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Repurposed and artificial heme enzymes for cyclopropanation reactions

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ABSTRACT

Heme enzymes are some of the most versatile catalysts in nature. In recent years it has been found that they can also catalyze reactions for which there are no equivalents in nature. This development has been driven by the abiological catalytic reactivity reported for bio-inspired and biomimetic iron porphyrin complexes. This review focuss es on heme enzymes for catalysis of cyclopropanation reactions. The two most important approaches used to create enzymes for cyclopropanation are repurposing of heme enzymes and the various strategies used to improve these enzymes such as mutagenesis and heme replacement, and artificial heme enzymes. These strategies are introduced and compared. Moreover, lessons learned with regard to mechanism and design principles are discussed.

1. Introduction

The last decades has seen a great effort towards developing biocatalysts for reactions that have no equivalent in nature. In this regard, it is no surprise that heme enzymes have received special attention. Heme enzyme such as the cytochrome P450s are some of the most versatile enzymes found in nature. Their ability to oxidize complex organic substrates with high regio- and stereoselectivity is unmatched. The heme cofactor, an iron protoporhyrin IX complex, is a key element of these enzymes catalytic activity as it can react with molecular oxygen to access a range of highly reactive species that makes it possible to oxidize substrates at unreactive C—H bonds [1–4]

The ferric heme complex, with a bound water ligand is the resting state. Upon binding of substrate, the water ligand is released and by addition of an electron from the reductase domain or an endogenous reductant, the ferrous heme complex is generated. This is the active state that can bind molecular oxygen. The oxidation activity of P450 enzymes is not solely due to the heme cofactor, other elements of the structure such as the axial ligand to the iron center and the proton transfer network are also important to achieve activation and subsequent bond cleavage of dioxygen by modulating its electronic structure. [4] However, the reactivity of iron porphyrins is not limited to oxidation catalysis as has been shown in extensive bio-inspired studies on synthetic and biomimetic iron porphyrins. A range of "abiological" catalytic activities have been reported. Of particular interest here is the work on iron porphyrin catalysed carbene transfer reactions, notably cyclopropanations. Cyclopropanations are reactions of alkenes with carbenes or metal-carbenoids (Fig. 1). The latter are often generated via reaction of a metal complex with a diazo compound, often a diazo ester such as ethyl diazoacetate (EDA).

Classic studies by Woo and Kodadek established the principle, [5] which later resulted in highly active and synthetically applicable Fe-tetraphenylporphyrin (Fe-TPP) complexes for cyclopropanation, also in water as solvent. [6–8] However, due to the planar nature of the porphyrin ligand and the synthetic challenge of making derivatives, it has proven difficult to achieve enantioselective catalysis.

These results suggest the potential of heme enzyme for catalysis of cyclopropanation reactions. But, despite the plethora of heme enzymes available in nature, none are known to have cyclopropanation as its biological activity. In this mini review I will describe the efforts made in recent years to develop heme enzymes for cyclopropanation reactions. The focus will be on describing and comparing the different approaches used and some general lessons concerning the mechanisms involved and the design of heme "cyclopropanase" enzymes will be given. This review is not meant to give an exhaustive overview of the synthetic applications. For this, the reader is referred to other reviews on the various approaches. [9–12]

2. Repurposed heme enzymes

A powerful approach is the repurposing of existing heme enzymes for cyclopropanation reactions. This involves screening heme enzymes for a desired catalytic activity, in this case cyclopropanations, and those enzymes that show a basic level of activity are then further engineered and optimized for the reaction of interest.

In a landmark paper, Coelho and Arnold reported the first

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biocatalysis of cyclopropanation reactions. [13] Their efforts were inspired by the studies on biomimetic Fe-porphyrin complexes and their similarity to the heme cofactor, as well as the presumed similarity –at least at first glance- of the iron porphyrin derived carbenoid intermediate to the active oxene intermediate responsible for heme enzyme catalysed oxidation. Several heme proteins were screened for cyclopropanation activity on styrene using ethyl diazoacetate (EDA) as carbene donor and sodium dithionite as reductant, needed to convert the Fe (III)-heme to its Fe(II) form, which is required for catalysis. NADPH could also be used as reductant, which raises the spectre of in vivo applications (see below). The reactions had to be performed under inert atmosphere to because of competing oxidation processes and oxidative inactivation. Common heme proteins such as horseradish peroxidase, myoglobin, cytochrome *c* and P450BM3 all showed multiple turnovers, but only in case of P450BM3 a change in diastereolectivity compared to hemin alone and a small enantiomeric excess (ee) was found. Hence, this enzyme was selected for further study.

Benefitting from their extensive experience with engineering of this enzyme, a panel of 92 different variants was selected and tested in the cyclopropanation of styrene. This led to the discovery of multiple variants that showed increased catalytic activity and (*enantio*)selectivity. One variant, called H2–5-F10, which has 16 mutations compared to the wildtype P450BM3, showed a 50 fold increase in turnover numbers and gave the trans cyclopropanation product of styrene in 95% ee. Other variants, showed preferential formation of the cis product, which is normally the more difficult product, also with an ee of 95%. Analysis of the successful variants showed them to be rich in alanine mutations in the active site, which led to exploring active site mutations. This resulted in a variant P450BM3 CIS T438S that showed excellent cis selectivity with up to 97% ee for the cyclopropanation of styrene. Analysis of individual mutations also showed T268A to be important: this mutation, which removes a residue important for assisting in the activation of dioxygen in its natural activity, dramatically increases catalytic rate not only in this but also in other P450s. [14,15]

Next, mutation of the axial ligand was explored. P450 enzymes characteristically are ligated by cysteine. Replacing the cysteine with serine, the mutation C400S, in combination with the 13 mutations previously introduced in the CIS variant, raised the reduction potential of P450BM3 CIS enough so that the initial reduction of Fe(III) can be achieved with the biological reductant NAD(*P*)H, even in the absence of substrate. [16] The new variant, which was dubbed P411 because of the shifted Soret band of the CO adduct, allowed for application in vivo. Indeed, using whole cells expressing P411BM3 CIS, more than 60,000 turnovers could be achieved, resulting in the cis cyclopropanation product of styrene with an ee of 99%. This allowed for gram scale scale reactions, an important step towards synthetic application.

The P411 enzymes have proven to be a good starting point for the development of cyclopropanation enzymes for wide variety of substrate classes. This is most likely due to a combination of their promising reactivity as well as the fact that these enzymes can be tested in whole cells, which greatly facilitates the screening of mutants. This has resulted in a series of highly active P411-based enzymes for the synthesis of, often all stereoisomers of, cyclopropane products from a variety of different substrate classes. This includes some for which there are no solutions using small molecule catalysts, and for the synthesis of important pharmaceutical intermediates. [17–23]

P450BM3 was compared to other P450s. [24] Indeed, other P450 enzymes such as P450cam (CYP101) from Pseudomonas putida, CYP119 from *Sulfolobus solfataricusm*, TxtE enzyme from *Streptomyces scabies* and CYP153A6 from *Mycobacterium* sp. *HXN-1500* all proved to be capable of catalysis of cyclopropanation, with different patterns in stereoselectivity in the products. The axial Cys to Ser mutation was generally found to give rise to more active cyclopropanation enzymes. The only exception being P450cam, which did not show an improvement upon introducing the C357S. However, UV vis spectroscopy showed that, in contrast to the other P450 variants investigated, the Fe(II)-CO adduct showed a Soret band at 421 nm instead of 407–413, suggesting another residues such as histidine acts as axial ligand in this protein. Also hemin bound to bovine serum albumin (BSA) was shown to be active, but then with preference for the trans product in cyclopropanation of styrene. This suggests the importance of a hydrophobic binding pocket for the reaction.

The discovery that replacing the axial cysteine ligand for serine resulted in a more active cyclopropanation catalyst, combined with the high activity observed with myoglobin catalysts, which feature a histidine as axial ligand, by Fasan et al. (vide infra), [25] spurred a study on the effect of this axial ligand. [26] Systematic variation of C400 with H, M, Y and A revealed the histidine ligated variant to most active (Fig. 2). Mutation of the distal residue T268, which is involved in proton transfer required for oxygen activation, to alanine also increases the cyclopropanation activity, while reducing the oxygen activation activity.

Further site saturation mutagenesis studies resulted in a highly active variant named BM3 Hstar which contained an additional three mutations in the active site. This variant is active on electron poorer alkenes and was used for the biocatalytic synthesis of Levomilnacipran (Fetzima). The histidine ligated variant also was shown to work in cells.

The fact that His ligated heme enzymes provide highly active cyclopropanation catalysts dramatically expands the range of heme proteins that could potentially be used, since His ligated hemes are prevalent in many classes. Initial results suggested that His ligation alone is not enough, evident from the fact that horseradish peroxidase is inactive. However, this most likely is partly related to the fact that a sufficiently large substrate binding pocket needs to be present. Indeed that has proven key in the development of His ligated heme enzymes for cyclopropanation and other carbene transfer reactions from diverse classes, for example based on cytochrome *c*, nitric oxide dioxygenase and oxygen carrier proteins, such as myoglobin. [9,17,27–31]

In case of a triple mutant of the thermostable *Rhodothermus marinus* (Rma) cytochrome *c*, named Rma cyt C TDE or Rma TDE, where TDE denotes the V75T M100D M103E mutations, the x-ray structure of the iron porphyrin carbene intermediate (IPC) formed upon reaction with ethyl diazopropionate could be determined (Fig. 3). [32] The carbene

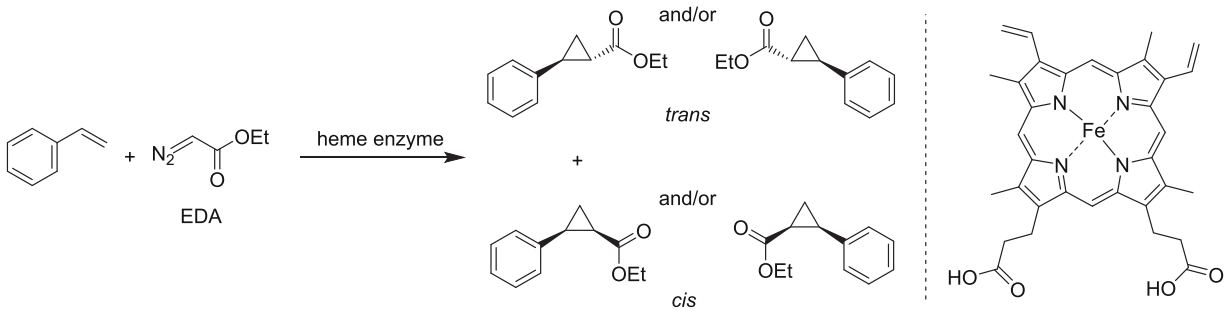

Fig. 1. Left: cyclopropanation of styrene with EDA catalysed by heme enzyme to give the trans and cis cyclopropane products; right: structure of iron-protoporhyrin IX (heme).

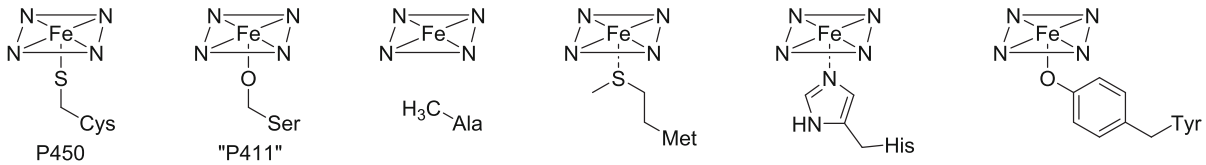

Fig. 2. Systematic variation of the axial ligand by mutagenesis.

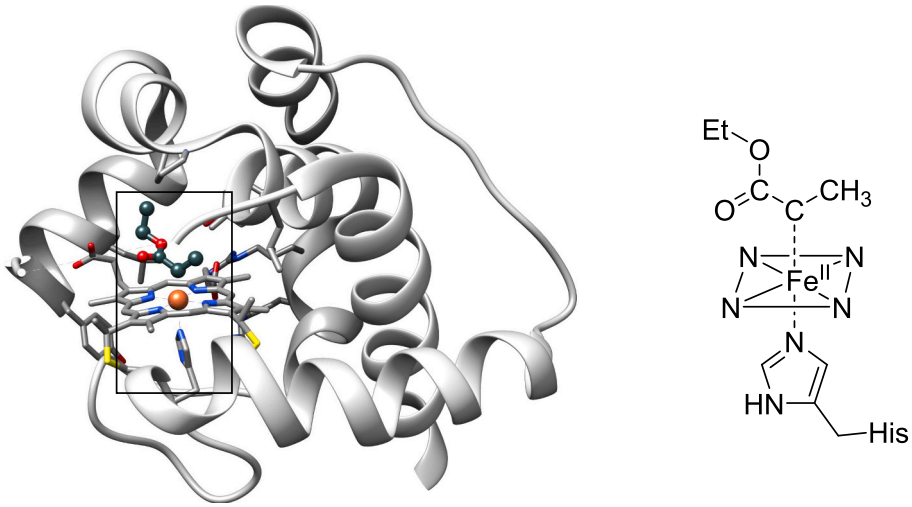

Fig. 3. Left: X-ray crystal structure of the Rma TDE carbene adduct (PDB: 6CUN) with the IPC in the box; right: schematic representation of the structure of the IPC.

group was found to coordinate in an end-on fashion with an Fe-$C_{carbene}$ bond length of 1.9 Å. From a combined spectroscopic, e.g. EPR and Mössbauer studies, and computational study it was concluded that the iron centre in the IPC is a closed shell singlet species, which was in agreement with an earlier reported study on myoglobin catalysed cyclopropanation (see below). [33] The IPC seems to be favoured in the Fe(II) state instead of the initially proposed Fe(IV) state that was initially cited as source of inspiration for exploring P450s as cyclopropanation catalyst because of its similarity to the iron oxene intermediate. [13]

However, with regard to catalytic performance, myoglobin appears to be most versatile as was demonstrated by Fasan and co-workers. They reported myoglobin to have significant activity in the cyclopropanation of styrene with EDA under anaerobic conditions. [25] Also addition of a reductant such as sodium dithionite was required to generate the active iron(II) form of the enzyme. Under these conditions, the activity observed was comparable to that reported for P450BM3, albeit that no enantioselectivity was achieved. Based on analysis of the structure of myoglobin (Mb), several residues in the distal pocket were selected for mutagenesis. This gave rise to a double mutant Mb(H64V,V68A) capable of tens of thousands of turnovers, with initial rates in the range of 1000 min^{-1}. This Mb variant and mutants thereof has proven a powerful enzyme for a wide variety of carbene transfer reactions in addition to cyclopropanation. [34–39]

Based on a model derived from the structure of the H64V mutant, it was proposed that the H64V mutation increases the opening towards the distal pocket, thereby increasing the accessibility of the pocket for the styrene and EDA substrates. The V68A mutation increases the size of the pocket and was proposed to allow the carbene to bind in a more favourable orientation for (enantioselective) reaction with the styrene. [40] The Mb (H64V, V68A) mutant has been demonstrated to be a powerful biocatalyst for cyclopropanation of a wide variety of alkene substrates, using many different diazo carbene precursors, some of which are highly unstable and had to be prepared immediately prior to reaction. In many cases Mb (H64V, V68A) itself proved to be a good catalysts. But it also served as convenient starting point for further mutants that were tailored for specific reactions and to achieve stereodivergent catalysis. [41–47] In another study, by computational design using RosettaMatch, a thermostable variant of Mb (H64V, V68A) was created using thioether bond-forming reaction between cysteine and O-2-bromoethyl-tyrosine, introduced at judicious positions via amber stop-codon suppression methodology. [48] This "stapling" resulted in an increase of 18 °C in melting temperature and gave increased stability towards denaturants and organic co-solvents, which can be an advantage for synthetic applications.

Also for Mb, a systematic study of the role of the axial ligand was performed. The activity in cyclopropanation was evaluated for variants containing both proteinogenic, i.e. Cys, Ser, Asp. Tyr, as non-proteinogenic amino acids (3-(3′-pyridyl)-alanine, *p*-amino-phenylalanine, β-(3-thienyl)-alanine and *N*-methylhistidine (NMH) as axial ligand. [49,50] The latter category were introduced using stop codon suppression methodology, which allows for specific incorporation of non-canonical amino acids at the position genetically encoded by the amber stop codon.

In case of the proteinogenic amino acids, the present strategy proved effective for obtaining a Mb catalyst, the variant Mb(H64V,V68A,H93D) was found to be an efficient olefin cyclopropanation catalysts under non-reducing conditions. This was attributed to the ability of this Mb variant to form the ferrous form, which is required for carbenoid formation, through reaction with the diazo substrate, without requiring an external reducing agent.

Mb(H64V, V68A) variants containing a non-proteinogenic amino acid as axial ligand were found to be active in cyclopropanation reactions. [49–51] A variant containing NMH was shown to be as active as Mb(H64V, V68A) without requiring external reductant. [49] It was suggested that maybe in this case the Fe(III) species itself is active, albeit that needs further experimental support.

Interestingly, a crystal structure of the NMH variant with the carbene bound was obtained (Fig. 4). [49] Unexpectedly, the carbene proved to be bridged between the Fe centre a nitrogen from one of the pyrrole rings. While this has been proposed to be the first step in deactivation,

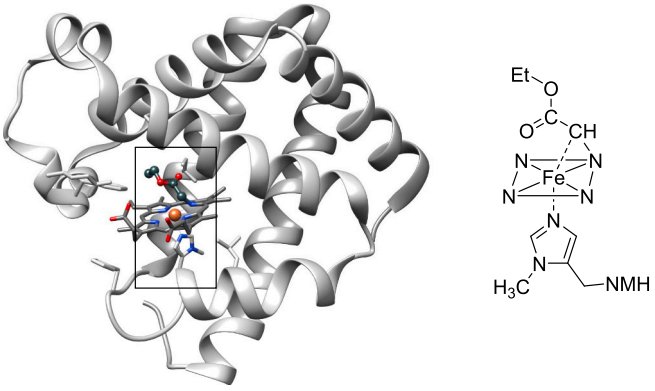

Fig. 4. Left: X-ray crystal structure of the Mb carbene adduct (PDB: 6G5b) with the heme carbene adduct in the box; right: schematic representation of the structure of the bridged carbene intermediate. NMH = *N*-methylhistidine.

this was not observed in this case. Indeed, based on quantum chemical calculations, the Fe-C-$N_{pyrrole}$ bridged species, which was proposed to be inert, was found to be in rapid equilibrium with the end-on carbene species. The latter species then is responsible for the cyclopropanation to occur.

2.1. Heme replacement

A key element that also can be considered for optimization is the heme cofactor, as this is the actual catalytic group. Above examples of repurposed enzymes all still rely on the natural heme cofactor. However, other metalloporphyrin dependent enzymes can be generated by removing the natural heme and reconstituting with a heme analogue. This methodology has found many applications in supramolecular chemistry, notably by the Hayashi group. [52,53] In recent years, this methodology has also been applied to generate novel cyclopropanation enzymes.

Fasan and coworkers reconstituted Mb(H64V, V68A) with a variety of metal protoporphyrin IX (ppIX) and mesoporphyrin IX (mpIX) complexes (Fig. 5). The reconstitution can be achieved in vitro, but also the enzymes could be assembled in vivo, using *E. coli* cells expressing the ChuA outermembrane transporter in combination with the chaperone complex GroEL/ES. While none of these outperformed the heme enzymes, some of these, such as the [Ir(Me)(mpIX)] enzyme did show activity even in presence of oxygen. [54] Interestingly, though, the enzyme created from Co-ppIX Mb(H64V, V68A, H93S), so with the axial histidine ligand replaced by serine, did show a strong preference for cyclopropanation over Y—H (Y = Si, N) insertion, which is opposite to the selectivity observed for iron and other metal containing Mb variants. [55]

In a follow-up study, Mb(H64V,V68A) was reconstituted with the iron-chlorin e6 cofactor. [56] The resulting enzyme proved highly active in the cyclopropanation, with total turnovers up to ~7000 and rates of >2000 min^{-1} and also gave rise to excellent enantio- and diastereoselectivities. The enzyme is active in the presence of oxygen. Interestingly, it was reported that the enzyme is active in both the ferrous and ferric forms.

The enzyme was functional in bacterial cells, allowing for whole-cell biocatalytic transformations. Interestingly, assembly in the cells occurred spontaneously, without requiring engineering of the cells, i.e. introducing heme transporters.

Oohora and Hayashi reconstituted Mb with iron porphycene (FePc). [57] This enzyme proved to have a 35-fold higher turnover frequency in the cyclopropanation of styrene with EDA than Mb. Focussing only on the reaction with EDA, then the MbFeFc enzyme was even >6oo times faster than Mb. This was further investigated by DFT calculations on the reaction of EDA with a heme-imidazole and an FePc-imidazole complex (Fig. 6), which suggested that this difference can be attributed to a difference in spin state of the resting states of Mb and MbFePc, which were found to be quintet and triplet, respectively. Since the metallocarbene species are proposed to be singlet, it is proposed that MbFePc is more efficient because it has to undergo only one intersystem crossing event, compared to Mb which requires two intersystem crossings.

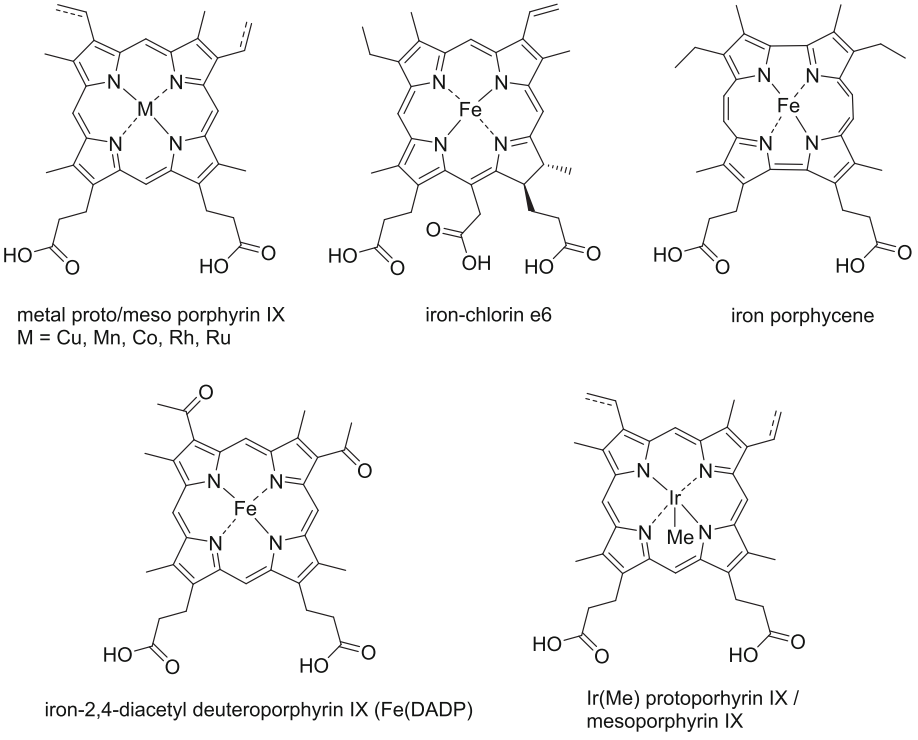

Fig. 5. Alternative porphyrin cofactors used in heme replacement.

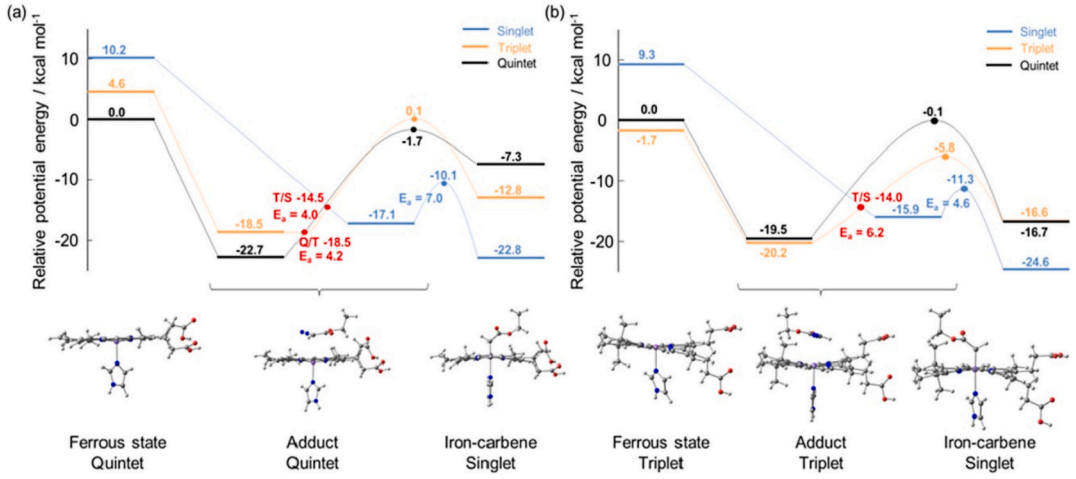

Fig. 6. Potential energy diagrams for the reactions of (a) the native heme-imidazole complex with EDA and (b) the FePc-imidazole complex with EDA. Belwo the diagrams are shown the optimized structures of the intermediates in different spin states at each step in the reaction. Reproduced with permission from Oohora et al. [57] Copyright 2017 American Chemical Society.

A combination of heme replacement with iron-2,4-diacetyl deuteroporphyrin IX (Fe(DADP) in combination with exchanging the axial histidine for the non-canonical amino acid *N*-methyl histidine resulted in an Mb-based enzyme that is active in cyclopropanation of both electron rich and electron poor alkenes. This is due to a switch in mechanism, from a concerted mechanism that is general for most iron heme enzymes to a radical mechanism. [58]

Hartwig and coworkers devised an expression protocol that allowed for production of apo myoglobin. Inserting a range of metallo mesoporhyrin IX complexes in vitro and testing in catalysis resulted in the discovery of the Ir –heme as the most active in cyclopropanation. This metal complex does not require axial ligation since it carries an axial methyl group. Hence, the axial histidine was mutated to alanine or glycine. Evolved variants of these enzymes even proved to have some activity on internal alkenes, which are challenging substrates for cyclopropanation. [59]

Replacing heme in the P450 enzyme CYP(119) gave rise to even more active enzymes, capable of cyclopropanation of a wide range of structurally diverse alkenes, including internal alkenes, with high activities and selectivities. [60]

3. Artificial heme enzymes

Heme enzymes have been the subject of rational design approaches for many years. [61–64] One of these approaches involves de novo designed tetrahelical proteins called “maquettes”. The Anderson group reported the design of “C45”, a thermostable maquette protein that could be assembled in vivo. It contains a covalently linked heme C which is uses a histidine residue as the axial ligand. [65]

C45 proved an active cylopropanation enzyme, giving high turnover numbers. [66] In addition, moderate to good enantioselectivities were reported. Stopped flow experiments were used to generate and spectroscopically study the metallocarbenoid intermediate.

Roelfes and coworkers created an artificial heme enzyme by binding of hemin to the lactococcal multidrug resistance regulator (LmrR). This protein, which has no natural catalytic function, [67] has proven a versatile scaffold for the creation of artificial metalloenzymes. [68] Among the approaches that have been used supramolecular binding of metal cofactors in the hydrophobic pore at the protein dimer interface has proven particularly successful. [69] It was shown that LmrR has nanomolar affinity for hemin. [70] The artificial heme enzyme proved active in cyclopropanation of styrene, giving hundreds of turnovers but a low ee of the product. By introducing a M8A mutation, in the centre of the pore, the activity and enantioselectivity was increased, up to 51% ee was obtained. X-ray crystallography showed the heme group the be located in the hydrophobic pocket, sandwiched between the two tryptophan moieties that are key to LmrR’s ability to bind guest molecules (Fig. 7). The planes of the indole moieties are right above and below the iron centre of the heme and, consequently, no axial ligand was bound to the heme iron. This is an unusual arrangement for a heme protein and the x-ray structure suggested there to be no room for substrates to approach the catalytic iron centre. This was puzzling in view of the observed catalytic activity. Hence, a molecular dynamics (MD) study was performed, which showed that significant amounts of time during the simulation, the structure opens up by movement of the front α-helix, accompanied by a move of one of the tryptophans towards the solvent exterior. This creates a pocket distal to the iron centre of the heme that is large enough to accommodate the substrates and even the transition state of the reaction, as calculated with DFT. This result emphasized the importance of structural dynamics in designed enzymes.

4. Artificial heme enzymes based on DNA

A priori, there is no reason why an enzyme has to be constructed from a protein. Any biomolecular scaffold that can contribute to binding and activation of substrates through second coordination sphere interactions is in principle suitable for creation of artificial (metallo-)enzymes. [71,72]

Our group and others have shown that double stranded DNA is an attractive scaffold for bio-inspired catalysis. [73–75] We have found that salmon testes DNA in combination with cationic iron porphyrins could give rise to cyclopropanation of styrene. [76] The best catalysts were iron meso-tetrakis(N-alkylpyridyl)porphyrins, in particular with the *N*-methyl substituents at the ortho position with respect to the porphyrin ring (Fe-2TMepyP, Fig. 8). Tens of turnovers and a moderate ee up to 53% was achieved. Notable features were that no requiring external reducing agents were required to reduce the iron(III) centre, while the reactions could be performed under ambient atmosphere, i.e. in the presence of oxygen. This could suggest that also in this case the reaction does not involve an iron(II) active species, albeit that more research is required to establish this. Another notable observation was that the reaction in the presence of DNA was much faster than the reaction with the iron porphyrin alone. This DNA accelerating effect was especially observed for the metal carbenoid formation step and was attributed to effective molarity effects as a result of local concentration of the reagents in hydrophobic cavities generated between the DNA and the bound iron porphyrin.

In addition to double stranded DNA, also other DNA architectures

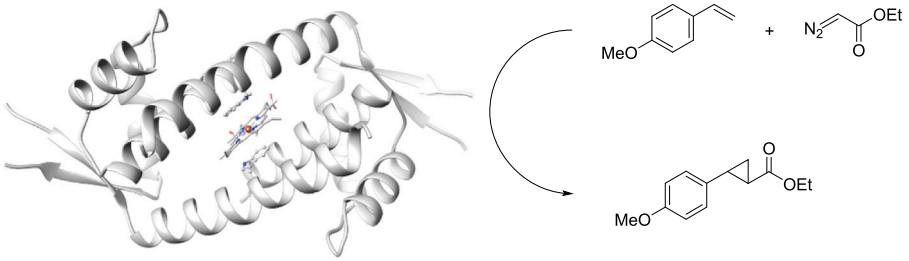

Fig. 7. X-ray crystal structure of heme bound to LmrR (PDB: 6FUU) and catalysed cyclopropanation reaction.

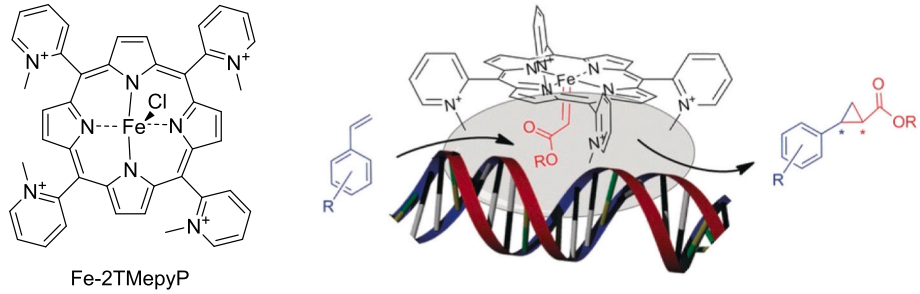

Fig. 8. Structure of iron porphyrin used and DNA-based catalytic cyclopropanation. Adapted with permission from Rioz-Martínez et al. [76] Copyright 2016 John Wiley and Sons, Inc.

such as G-quadruplexes have found application in DNA-based catalysis. [77] Notably, heme DNAzymes, which feature a heme bound on top of a DNA G-quadruplex structure, have found application is many field because of its peroxidase activity. Sen and co-workers used these heme dependent structures for cyclopropanation of styrene, showing good activity but low enantioselectivity. [78] All catalysts investigated contain an overhang of at least one nucleotide, which may contribute to binding the substrates, but is insufficient to provide a chiral architecture that will give rise to enantioselectivity. This DNAzyme was most efficient when using sodium dithionite as reductant, under inert atmosphere. Low, but significant, levels of activity were observed without dithionite, but the presence of oxygen was detrimental. Switching from heme to iron meso-tetrakis(N-alkylpyridyl)porphyrins (FeTMPyP4) did not give rise to good results in catalysis in this case.

In contrast, Li and co-workers reported that using another G-quadruplex structure, derived from the well-known thrombin aptamer in combination with FeTMPyP4, efficient cyclopropanation was observed (Fig. 9). [79] By introducing judicious mutations, two quadruplex structures, mG7A9C and TmC4T, that were capable of 400–500 turnovers and ~ 80% ee were obtained. Interestingly, these two heme-DNA G-quadruplex structures proved enantiocomplementary: the opposite enantiomers of the cyclopropanation product were obtained. A spectroscopic study showed that the Fe(III) centre in the FeTMPyP4 is reduced to its Fe(II) form and it was proposed that this is the active form.

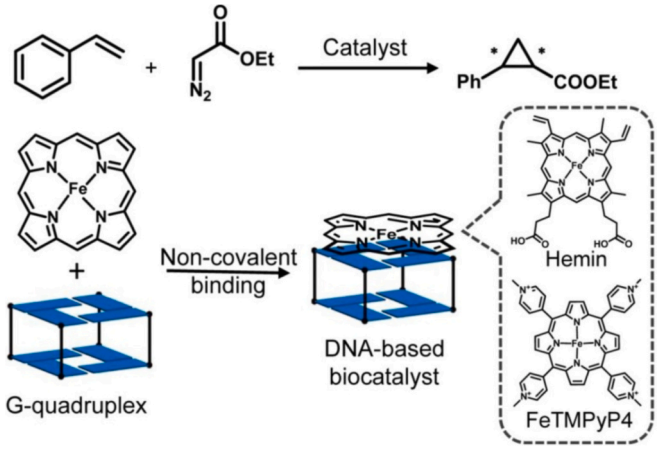

Fig. 9. DNA G-quadruplex / iron porphyrin catalysed cyclopropanation. Adapted with permission from Hao et al. [79]. Copyright 2020 American Chemical Society.

5. General considerations for the design of artificial heme enzymes for cyclopropanation

With the large diversity of repurposed and artificial heme enzymes that are active in cyclopropanation reactions, as described above, it is worthwhile to try to discern design trends that may help in future enzyme design undertakings. Zhang and Fasan reported a combined computational and experimental study that helps in understanding and rationalizing the results observed with the different designs, even though it only investigated a small subset of catalysts. [33]

The stated inspiration for the early studies towards using heme enzymes for cyclopropanation reactions was the analogy between iron carbene species with the iron oxene active species in P450 catalysed oxidations (Fig. 10). However, a consensus has emerged that in case of P450BM3 and Mb and many other cases, the iron(II) resonance form of the metal carbenoid is the actual active species (Fig. 11). [33] Additionally, an iron(III) radical based pathway, analogous to that proposed for cobalt-porphyrins, was ruled out for these heme enzymes based on the combination of computational and experimental results. The excellent stereoselectivity (cis/trans) in the cyclopropanation was considered

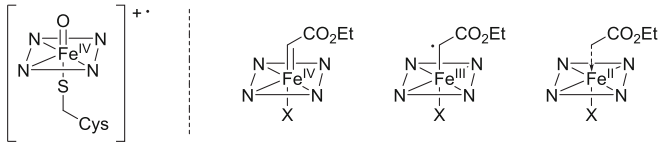

Fig. 10. Comparison of the iron oxene intermediate proposed for P450 catalysed oxidations with possible structures of the iron porphyrin carbene intermediate.

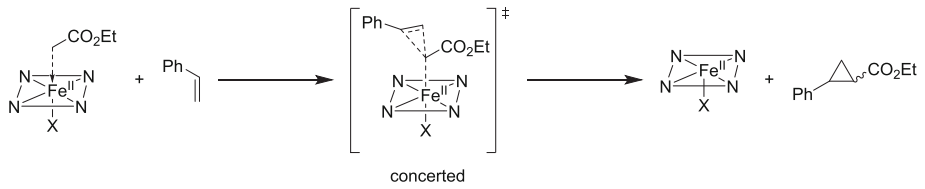

Fig. 11. Proposed concerted mechanism for P450 and Mb catalysed cyclopropanation of styrene.

to be in agreement with a concerted non-radical mechanism.

Another notable general observation is the important role of the axial ligand. In the initial P450 studies, the mutation of the axial cysteine ligand to a serine was the key to creating an active cyclopropanation catalysts. These P411 enzymes proved successful in a wide variety of cyclopropanation reactions and other carbene transfer reactions. Histidine as axial ligand seems to be even more preferred, as especially illustrated by the success of the myoglobin-based catalysts. This was confirmed by the computational study, which showed that of the proteinogenic amino acids, histidine is the best activator. The imidazole ligand was proposed to have a double, synergistic effect in that it reduces the activation energy towards both the carbene formation and actual cyclopropanation step. The latter appears to be related to the increased electrophilicity of the carbene carbon compared to thiolate- or non-ligated heme catalysts. Other axial ligands do give rise to similar effects, but to a significantly smaller extent. Also in agreement with this is that cyclopropanation enzymes that do not provide an axial ligand for the iron centre are generally much less active than those who do. Finally, non-proteinogenic amino acids as axial ligand also offer interesting further possibilities to modulate reactivity of these heme enzymes, as was in particular demonstrated in Mb containing NMH as axial ligand.

Similarly, changes to the porphyrin have an effect. When more electron withdrawing groups are present, also the electrophilicity of the carbene is increased which is reflected in an increased reactivity, as was shown also in the heme replacement studies.

Moreover, both the axial ligand and the porphyrin structure influence the reduction potential of the iron centre, determining whether external reductants are required. For example, the serine axial ligand in the P411 enzymes made application in cells possible because it increased the reduction potential, allowing for reduction of the iron(III) centre to the catalytically active iron(II) state by NADH instead of sodium dithionite.

This can also give rise to a change in mechanism. For some of the cyclopropanation enzymes described above, e.g. the Mb with NMH as axial ligand or iron chlorin e6 instead of heme as cofactor and the double stranded DNA based artificial heme enzyme using an iron meso-tetrakis (N-alkylpyridyl)porphyrin cofactor, iron(III)-based pathways have been suggested, because they do not require addition of external reductant and are stable towards oxygen. Indeed, using a combination of a more electron poor porphyrin ligand and NMH as axial ligand, as is the case for Mb(H64V,V68A,H93NMH)[Fe(DADP)], it was demonstrated that the reaction proceeds via a radical mechanism (Fig. 12).

Finally, a key feature of enzymatic catalysis is of course the structure of the active site. Comparison of the various heme enzymes shows that a hydrophobic pocket of sufficient size, distal to the iron porphyrin, is key to achieving efficient catalysis. This suggests that, in addition to the electronic effects exerted by porphyrin and axial ligand, effective molarity effects are an important contributor to the observed rate accelerations in cyclopropanation catalysis. Indeed, also when using iron porphyrins in combination with micelles, significantly enhanced cyclopropanation activity is obtained, underlining the importance of hydrophobic effects. [80] This could explain why systems which do not have precisely shaped active sites, such as for example the DNA based catalysts, still can achieve significant rate accelerations compared to the iron-porphyrin alone. Engineering the pocket allows for tailoring the enzyme for specific transformations and achieving high selectivities.

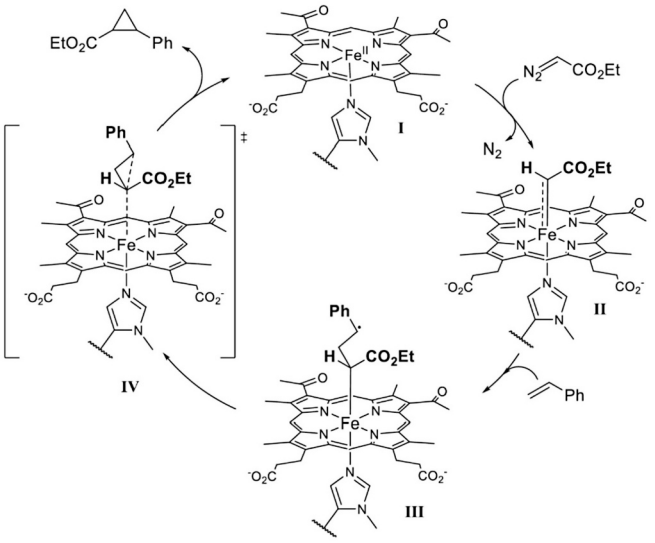

Fig. 12. Radical based mechanism for cyclopropanation of styrene catalysed by Mb(H64V,V68A,H93NMH)[Fe(DADP)]. Reproduced with permission from Carminati et al. [58] Copyright 2019 American Chemical Society.

The optimized pockets are usually rich in apolar hydrophobic residues, suggesting that London dispersion forces and shape complementarity are of key importance. Noteworthy is that many of the mutations that are beneficial for cyclopropanation actually are detrimental to the natural activity, i.e. oxidation. This shows that even though the initial studies were based on the perceived similarity of the reactive intermediates involved, actually the species and mechanisms involved are quite different and have different requirements with respect to the protein environment.

6. Conclusions

The combined work in the past years has shown the power of repurposed and artificial heme enzymes. Especially repurposed enzymes are quickly becoming valuable addition to the synthetic chemistry toolbox, expanding from simple model reactions such as cyclopropanation of styrene to more economically important targets and transformation that are very difficult to achieve using small molecule catalysts. Moreover, developments have not stopped with cyclopropanation, but heme catalysis has been expanded to wide variety of other important carbene transfer reactions.

In comparison, the artificial enzymes that were not derived from natural heme proteins, such as the LmrR and DNA-based artificial enzymes, to date are relatively modest catalysts. This may be because the important parameters for activity and selectivity, that are axial ligand and well-defined structure of the active site, are not yet optimal. But the construction of these artificial enzymes holds important lessons with regard to enzyme design and, because of the design freedom, may unlock novel chemistry.

Combined, these repurposed and artificial heme enzymes illustrate how the catalytic power of the heme and related iron porphyrin

cofactors can be harnessed to achieve novel biocatalysis. Hence, they may be a key component of the transition to a more green and sustainable approach to chemical synthesis.

Author statement

Gerard Roelfes researched the literature and wrote the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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