Nitrene transfers mediated by natural and artificial iron enzymes

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Abstract

Amines are ubiquitous in biology and pharmacy. As a consequence, introducing N functionalities in organic molecules is attracting strong continuous interest. The past decade has witnessed the emergence of very efficient and selective catalytic systems achieving this goal thanks to engineered hemoproteins. In this review, we examine how these enzymes have been engineered focusing rather on the rationale behind it than the methodology employed. These studies are put in perspective with respect to *in vitro* and *in vivo* nitrene transfer processes performed by cytochromes P450. An emphasis is put on mechanistic aspects which are confronted to current molecular knowledge of these reactions. Forthcoming developments are delineated.

Keywords

nitrene transfer
hemoprotein
iron enzyme
nitrene precursor
mechanism
catalysis

Highlights

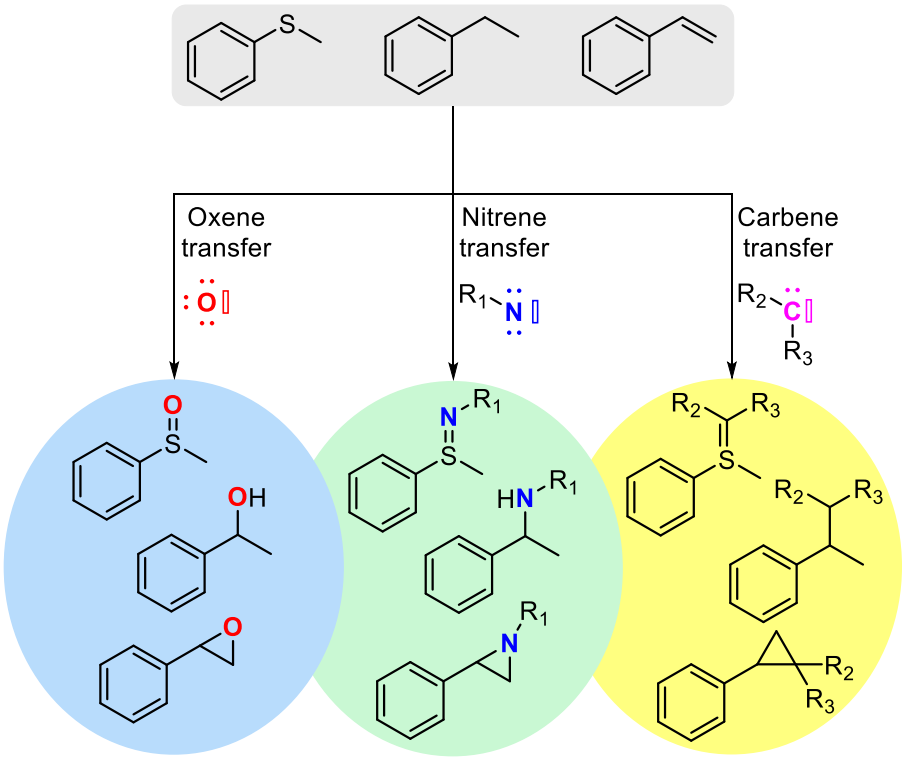
Historical coverage of biological nitrene transfer by hemoproteins

Mechanistic understanding of generation of the nitrenoid species from nitrene precursors

Comprehension of the electronic aspects of nitrene transfer reactions

1. Introduction

Contemporary chemistry faces the challenge to become ever more sustainable.[1] Biocatalysis is no doubt likely to play a major role in this endeavor especially for the synthesis of pharmaceuticals.[2] A particular issue faced by this industry is the synthesis of amines which are essential components of many biological molecules and drugs.[3,4] Organic chemistry has developed an immense repertoire of amine syntheses based on C – N metal-catalyzed coupling methods, where Buchwald-Hartwig palladium coupling occupies a prominent place.[5–7] However, an alternative and more direct route based on C – H functionalization was proposed.[8–13] This route takes advantage of the isolobal analogy of oxene (O:), the oxygen atom transfered by many oxygenating reagents including oxygenases, with carbene (R_2C:) and nitrene (RN:). Indeed, it is well established that oxenes, nitrenes and carbenes can be similarly transfered to variously functionalized hydrocarbons and heteroatoms as illustrated in Scheme 1.



Scheme 1. Examples of oxene, nitrene and carbene transfers on representative phenyl derivatives.

Moreover, there is a strong mechanistic similarity of their reactions, which makes cytochrome P450 the universal mechanistic paradigm for these transfer reactions. This is especially obvious in the case of bio-relevant nitrene transfers. As a matter of fact, in the early 1980s,

the pioneering work of the laboratories of Breslow[14,15] and Mansuy[16,17] using metal porphyrins paved the way to the first "*in vitro*" enzymatic amination catalyzed by cytochrome $P450_{BM3}$ reported by Breslow, Dawson and coll. in 1985.[18] However, whereas metalloporphyrin-based nitrene transfer catalysts florished,[19–23] the enzymatic field staid dormant for almost thirty years until F. H. Arnold and R. Fasan engineered cytochrome P450s and other hemoproteins achieving impressive activities and selectivities for a wide range of nitrene transfer reactions.[24] Last but not least, the first report of a true nitrene transferase was published by Ohnishi, Katsuyama and coll. in 2018.[25]

A few reviews covering nitrene transfers by engineered enzymes were reported in the last years and focused on the organic aspects.[22,26,27] In this review, we will briefly highlight the key mechanistic features of cytochrome P450 of interest in the present context of nitrene transfer. Then, we will underline how cytochrome P450 has been engineered to perform efficient and selective nitrene transfers, focusing more on the rationale behind it than on the methodology to achieve it, which has been amply and excellently described.[28,29] In a next section, we will describe the various nitrene transfer reactions developed so far with heme-based engineered enzymes and the emerging studies of non-heme engineered enzymes. After commenting on the peculiarity of the generation of the species active in nitrene transfer, we will highlight some future directions of the field.

2. The cytochrome P450 mechanistic paradigm

Owing to their central role in chemical toxicology,[30,31] the mechanism of action of cytochromes P450 has been thoroughly investigated both experimentally[32,33] and theoretically.[32,34] It has been definitely established thanks to cryocrystallographic[35,36] and spectroscopic[37,38] identification of key intermediates (Figure 1).

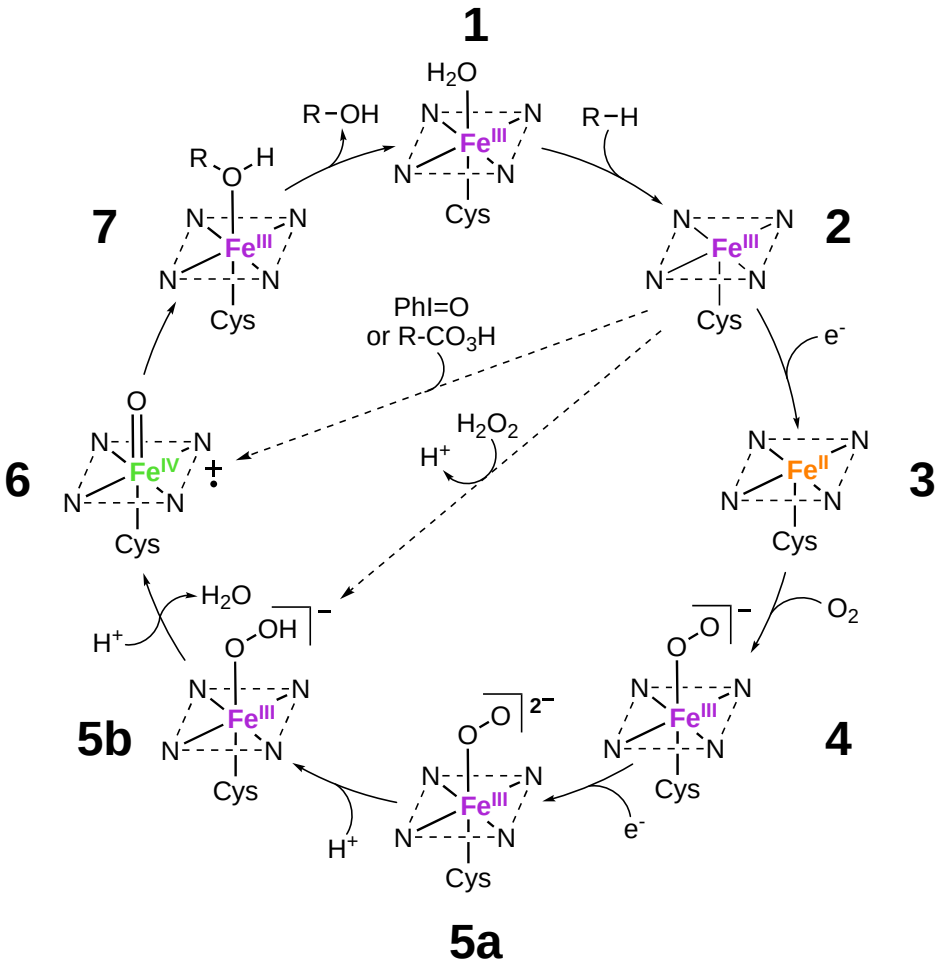
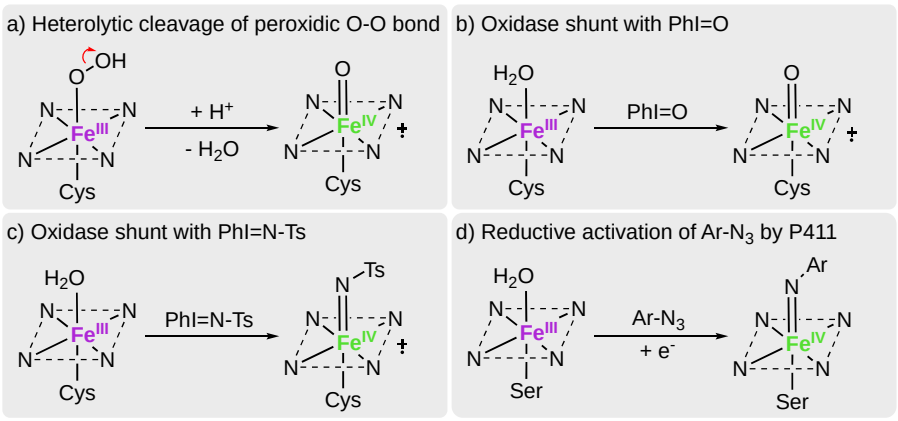


Fig. 1. Catalytic cycle of cytochrome P450. Adapted from [36] with permission.

Starting from the resting state **1**, substrate binding initiates the reductive activation of bound dioxygen to the hydroperoxide intermediate **5b** which is the precursor of the active species **6,** the "so-called" Compound I, best described as an oxoiron(IV) complex of heme b radical cation. The electrons needed for generating the ferrous heme **3** and the peroxo intermediate **5a** are provided by a reductase (iron-sulfur or FMN/FAD-dependent). In flavocytochrome $P450_{BM3}$, the heme and reductase domains are associated together on a single peptide chain.[39] The required cleavage of the hydroperoxide O — O bond (Scheme 2a) is assisted by the strong push-effect of the axial cysteinate ligand[40] (C400 in the numbering of cytochrome $P450_{BM3}$) and the pull-effect of protonation of the distal oxygen by a threonine residue (T268).

As illustrated in the catalytic cycle of Fig. 1, this activation process can be short-circuited by providing the enzyme with activated forms of dioxygen. Reacting the oxidized state **2** with hydrogen peroxide produces **5b** (reaction known as "peroxide shunt") which then transforms into Compound I. The latter can be obtained directly from **2** through reaction with an oxygen

donor (Scheme 2b), i.e. a peracid[41] or iodosylbenzene PhI=O.[42] This reaction known as "oxidase shunt" was used also to generate the species active in nitrene transfer from an aryltosyliminoiodinane ArI=NTs (Scheme 2c).[18]

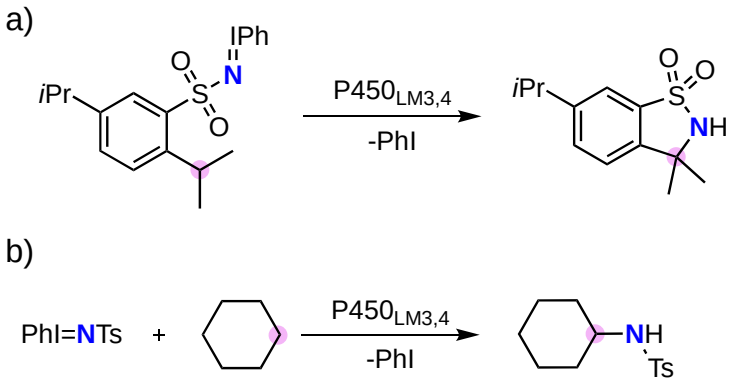


Scheme 2. Obtention of cytochrome P450 active forms: (a) heterolytic cleavage of peroxidic O-O bond, (b) oxidase shunt with iodosylbenzene, (c) oxidase shunt with tosyliminophenyliodinane and (d) reductive activation of an aromatic azide by cytochrome P411. ArN_3 is an arylsulfonylazide substrate or reagent (see text).

3. *In vitro* and *in vivo* nitrene transfers by cytochromes P450

3.1 In vitro nitrene transfers by cytochromes P450

In the wake of the first reports that iron porphyrins could catalyze nitrene transfer, the reaction was attempted with rabbit liver microsomal cytochrome $P450_{LM2}$ using PhI=NTs as nitrene precursor.[43] For all substrates (cyclohexane, methylcyclohexane and *p*-xylene) only the corresponding alcohols were obtained. It was proposed that a transient nitrene compound I analog $(Por^{\bullet+})Fe^{IV}$=NTs formed and was hydrolyzed to the corresponding compound I $(Por^{\bullet+})Fe^{IV}$=O which performed the analogous hydroxylation. Subsequently, Dawson, Breslow and coll. used purified cytochrome $P450_{LM3,4}$ to attempt the intramolecular amination of [(2,5-diisopropylphenyl)sulfonyl]iminophenyliodinane (Scheme 3a).[18] The benzosultam product 2,3-dihydro-3,3-dimethyl-6-isopropyl-1,2-benzisothiazole-1,1-dioxide was obtained with a turnover frequency of 2.2 nmol product/nmol enzyme/min. In addition, the formation of *p*-toluenesulfonamide was noted. Moreover, the intermolecular amination of cyclohexane could be performed by the same cytochrome P450 isozyme albeit less efficiently (Scheme 3b), a significant result with respect to biocatalysis. This reaction was shown to be isozyme dependent since it was not catalyzed by cytochrome $P450_{LM2}$.[18]

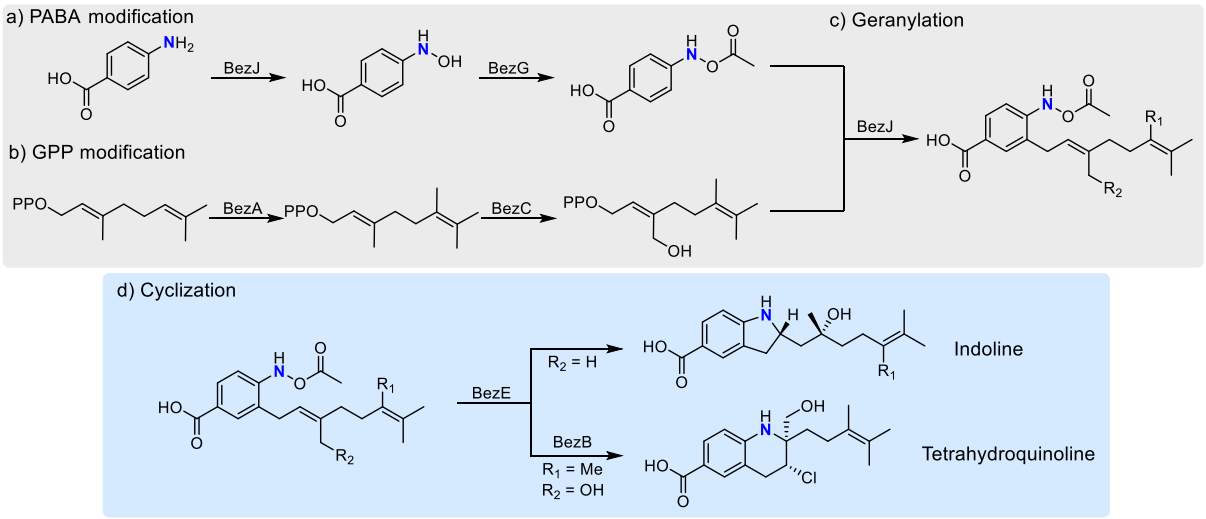


Scheme 3. *In vitro* enzymatic aminations by cytochrome $P450_{LM3,4}$.[18]

This system operated as a true cytochrome P450 through the so-called "oxidase shunt", where the active intermediate is a compound I analog ($(Por^{\bullet+})Fe^{IV}$=NTs) formed directly from the oxidized enzyme by reaction with an iminoiodinane (Scheme 2c).[18] This view was supported by a computational study which contributed to characterizing this species and highlighting its electronic differences with compound I.[44]

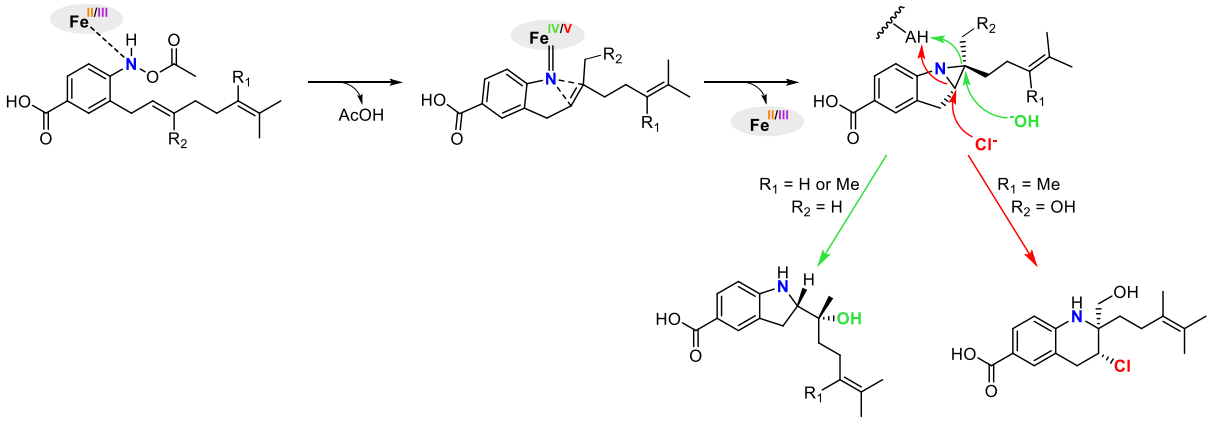
3.2 In vivo nitrene transfer by cytochromes P450

The first and only biological nitrene transfer was reported very recently by Ohnishi, Katsuyama and coll..[25] It is catalyzed by a cytochrome P450 which is part of the BezA-J complex comprising 10 enzymes which are involved in distinct complementary processes. In a first process (Scheme 4a), *p*-aminobenzoic acid (PABA) is oxygenated by the oxygenase BezJ and acetoxylated by BezG to give the acetoxyaniline which is the actual nitrene precursor. In parallel (Scheme 4b), geranyl pyrophosphate (GPP) was methylated (BezA) and hydroxylated (BezC). Then (Scheme 4c), the modified PABA and maturated geranyl are condensed at the aniline ortho position by BezF which is a prenyl transferase. The condensation product resulting from geranylation of modified PABA is transformed, through a nitrene transfer by the enzyme BezE which is a cytochrome P450, into an aziridine whose nucleophilic attack produces variously substituted benzastatins (Scheme 4d).[25]



Scheme 4. Proposed biosynthesis of some benzastatins illustrating the principal enzymatic steps: (a) acetoxylation of PABA, (b) modification of geranyl pyrophosphate, (c) geranylation of modified PABA and (d) nitrene transfer and cyclization. Adapted from [25] with permission.

To explain the cyclization reaction (Scheme 4d), it was proposed that, by elimination of AcOH, BezE generates an Fe-bound nitrene that realizes the intramolecular aziridination of the geranyl branch (Scheme 5). The ensuing aziridine is then opened by an incoming anion (OH^-, Cl^-) to form either an indoline (green pathway) or a tetrahydroquinoline (red pathway, with assistance of a protic residue). It is noteworthy that, in contrast to the above *in vitro* experiments, the nitrene precursor is enzymatically generated onto the substrate through the specific BezG BezJ machinery. The underlying mechanism of nitrene generation will be discussed in a later section.



Scheme 5. Proposed mechanisms for aziridination and cyclization leading (with assistance of a protic residue) to indoline (green pathway) and tetrahydroquinoline (red pathway). Adapted from [25] with permission.

4. Engineered hemoproteins

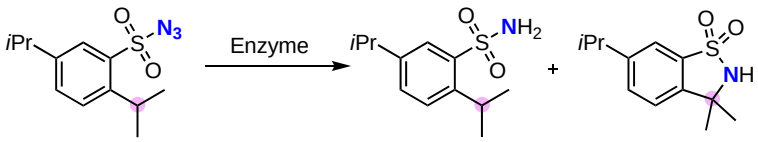
4. 1. Engineering approaches

Directed evolution emerged three decades ago as an extremely powerful tool to select and optimize biocatalysts for a wealth of organic transformations and it is continuously developed to include high-throughput assays enabling large libraries of biocatalysts to be screened.[29,45,46] From the onset, cytochromes P450 have been engineered to develop highly efficient and selective oxygenases through the generation of specific libraries[47,48] potentially including nonnatural amino acids.[49,50] The latter could later serve as an entrance point in the development of biocatalysts devoted to related activities such as carbene and nitrene transfers.[24] This approach is validated by the known catalytic promiscuity of most enzymes[51] and justified by the above mentioned isolobal analogy of the groups transfered.

Extensive site-saturated mutations have been achieved to optimize both the activity and the selectivity of the engineered enzymes for specific nitrene transfer reactions through the directed evolution methodology.[52,53] Moderate activities were detected for several wild-type hemoproteins (cytochrome P450 (P450), myoglobin (Mb), horse radish peroxidase, catalase and cytochrome c) but the engineering efforts concentrated mostly on cytochrome P450 and Mb.[24] Mutations designed to enhance selectivity will be outlined for each reaction, because they are specific to each substrate. Those devoted to improving overall efficiencies of cytochrome P450-based engineered enzymes are briefly analyzed below because they apply to all.

This engineering endeavor is rooted in the consideration that to carry out the reductive activation of dioxygen, cytochrome P450s have incorporated a few features that prove to be detrimental to other non-natural activities. These features comprise (i) the reduction system which delivers the two electrons required to generate the peroxo intermediate (Fig. 1), and (ii) the combination of the axial Fe cysteinate ligand (C400) and the proton relay including a threonine residue (T268) to enable the heterolytic cleavage of the hydroperoxo O–O bond (Fig. 1 and Scheme 2a). Both the proton relay and the electron flow systems were tacked by the groups of Arnold and Fasan. To calibrate enzymatic efficacies, they exploited the intramolecular synthesis of benzosultams introduced by Breslow (Scheme 3a)[14] with the 2,4,6-trisethylbenzenesulfonylimine derivative (Scheme 6). But, as nitrene precursor, instead

of the arylsulfonyliminophenyliodinane, they used the arylsulfonylazide derivative which is more suited to the conditions of enzymatic reactions (Scheme 6).[54] As shown in Scheme 6, in addition to the benzosultam, the reaction produces the arylsulfonamide degradation product.[24] The yield / Total Turnover Number and selectivity in the benzosultam at the expense of the arylsulfonamide were used to assess the nitrene transfer capacity of the variants.



Scheme 6. Intramolecular amination forming the arylsulfonamide and the benzosultam.

4. 1. 1. Iron axial ligation and screening of hemoproteins

The use of organic azides is of consequence in terms of mechanism because they are activated only by the heme-Fe^{II} state[55,56] and therefore a reductant is required to adapt the resting state of the protein.[54,57] The species active in nitrene transfer is thus an iminoiron(IV) heme complex (Scheme 2d) which is one oxidation state lower than cytochrome P450 Compound I[37] (Scheme 2b) and the active species in Dawson's *in vitro* experiments[18] (Scheme 2c). Hence the reactions are currently performed with a large excess of reducing agent (NADPH or sodium dithionite) which may be detrimental since it can react with the iminoiron(IV) active species (see below).

Cytochrome P450 is redox-activated for monooxygenase activity by the incoming substrate which causes the release of the axial water ligand and resultant rise in redox potential of the heme-Fe^{III}/Fe^{II} couple from -427 to -289 mV_{NHE}. This allows the reduction of the heme-Fe^{III} by NADPH (E_{NADPH} = -326 mV_{NHE}). The group of Arnold noted that mutation of the axial cysteinate residue of cytochrome $P450_{BM3}$ by a serine has the same effect.[58] As a matter of fact, apart shifting the absorption of the CO adduct to 411 nm, it increases the heme-Fe^{III}/Fe^{II} couple from -430 to -293 mV_{NHE} in P411 (C400S).[59] Concurrently, these mutations led to a 15-fold increase in the total turnover number (TTN) of benzosultam formation.[54]

A similar observation of the redox effect was made by the group of Fasan[57] who investigated the efficacy of various hemoproteins in the synthesis of benzosultam. They noted that horse radish peroxidase and myoglobin with an histidine axial ligand (E = -250 and + 50 mV_{NHE},

respectively) are more than one order of magnitude more active than catalase with a tyrosinate axial ligand ($E_{FeIII/FeII}$ = -500 mV_{NHE}) and wild type cytochrome $P450_{BM3}$. These differences in activities parallel the difficulty in oxidizing the heme-Fe^{II} state: the higher the heme-Fe^{III}/Fe^{II} couple, the higher the nitrene transferase activity.[57] The same influence of redox potentials was recently evidenced for simple Fe catalysts[60] and assigned to an increased electron affinity of the resulting imidoiron active species.[61]

4. 1. 2. Competition between arylsulfonamide and benzosultam formation

As illustrated in Scheme 6, the formation of the benzosultam is in competition with that of the arylsulfonamide derived from reduction and protonation of the starting azide or the active compound I analog ($(Por^{\bullet+})Fe^{IV}$=NTs). That arylsulfonamide formation depends on the efficacy of the nitrene transfer is illustrated by sulfimidation studies of *p*-substituted thioanisoles (*p*-X-Ph-S-Me, X = H, Me, OMe) by *p*-tosylazide catalyzed by a cytochrome $P450_{BM3}$ variant (see below Section 4.4).[62] Indeed, replacement of H by Me and by OMe caused a substantial increase in the production of the sulfimide product from 30 to 190 to 300 TTN, respectively, while the formation of *p*-tosylamide decreased from 510 to 400 to 270 TTN. As a matter of fact, owing to the electrophilicity of the active species, electron-releasing substituents on the substrate accelerate the nitrene transfer which then outcompetes sulfonamide formation. A similar tendancy was observed by Arnold's group when considering the aziridination of styrenes (see below Section 4.5).[63] In spite of these marked improvements, sulfonamide formation remained substantial which justified more specific approaches.

Arylsulfonamide production besides nitrene transfer results from reduction and protonation of the active Fe-nitrene species. In the case of cytochrome P450-derived catalysts, the most likely origin of the arylsulfonamide is to be found in excess electrons provided by the reductase associated with cytochrome P450 together with the protons provided by the proton relay system. Two approaches can therefore be used to shift the balance toward product formation, either slow down arylsulfonamide formation by cutting proton and electron flow in cytochrome P450 variants or accelerate the nitrene transfer.

4. 1. 3. Suppression of the proton relay system

Both Arnold and Fasan groups have addressed this matter and mutated residues potentially involved in cytochrome P450 proton relay system.

4. 1. 3. 1. Arnold laboratory

Starting from cytochrome P450 variants developed for monooxygenation and cyclopropanation[59] reactions, the group of Arnold[54] observed that mutation of threonine T268 by an alanine in wild-type cytochrome $P450_{BM3}$ (variant $P450_{BM3}$-T268A) increased the efficacy of benzosultam formation (Scheme 6) by 7 fold (TTN 15 vs 2.1). Similarly, mutation of the axial cysteine by a serine in wild-type cytochrome $P450_{BM3}$ (variant $P411_{BM3}$) ameliorated the nitrene transfer efficacy by 15 fold (TTN 32 vs 2.1). Combining both mutations in variant $P411_{BM3}$-T268A brought an overall 57 fold enhancement (TTN 120 vs 2.1). Eventually, the variant $P411_{BM3}$-CIS-T438S (Fig. 2a) incorporating a dozen other mutations proved 182 fold more active than wild-type cytochrome $P450_{BM3}$, reaching a TTN of 383.[54]

When considering the aziridination of styrenes, they noted that the variant $P411_{BM3}$-CIS-T438S exhibited a moderate activity for parent styrene and its *p*-Me and *p*-OMe derivatives (TTN 5, 8 and 15, respectively). Further studies of the reaction of *p*-Me-styrene showed that the corresponding aziridine was obtained with an 1.1 % yield and 25 % ee whereas the arylsulfonamide was formed with a yield of 95 %. A single further mutation I263F drastically increased both the yield and ee of the aziridine to 40 % and 55 %, respectively, bringing arylsulfonamide formation down to 55 %. Two subsequent mutations at additional active site positions (A328V and L437V) improved even more both yield (55 %) and ee (99 %), further reducing arylsulfonamide formation to 43 %.[63]

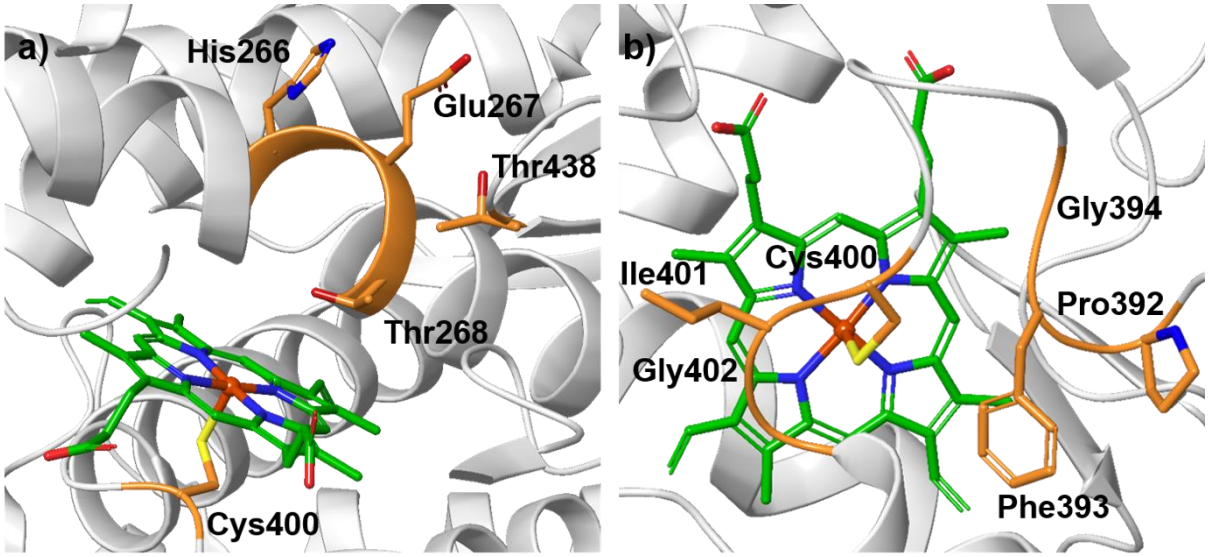


Fig. 2. Important residues in the heme distal (a) and proximal (b) regions of cytochrome $P450_{BM3}$. Adapted from [64] with permission.

4. 1. 3. 2. Fasan laboratory

Initially, the group of Fasan[65] also investigated T268A mutation in cytochrome $P450_{BM3}$ and a series of available variants. In every variant, the mutation proved beneficial for benzosultam formation (Scheme 6), albeit the extent of the enhancement depended on the parent enzyme. At the same time, arylsulfonamide formation was significantly reduced, which highlights the negative influence of protonation. A more global approach was recently developed which includes combined mutations of T268 and close residues E267, H266 and T438 (Fig. 2a).[64] Starting with the variant FL#62, a variant selected for its high and broad oxygenase activity,[66] (TTN 460), each single mutation H266F and T268V improved the activity by 3.6 fold and their combination by 5.5 fold. A further improvement (up to 7.3 fold, TTN 3360) was brought about by mutating (into a glycine) proline 392 that is close to cysteine 400, the axial heme ligand on the proximal side (Fig. 2b).

4. 1. 4. Suppression of the electron flow

Enhancing nitrene transfer proved efficient also in indole amidation studies to reduce sulfonamide formation to 14 %. To bring it even lower Arnold's group targetted the FMN-FAD reductase domain.[67] Deletion of the complete domain or the FAD subdomain failed to provide an improvement. However, they identified residue W1046 in the electron transfer chain, the mutation of which was beneficial. Indeed, the mutant W1046P enhanced indole amidation by

10 % while decreasing sulfonamide formation by 6 %, an overall increase in selectivity from 5:1 to 9:1.[67]

4. 2 General mechanism of nitrene formation and transfer

The overall cytochrome P450 mechanism of nitrene formation and transfer in benzosultam formation was studied in detail by the group of Fasan[64,68] through the determination of kinetic isotope effects (KIE) in various reaction conditions (Fig. 3).[64]

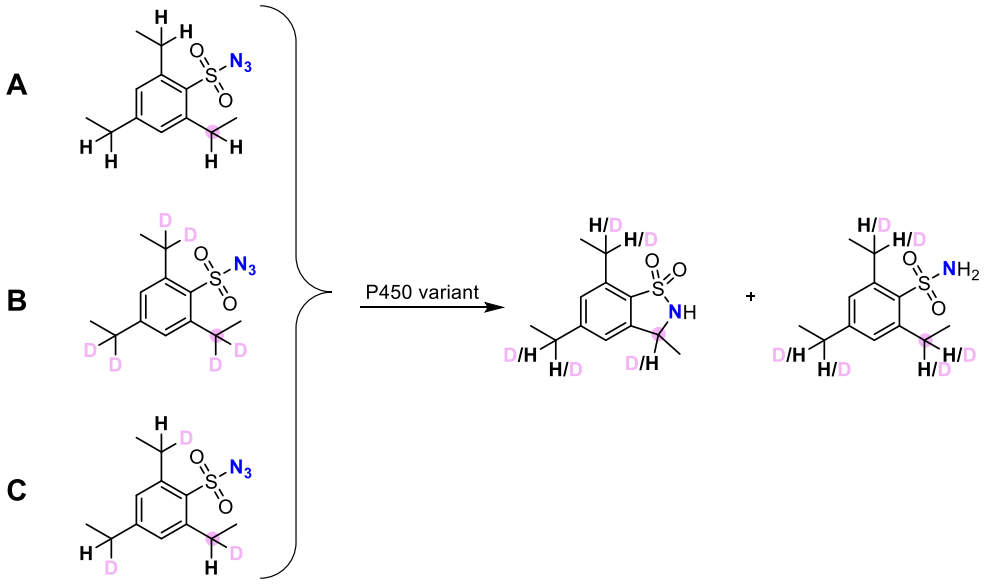


Fig. 3. Substrates used in Kinetic Isotope Effect experiments.[64] Experiment 1: the kinetic constants k_H and k_D of the reactions of A and B, respectively, were independently measured spectrophotometrically; experiment 2: the yields of the two products from the reaction of a 1:1 mixture of A and B were determined by ^{1}H NMR; experiment 3: the yields of the two products from the reaction of C were determined by ^{1}H NMR.

Using C with a mixed H/D composition (Figure 3), they determined the intramolecular KIE which is close to $KIE_{intra} \approx 4$, thereby indicating that the overall process involves an H• abstraction. By contrast, competition experiments using A and B (Figure 3) allowed them to quantitate the intermolecular KIE which is close to $KIE_{inter} \approx 1$, which suggests that the H• abstraction process is hidden by another reaction. This situation corresponds to a reaction profile where the C–H bond cleavage step is preceded by the rate determining step[69] which may be the binding or activation of the substrate. In the present case, binding of the azide substrate to the heme-Fe^{II}, a mere ligand binding or exchange, is likely to be fast. However, activation of the Fe^{II}-bound azide to form the active species, a two-electron rearrangement with N_2 extrusion, is known to require a substantial activation.[70–72] It is therefore the likely

rate determining step in this case, albeit it was noted for P411 variants that the heme pocket may facilitate this reaction.[73] The resulting active species is two-oxidation state higher than the azido heme-Fe^{II} precursor, and therefore potentially an heme-Fe^{IV} imino complex [(P)Fe^{IV}=NSO_2Ar]. This electrophilic active species is able to abstract a benzylic H leading upon radical rebound to benzosultam formation. Alternatively, it can be reduced and protonated to form the arylsulfonamide by-product. These studies allowed the group of Fasan to propose the following mechanism (Fig. 4).[64]

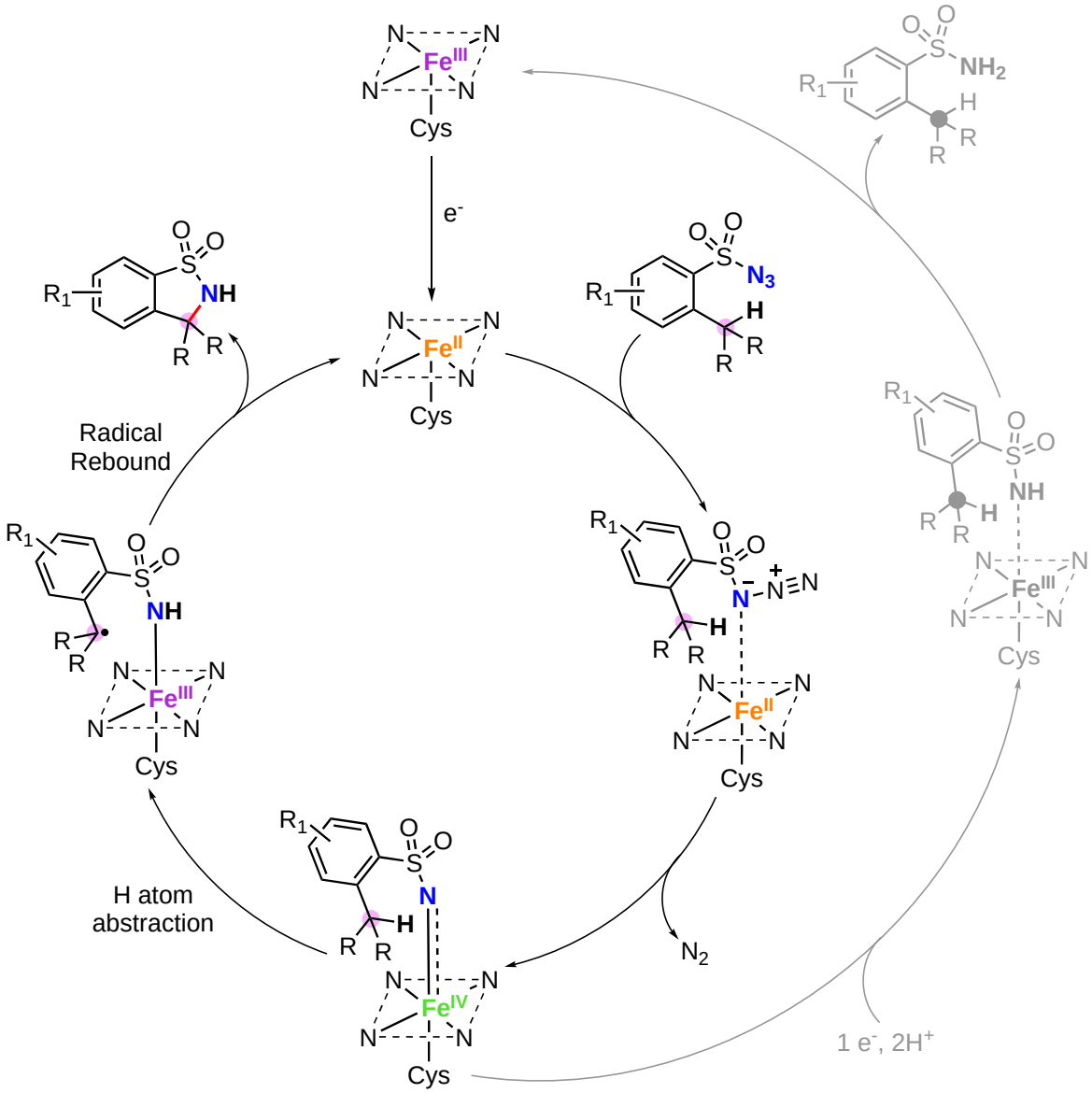


Fig. 4. Proposed mechanism for intramolecular C–H amination of sulfonyl azides catalyzed by cytochrome P450 and sulfonamide unproductive formation. Adapted from [64] with permission.

The occurrence of an electrophilic active species is consistent with the observations of the group of Arnold with P411 variants. As a matter of fact, a higher transformation of *p*-methoxy

substrates was noted in sulfimidation,[62] aziridination[63] and intermolecular amination[74] reactions. Very recent DFT and CAS computational studies concluded that the electronic structure of the corresponding active species of the P411 variant was best described as an imidyl ferric heme [(P)Fe^{III}–$^{\bullet}NSO_2Ar$],[72] as found for dipyrromethene complexes.[75]

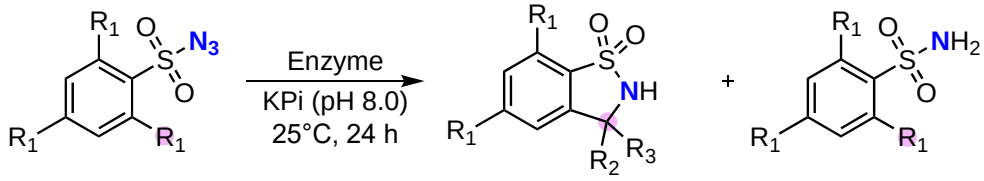
5. Catalytic reactions

5. 1 Aminations

In this section, we will present some remarkable results obtained in the transformation of substrates, emphasizing the regio- and stereoselectivity issues. It must be recalled that these amination reactions proceed through a H$^{\bullet}$ abstraction / radical rebound sequence which complicates the control of the stereoselectivity

5.1.1. Intramolecular aminations

5.1.1.1. Regio- and enantioselectivity in benzosultams syntheses



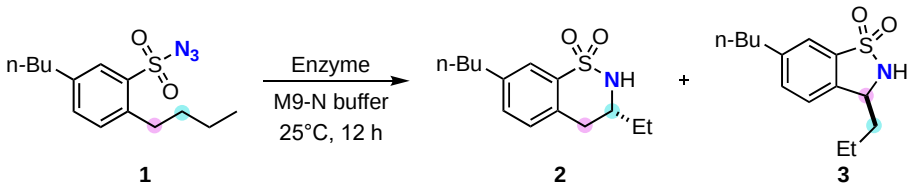
Variant	TTN	R_1	R_2	R_3	R_1 BDE (kcal mol^{-1})
$P411_{BM3}$-CIS	26	Me	H	H	89
$P411_{BM3}$-CIS	310	Et	Me	H	85
$P411_{BM3}$-CIS	36	i-Pr	Me	Me	85

Scheme 7. C-H Amination of 2,4,6-Trialkyl-benzenesulfonyl azides producing benzosultam and arylsulfonamide.

The group of Arnold compared the formation of benzosultam from arylsulfonylazides bearing Me vs Et vs iPr substituents (Scheme 7) using the $P411_{BM3}$-CIS variant.[54] The Et-substituted substrate was the most transformed (TTN 310) while those substituted by iPr and Me exhibited similar reactivities (TTN 36 and 26, respectively). The lower reactivity of the Me-substituted substrate is in line with the higher BDE of its benzylic C–H bond (BDE Ar-Me 89 kcal mol^{-1}, Ar-Et and Ar-iPr ca 85 kcal mol^{-1}).[76] These values cannot explain the low reactivity of the iPr derivative which likely results from steric constraints. Steric interactions are likely to play a major role not only on conversion but also on stereoselection and they can be driven

by site-directed mutagenesis. An illustration was provided by comparing the activities of intact *E. coli* cells expressing the P411$_{BM3}$-CIS variant and P411$_{BM3}$-CIS-T438S variants: while the threonine mutation drastically improved the enantiomeric excess (from 60 to 87 %), it impaired the conversion (from TTN 680 to 430).

This benchmarking reaction involves the amination of a benzylic-type bond with a BDE close to 85 kcal mol^{-1}. Amination of stronger C – H bonds is obviously more difficult and amination of strong C – H bonds in presence of weak ones is a real challenge. This was successfully addressed by the group of Arnold who reported on the preferential amination on the β-carbon of *n*-Pr and *n*-Bu substituted benzenes vs that on α-carbon in spite of their widely different BDE values 98 vs 85 kcal mol^{-1}, respectively (Scheme 8 for *n*-Bu derivatives).[77] Moreover, playing with different variants, they could obtain catalysts exhibiting > 90 % selectivity on α- or β-positions and > 99 % enantiomeric ratio.



1 **2** **3**

Variant	TTN	Selectivity (2:3)	er[a](2)	er[a](3)
P411$_{BM3}$-CIS-T438S-I263F	178	90:10	99.5:0.5	
P411$_{BM3}$-T268S-F87A	128	3:97		99.5:0.5

[a]er: enantiomeric ratio

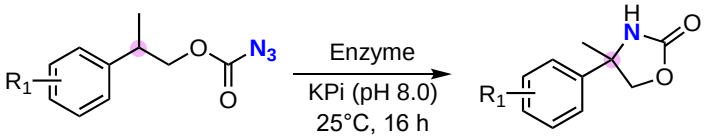
Scheme 8. Comparison of regioselectivities in benzosultam syntheses.[77] (er = enantiomeric ratio)

The mechanisms of these reactions have been studied in detail by QM/MM computational methods for a model methoxy ferrous heme and both mutants.[78] In each case, a two-step H• abstraction-rebound mechanism was identified. However, the differences in the respective activation energies of the two steps appeared crucial to orient the attack on the different sites. In variant A (P411$_{BM3}$-CIS-T438S-I263F), the regioselectivity of the attack is controled by the H• abstraction, while the rebound controls the stereoselectivity. By contrast, in variant B (P411$_{BM3}$-T268A-F87A), both are controlled by the rebound process. The optimized geometries of the active nitrene species reveal in variant A the presence of an H bond between G265 and an oxygen from the Fe-bound substrate sulfonimide. This probably contributes to lower activation energies and more restricted transition states in variant A than in variant B.

5.1.1.2. Other cyclizations through benzylic amination

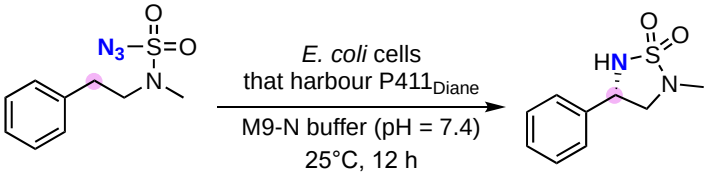
As already mentioned, nitrogen heterocycles bear a special interest as targets as well as synthetic intermediates. The group of Fasan has tested the possibility to perform cyclization reactions starting from a 2-phenylpropanol scaffold (Scheme 9a) using different nitrene precursors, acetoxy- and aryloxycarbamates and carbonazidate.[68] The latter proved more efficient for this reaction using the cytochrome P450 variant FL#62 and provided the oxazolidinone with a moderate TTN 74 which is enhanced to 100 by *p*-methyl substitution. However little chiral induction was noted, which suggests the intermediacy of a long-lived radical. Consistently, measurements of KIE (k_H/k_D ca 3) support a H• abstraction process as part of the rate-determining step.

a)



Variant	TTN	R_1
FL#62	74	H
FL#62	100	*p*-Me

b)



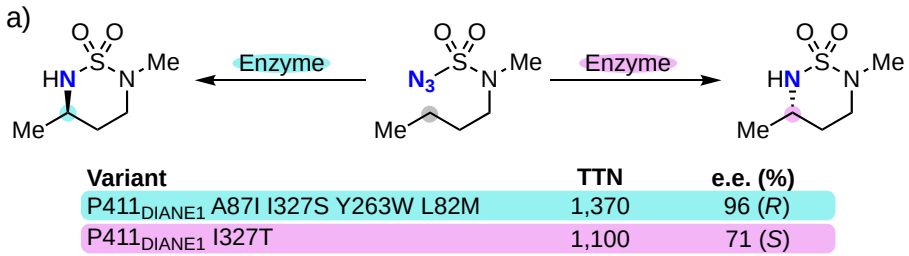
Variant	TTN	e.e (%)
$P411_{Diane1}$	450	94
$P411_{Diane1}$ I327P	3,490	99.9
Y263W Q437F ($P411_{Diane2}$)	72,000	99.9

Scheme 9. Cyclization reactions catalyzed by enzymatic systems

The same kind of approach was used by the group of Arnold to address the asymmetric synthesis of cyclic diamines which are important biological compounds.[79] An initial screening of cytochrome P450 variants showed that P411 lacking the FAD domain (P_{Diane1}) is a potent catalyst for diamine synthesis (TTN 450, ee 94 %, Scheme 9b). Directed evolution led to a significant improvement of the biocatalyst activity ($P411_{Diane2}$ = P_{Diane1} I327P Y263W Q437F, TTN 3,490, ee 99.9 %, Scheme 9b). Eventually, whole-cell biotransformations using low cell density further enhanced the activity to 72,000 TTN.

5.1.1.3. Cyclizations through amination of unactivated C(sp^3) – H bonds

Building on these successes, they addressed more challenging problems both in terms of reactivity and selectivity, as detailed in the following two examples (Scheme 10). Indeed, these biocatalysts proved efficient for the amination of unactivated secondary or tertiary C(sp^3)-H bonds. In the first series of experiments they could obtain cytochrome P450 variants able to perform divergent efficient and steroselective aminations of the same substrate (Scheme 10). In the last example described below, they achieved the enantioconvergent formation of a tetrasubstituted "methyl-ethyl" stereocenter starting from a racemic substrate. The 82 % yield and 87 % ee of the transformation were explained by a two-step mechanism involving a stereoablative H• abstraction followed by a stereoselective C-N bond formation.



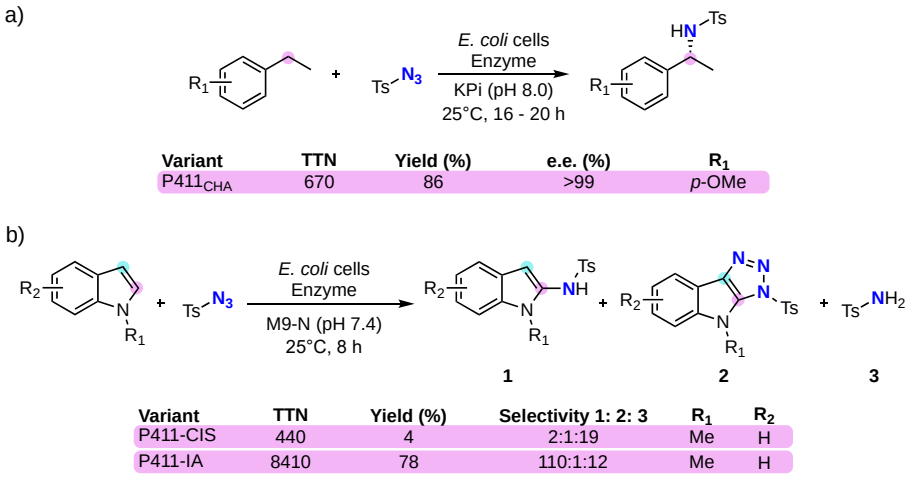
Variant	TTN	e.e. (%)
$P411_{DIANE1}$ A87I I327S Y263W L82M	1,370	96 (*R*)
$P411_{DIANE1}$ I327T	1,100	71 (*S*)

Variant	TTN	Yield (%)	e.e. (%)
$P411_{DIANE1}$L82C L181V I327T A330M Q437G	2,120	82	87

Scheme 10. Cyclization reactions catalyzed by cytochrome P450 variants[79]

5.1.2. Intermolecular aminations

Intermolecular aminations are far more challenging than intramolecular cyclizations described above because the alkane substrate must be aminated before the generated nitrene be consumed by reduction to the corresponding arylsulfonamide (Scheme 3b and Fig. 4), owing to the strongly reducing medium. From hemoproteins screening, the group of Arnold found that some variants developed for thioanisole sulfimidation (see below) were active also for ethylbenzene amination (Scheme 11a).



Scheme 11. Intermolecular aminations catalyzed by cytochrome P450 variants.[67,74]

Mutations of four key aminoacids (A82L, A78V, F263L, and E267D, Fig. 5) gave the variant $P411_{CHA}$ able to aminate 4-methoxyethylbenzene with tosylazide in > 1000 TON and 99 % ee.[74] The activity is highly sensitive to electronic effects with a decrease in yield from 86 to 53 and 15 % when the *p*-OMe substituent is replaced by Me and then H. It is also highly sensitive to steric influence: whereas the yield of amination of 2-ethylnaphthalene is 81 %, that of 1-ethylnaphthalene is only 6 %. This observation highlights the crucial role of the heme pocket on the reaction. These impressive results motivated several groups to investigate the mechanism of the reaction by QM/MM.[72,73] It was found that the triangular arrangement of A82L, A78V and F263L mutations reduces the size of the heme pocket (Fig. 5) and favors the binding of the incoming tosylazide to the heme Fe. As a result, the activation energy to generate the Fe-bound tosylnitrene is diminished to the point that it becomes smaller than that of the H• abstraction process, as opposed to the situation found in other enzyme catalysts[64] or synthetic ones.[72] Moreover, the activation energy of H• abstraction is modulated by the electron releasing strength of the *p*-substituent of ethylbenzene, the more donating the substituent, the lower the barrier, as found experimentally. Lastly, the calculations also explained the preference observed for the (R)-enantiomer by a lower activation energy of the H• abstraction process and traced it to a steric clash of the substrate terminal methyl group with A264 residue in the reaction leading to the (S)-enantiomer.[73]

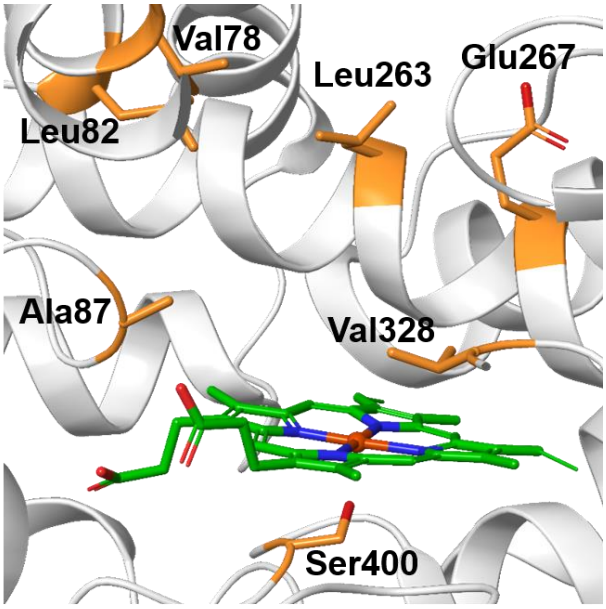


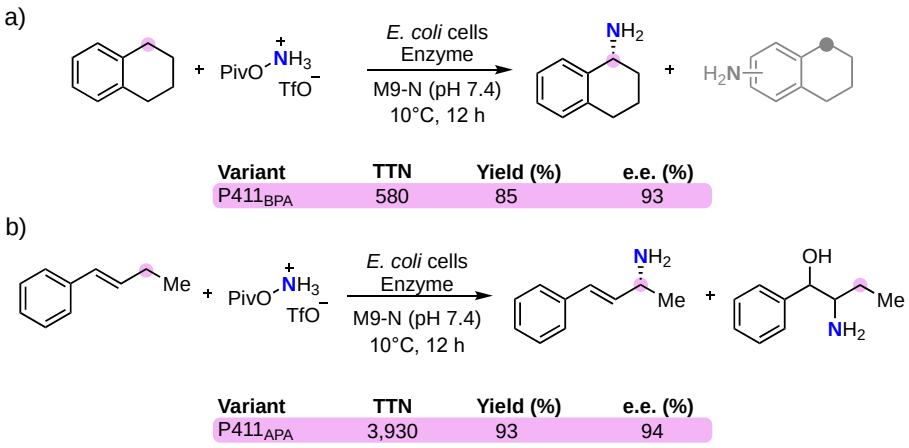
Figure 5. X-ray structure of the cytochrome P411 A82L A78V F263L variant showing important amino acids. Adapted from [74] with permission.

Intramolecular aromatic amination is currently used to access aromatic N heterocycles and classical procedures generally afford N-substituted compounds. However, intermolecular aromatic C(sp^2)-H amination is more arduous. It was elegantly achieved by Arnold's group[67] who observed that reaction of 1-indole with tosylazide catalyzed by a P411 variant gave the targetted product 1-methy-2-tosylaminoindole (Scheme 11b) but faced two problems: (i) a major formation of the sulfonamide reduction product and (ii) the formation of a triazole due to cycloaddition of the azide with the substrate. On one hand, to orient the reaction towards amination vs cyclization they mutated several residues within the enzyme pocket. On the other hand, reasoning that formation of tosylamide was due to excess reduction they introduced mutations in the reductase associated to P411 variant to slow down the electron flow. Combination of the two effects allowed them to turn the ratio indoleamine : triazole : tosylamide from 2 : 1 : 19 initially to 110 : 1 : 12 with a yield of 78 % and TTN 8410 for 1-methylindole.

5.1.4. Primary aminations of C(sp^3)-H bonds

All the above reactions produce N-substituted or N-protected amines which must be transformed to obtain primary amines usable in further derivatizations. To avoid these steps, primary amination of C(sp^3)-H bonds thus appears highly desirable, but requires a nitrene precursor able to deliver an unsubstituted nitrene. As a matter of fact, such a reagent, the

triflate salt of hydroxylammonium pivalate ester PivO-NH_3^+,TfO^-, was very recently introduced by the group of Morandi and shown to be active in nitrene transfers catalyzed by iron complexes.[80] Using this reagent, the group of Arnold[81] engineered enzymatic catalysts for benzylic and allylic primary aminations, $P411_{BPA}$ and $P411_{APA}$, respectively. In a first step, they showed that the variant P411-B2, which proved efficient in benzylic amination using tosylnitrene, was able to aminate tetrahydronaphthalene on one benzylic position using PivO-NH_3^+,TfO^- as nitrene precursor (Scheme 12a). This reaction was selective and minute amounts of the aniline side-product resulting from C(sp^2) amination were detected. After site-saturation mutations of five residues belonging to a α-helix capping the heme proximal site (V78M G266P E267D T327F L437F S438G), they obtained the variant $P411_{BPA}$ which exhibits high reactivity (TTN 750) and enantioselectivity (ee 93 %) for this reaction.[81]

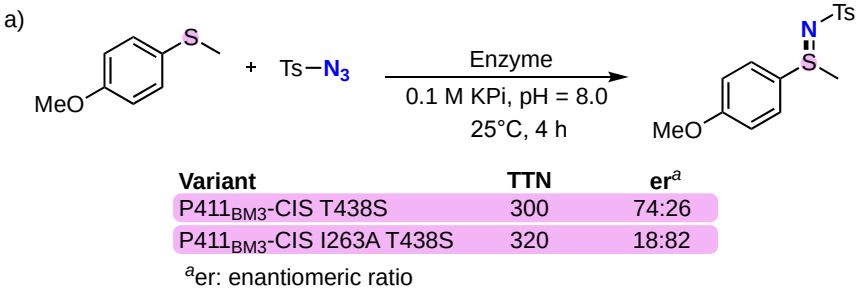


Scheme 12. Intermolecular primary aminations catalyzed by cytochrome P450 variants.[81] Reaction by-products are depicted in gray.

Allylic and benzylic C—H bonds have very similar Bond Dissociation Energies, 82 and 85 kcal mol^{-1} respectively.[76] This makes the above variants potential catalysts for allylic amination of styrene derivatives, provided that the olefin attack leading to aziridine or aminonucleophile substitution not be prefered. They were thus able to engineer $P411_{APA}$ a close relative of $P411_{BPA}$ with one additional mutation (V78M G266P E267D T327F N395R L437F S438A) that is very efficient (TTN 3930) and stereoselective (ee 94 %) in the primary amination of allylic substrates (Scheme 12b).[81]

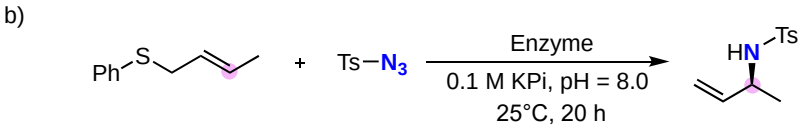
5. 2. Sulfimidation

Chiral sulfimides are useful molecules for various applications and their synthesis has attracted interest for many years. In contrast to sulfoxidation which is catalyzed by many enzymes there was no example of analogous sulfimidation reactions. The group of Arnold[62] developed efficient and selective catalysts for this reaction by engineering cytochrome P450s. Starting from the $P411_{BM3}$-CIS variant including the C400S mutation, they found that T438S mutation on the proximal side afforded an efficient sulfimidation of thioanisole derivatives (TTN 300) with a good enantiomeric ratio (er 74:26) using tosylazide as nitrene precursor (Scheme 13a). Quite remarkably, introducing I263A mutation in this variant maintains the activity but inverses the enatiomeric selectivity (er 18:82).



Variant	TTN	er[a]
$P411_{BM3}$-CIS T438S	300	74:26
$P411_{BM3}$-CIS I263A T438S	320	18:82

[a]er: enantiomeric ratio



Variant	TTN	Yield (%)	e.e. (%)
$P411_{BM3}$-CIS T438S I263F V87A A328V A268G A82I	2,200	77	68

Scheme 13. a) Intermolecular sulfimidations catalyzed by cytochrome P450 variants.[62] b) Intermolecular sulfimidation of phenylcrotyl sulfide followed by [2,3-sigmatropic] rearrangement to phenylthiosulfinamide catalyzed by cytochrome P450 variant and final tosylallylamine formation.

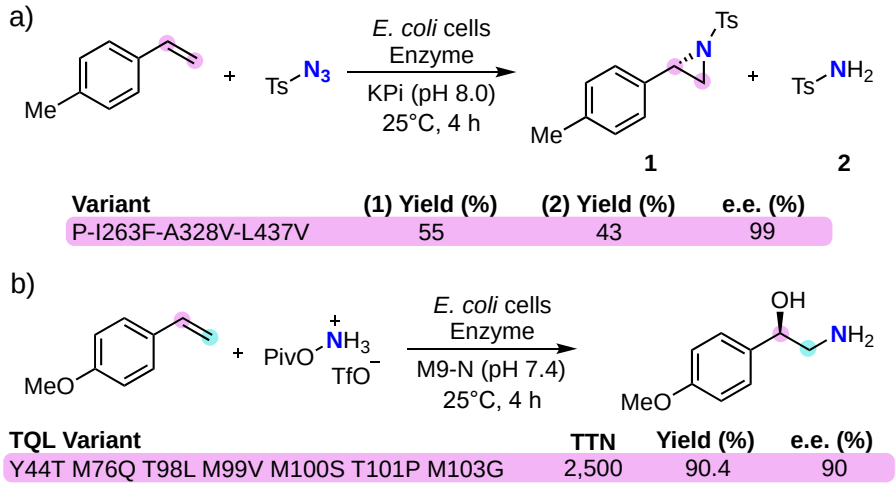
As mentioned in Section 4.1.2, the reaction proved to be very sensitive to electronic effects of thioanisole substituents. The *p*-methoxy substituted thioanisole was the most reactive substrate, which indicates an electrophilic active species and a Hammett correlation revealed a slope $\rho = -4.0$. This behavior is consistent with an electron transfer being the first step of the nitrene transfer reaction.[82] Consistently, it was observed that arylazides were not reactive. As a matter of fact, it has been shown that the oxidation potential of an [Fe^{IV}=NTs] active species is lowered by ca 850 mV in [Fe^{IV}=NTol] where the sulfonyl group is absent.[61]

The group of Arnold took advantage of the efficient sulfimidation they had set up to design an access to allylic amines through a [2,3-sigmatropic] rearrangement of allylic sulfimides in

which an N–S bond is exchanged for an N–C bond (Scheme 13b).[83] In whole cells conditions, the resulting phenylthiosulfinamide was reduced to allylic amine by cellular reductants. The variants developed for the sulfimidation (see above) proved essentially unactive on phenylcrotylsulfide owing to the larger size of the crotyl chain. Introducing the already known I263F mutation confered minimum activity suitable for a directed evolution round that identified efficient A328V and V87A variants (TTN 490). Mutation A268G which enlarges the substrate pocket proved conclusive (TTN 1800) and this effect was further enhanced by mutation A82I (TTN 2200). Eventually, working in whole cells afforded the allylic amine 79 % isolated yield and TTN 6100 in aerobic conditions. A moderate enantiomeric excess 68 % is noted that is due to imperfect stereoretention in the rearrangement.

5. 3. Aziridination

Direct aziridination of olefins is particularly attractive for its production of useful synthetic intermediates. The development of aziridination biocatalysts was addressed by Arnold's group (Scheme 14a).[63] They noted that the variant $P411_{BM3}$-CIS-T438S exhibited a moderate activity for parent styrene and its *p*-Me and *p*-OMe derivatives (TTN 5, 8 and 15, respectively). Further studies of the reaction of *p*-Me-styrene showed that the corresponding aziridine was obtained with an 1.1 % yield and 25 % ee whereas the arylsulfonamide was formed with a yield of 95 %. Several mutations within the substrate pocket drastically improved these results. A single further mutation I263F (Fig. 5) increased both the yield and ee of the aziridine to 40 % and 55 %, respectively, bringing arylsulfonamide formation down to 55 %. Two subsequent mutations (A328V and L437V) in the active site improved even more both yield (55 %) and ee (99 %), further reducing arylsulfonamide formation to 43 %.[63] As mentioned in section 4.1.2, a strong electronic influence on styrenes aziridination was observed in line with a strong electrophilicity of the putative nitrenoid active species.



Scheme 14. a) *p*-Methylstyrene aziridination and arylsulfonamide formation (a) and *p*-methoxystyrene aminohydroxylation (b) catalyzed by cytochrome P450 variants.

5. 4. Aminohydroxylation

Chiral 1,2-amino alcohols are present in numerous bioactive compounds and find use as chiral auxiliaries for many purposes, which explains why their synthesis is attracting strong interest. Using directed evolution, the group of Arnold[84] has been able to devise a direct route to unprotected enantioenriched amino alcohols (Scheme 14b) taking advantage of new aminating agents based on substituted hydroxylamines which give access to unprotected amines (see Sections 5.1.4 and 6). Among various hemoproteins evaluated for the reaction, wild type cytochrome c from *Rhodothermus marinus (Rma)* emerged as the most efficient, transforming 4-vinylanisole in the amino alcohol with 40 TTN and 32 % *ee*, and was chosen for directed evolution. The methionine Fe ligand (M100) and close residues on the distal side (M99, T101, M103) where mutated to open a reaction site and nearby residues (Y44, M76 and T98) were changed to provide access to it (Figure 6). Using PivO-NH_3^+,TfO^- as nitrene precursor, the resulting *Rma* Cytc (M100S M103G T101P M99V T98L M76Q Y44T) "TQL variant" converted 4-vinylanisole to the amino alcohol with 2500 turnovers and 90 % *ee*. Notably, this variant tolerates various substituents on the aromatic nucleus and the aliphatic chain. Preliminary investigations do not support a mechanism involving successive formation and opening of the aziridine, but rather water interception of an intermediate benzylic cation. It is worth noting that related solvent interceptions of intermediates during nitrene transfer reactions have been very recently documented.[85,86]

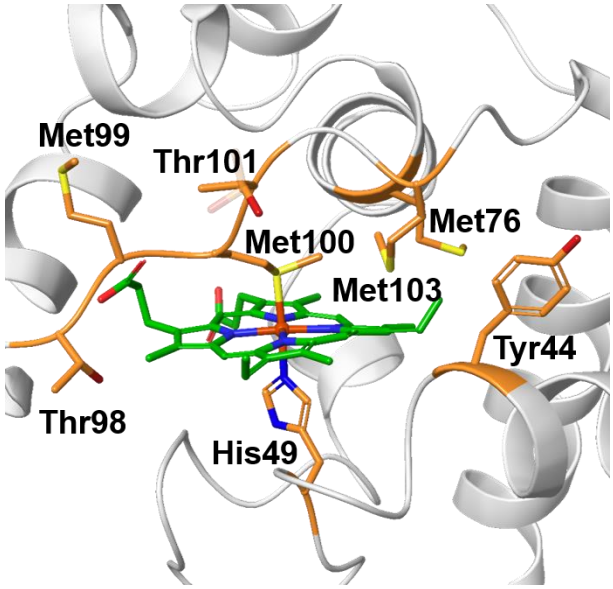


Figure 6. X-ray structure of the active site of *Rma* cytochrome c. Adapted from [84,87] with permission.

6. Nitrene precursors

This survey of biological nitrene transfer reactions performed by either natural or engineered enzymes has uncovered that over the years very different nitrene precursors were used. As a matter of fact, while pioneering studies used tosylphenyliminoiodinane,[18] this reagent was initially outcompeted by tosylazide,[54] but more recently new reagents were used such as acetoxy- and aryloxycarbamates and carbonazidates.[68] Such reagents were used for some time with synthetic catalysts.[88–91] All of them contain an N–O bond with the O atom attached to an electron withdrawing group and can be viewed as polarized hydroxylamines.[92] Eventually, hydroxylammonium derivatives[93] were used because of their utmost advantage to give access to primary amines.[80,84] In this respect, it is worth remembering that the only natural nitrene transferase known to date operates thanks to an hydroxylamine derivative (Scheme 5) that is formed by successive hydroxylation (BezJ) and acetylation (BezG) of the aniline substrate (PABA, Scheme 4a).[25] From a mechanistic point of view, generating the Fe-bound nitrene (or Fe imido species) is conceptually similar to generating the Fe-bound oxene (or Fe oxo species) from the hydroperoxo intermediate in cytochrome P450 catalytic cycle (intermediate 5b in Fig. 1). This requires the heterolytic cleavage of the N – X bond with X being an aryl iodide, or N_2, or an acyl-type group on the reagent, a process that is driven by the elimination of a highly stable molecule, such as iodobenzene, pivalic acid, or even N_2 and CO_2.

7. Very recent developments

In the past two years, new enzymatic systems have been successfully explored as potential nitrene transferases. A first set comprises heme proteins selected on the basis of the improved mechanistic understanding of the reaction,[64] while the other encompasses non-heme enzymes catalyzing diverse oxygenases reactions.[94,95]

7.1. Heme proteins

The improved mechanistic understanding of the enzymatic nitrene transfer (Section 4.2) allowed the group of Fasan to identify the cytochrome P450 of the explosive degrading enzyme XplA as a potential improved catalyst.[64] XplA comprises a heme domain fused to a flavodoxin domain. Its X-ray structure revealed that it lacks residues involved in proton transfer. The XplA truncated heme domain (XplA-heme) proved very active in benzosultam formation with a TTN of 1780 and selective as well with only 18 % of sulfonamide. As found by Arnold group,[54] mutation of heme axial cysteinate ligand C503 by a serine was instrumental also to increase the efficacy of the XplA-heme (C503S) variant without loss of selectivity (TTN 3830, 13 % sulfonamide). Both features could be improved further (TTN 8090, 4 % sulfonamide) by fusing Xpla-heme domain with the reductase domain of an other organism to mimick the arrangement of cytochrome $P450_{BM3}$. Eventually, they observed that wild type BezE, the only natural nitrene transferase reported so far,[25] exhibited an impressive activity for benzosultam formation (TTN 10880, 13 % sulfonamide).[64] This observation is significant and promising because the involved benzylamination strongly differs from its natural aziridination activity.

7.2. Non-heme proteins

Both the groups of Arnold,[94] and Fasan[95] reported very recently successful attempts to engineer non-heme iron dioxygenases into nitrene tranferases. From a screening of seven such enzymes against styrene aziridination by tosylazide, Arnold and coworkers found that *Pseudomonas savastanoi* ethylene-forming enzyme (Fig. 7a) exhibited a significant activity. This enzyme binds Fe through two histidines and an aspartate and requires α-ketoglutarate (α-KG) as cofactor to generate the oxo Fe^{IV} species active in oxygenation reaction. Reasoning

that the (α-KG) cofactor was no longer needed, they observed that its replacement by acetate or N-oxalylglycine enhanced the activity by 6.7 and 7.8 fold, respectively. Mutations of several residues in the substrate binding pocket further enhance the activity to 11 fold in presence of acetate. Furthermore, some of the variants developed for aziridination proved efficient to promote benzosultam formation (Scheme 6) with up to TTN 730 in presence of N-oxalylglycine.[94]

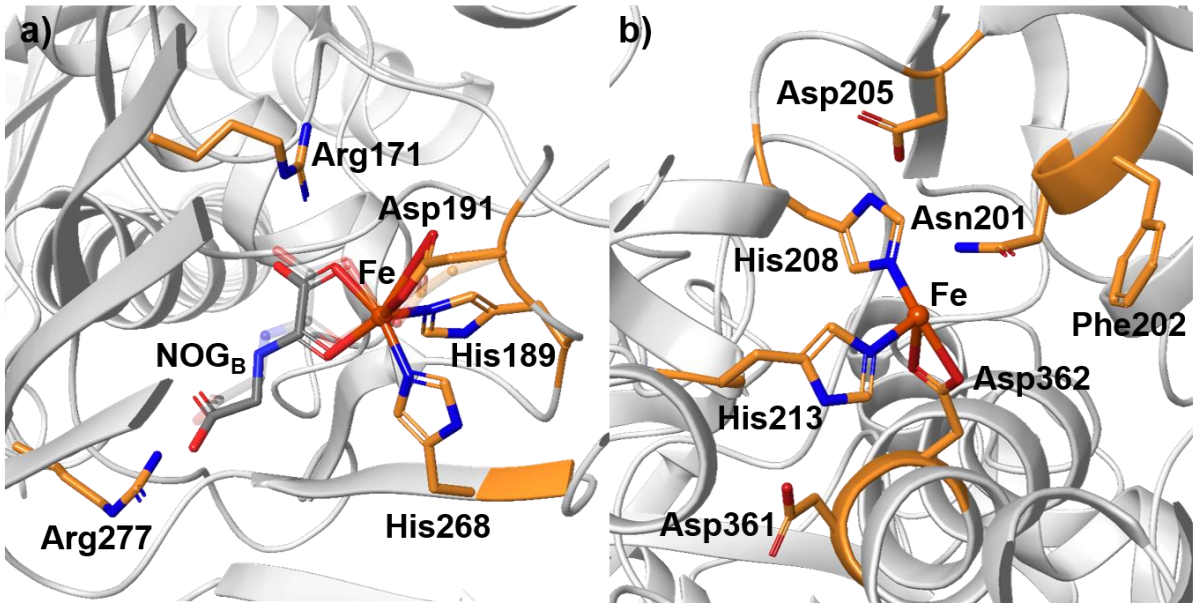
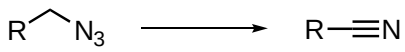


Figure 7. X-ray structures of the active sites of (a) *Pseudomonas syringae pv. phaseolicola PK2* ethylene-forming enzyme [96] and (b) *Escherichia coli* naphthalene dioxygenase.[97] Adapted with permission.

It was recently found that Toluene dioxygenase,[98] a Rieske enzyme, and a few α-KG-dependent enzymes[99] were able to transform organic azides into nitriles (Scheme 15) and in the former case an imido Fe^{IV} active species was proposed from DFT calculations.[98] Based on these results the group of Fasan screened a set of Rieske dioxygenases for nitrene transfer in benzosultam formation (Scheme 6).[95]



Scheme 15. Reaction performed by Toluene Dioxygenase and L-leucine and L-isoleucine hydroxylases.[98]

Toluene dioxygenase (TDO), Naphthalene dioxygenase (NDO, Fig. 7b) and Chlorobenzene dioxygenase (CBDO) expressed in *E. coli* strains exhibited detectable amination activities

which were further optimized for the latter two enzymes. A high selectivity toward benzosultam formation (as opposed to reduction to the sulfonamide) was observed, as well as a good dioxygen tolerance. In addition, α-KG-dependent hydroxylases and a chlorinase were assayed in whole cells for benzosultam formation and 2-phenylpropyl carbonazidate cyclization (Scheme 9b). Most of them revealed significant activities.[95] Further work is needed to establish mechanisms and develop these preliminary results.

8. Conclusions and Perspectives

To briefly summarize this journey over more than three decades, we started with cytochrome $P450_{LM3}$, a natural oxygenase, which exhibited very modest nitrene transfer activitity of a few turnovers and ended up with BezE, a natural nitrene transferase catalyzing an intramolecular aziridination, with activities surpassing 10^4 turnovers.[25,64] In the past decade, numerous cytochrome $P450_{BM3}$ variants have been engineered for most types of nitrene transfer reactions and reaching impressive activities in terms of both efficacy and selectivity. The viability of enzymatic catalysis of a wide range of nitrene transfer reactions, both intra- and intermolecular, has been established. A particular feature that must be emphasized is the amination of strong C–H bonds in the presence of weaker ones.[77] In addition, enantioselective syntheses of allylic amines[83] and aminoalcohols[84] were achieved. A strong contribution to this progress was provided by the continuously improved understanding of the respective requirements of oxene and nitrene transfers by hemoproteins, in particular for the generation of the Fe^{IV} oxo vs imido active species. Independently, the basic features of Fe nitrene transfer reactions were established thanks to the pioneering work of Shaik and coworkers[44] and the seminal work of Sastri, de Visser and coll.[82] supported and complemented by a few other groups.[61,100] Overall, a thorough knowledge of Fe nitrene transfer chemistry has been attained, which allows the design of efficient biocatalysts. These expand and complement the arsenal of biocatalytic systems used to form C–N bonds by carbene insertion in a N–H bond[101] or through an enzymatic cascade.[102]

A first line of development of new catalysts is currently based on cofactor substitution as initiated by the group of Hartwig who replaced the (natural) heme of *Sulfolobus solfataricus* cytochrome P450 CYP119 by a methyl iridium porphyrin Ir(Me)PIX.[103] The iridium complex was selected after screening of various metal porphyrins, including Ru and Co complexes, for

catalysis of benzosultam formation. Nevertheless, owing to the known propensity of Ru and Co porphyrins[22,104] to catalyze a wealth of nitrene transfer reactions, it cannot be excluded that other hemoproteins incorporating Ru PIX or Co PIX may exhibit interesting activities for amination reactions.[105] In addition, the introduction of non-natural aminoacids can boost or reorient these activities.[49,50]

Concurrently, an active search of new proteins, both heme[53,64] and non-heme[94,95] dependent, is developed. Engineered non-heme dependent enzymes have only recently emerged (Section 7.2) and the wide versatility of this class of enzymes for oxygen transfer and related processes[106,107] is promising for adaptation to nitrene transfer reactions owing to enzyme promiscuity[51,108] and previous successfull repurposing of several enzymic activities.[98,99,109] The engineering methodology and alternate strategies[110] to develop new artificial enzymes for nitrene transfer are fully mature and new examples, even more complex, are likely to abound in the future.[111]

Declaration of Competing Interest

None.

Acknowledgements

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Graphical abstract

Examples of nitrene transfer reactions catalyzed by natural and engineered hemoproteins.

