drug/drug-like substrates[423] and even fatty acids[245] (Supplementary Table 33). The variant includes 11 substitutions and displays greater activity on fatty acid substrates compared to wild-type P450 BM3.[220] Additionally,

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it can catalyze aromatic epoxidation reactions with styrene at a conversion exceeding 40%. However, the resulting product is formed in a near-racemic fashion.[326] This variant shows an ability to accommodate a broad range of substrates across many reaction classes.

9-10A. Derived from 139 to 3, 9-10A has been further adapted for small alkyl substrate hydroxylation, resulting in respective improvements of 3 and 2.2-fold over 139-3 for propane and octane hydroxylation (Supplementary Table 34).[426] The mutation-induced focus toward smaller substrates makes the variant 9-10A an ideal starting point for enzyme engineering purposes. This variant has been widely used as a foundation for advancing hydroxylation of small alkyl substrates,[437] as well as carbene[30] and nitrene transfer reactions.[52] Further mutation of 9-10A allows for the acceptance of large, drug-like substrates, which is not a capability of 9-10A (Supplementary Table 35).[217,334] The activities of carbene and nitrene transferases have been previously discussed by Yang and Arnold, concerning 9-10A derived variants.[135]

FL#62. Finally, variant FL#62, with 16 mutations compared to the wild-type P450 BM3, catalyzes the hydroxylation of terpenes, a nitrene transfer reaction with a variety of substrates, and the oxidation of alkyl azides to aldehydes.[51,53,54,60,217,442,453,462] The introduction of further substitutions in FL#62 can alter the activity and selectivity of these reactions, making it an excellent starting point for further enzyme engineering. Collectively, these variants possess well-defined properties and substrate preferences.

SUMMARY AND CONCLUSIONS

By conducting a broad search of the scientific literature for P450 BM3 variants, we have developed a powerful tool that categorizes variants by their catalyzed reactions and includes details about substrates to provide context about the reactions. This database of P450 BM3 variants is downloadable and includes instructions to maximize ease of gathering information. Only variants that exhibited a performance enhancement over the listed parent enzyme were considered. The resulting database, created using these criteria, comprises over 1500 variants with a significant portion being single, double, and triple variants. This indicates that in numerous instances, few substitutions suffice to produce a functionally valuable catalyst. Extra substitutions become necessary only when departing significantly from the original substrates and its corresponding reaction.

It is the innate versatility of wild-type P450 BM3 that has enabled scientists to engineer this enzyme to induce a broad range of reactions using a diverse array of substrates. There exists a significant gap between natural evolution and protein engineering: although most residues in or adjacent to the active site are highly conserved in nature, the database allows us to observe that they are among the most highly mutated by researchers. It is possible that the degree of natural conservation provides hints concerning the positional importance for the native reaction; when attempting to generate novel reactions, these highly conserved positions have the potential to be the most advantageous to mutate. The most impressive case reported to date involves C400 and T268, which are both essential for the native reaction and exhibit high conservation. However, mutations in these residues broaden the reaction possibilities to allow new-to-nature reactions, suggesting remaining untapped potential in the catalytic functionalization of this enzyme. To analyze the disparity between natural evolution and mutations made during protein engineering, a comprehensive database such as this is imperative.

The creation of this database allows rapid identification of common substitutions present in the literature, aiding researchers who are unfamiliar with the enzyme in identifying starting points for enzyme engineering. It should be noted that in some instances, a mutation's frequency in the database can be attributed to its inclusion in popular variants rather than any striking property. Nevertheless, researchers frequently target positions where substitutions have been shown to catalyze reactions in multiple classes and employ a range of substrates. Top variants identified during screening can be combined to generate improved properties over corresponding single variants; use of this database will facilitate identification of candidate positions and variants to combine and further investigate.

In summary, the database along with the review provides a user-friendly platform for researchers to understand, predict, and identify the attributes of various P450 BM3 variants. Additionally, it encourages the advancement of research on this enzyme toward new reactions.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.4c00086.

Database, including upload of raw data; instructions on how to use the database; substrate information; variant identities; formatted/sorted version of database; and evolutionary conservation of amino acids among P450 BM3 homologues (PDF)

Instructional video on how to use excel upload of database (MP4)

Excel upload of database (XLSX)

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D.J.F. performed data collection, data curation, database generation, creation all figures and tables, and wrote the original manuscript draft. D.J.F., J.N.B., and A.F. contributed to the design and organization of the database. All authors contributed to the review of the manuscript and have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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