Biocatalysis

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# Highly Diastereoselective and Enantioselective Olefin Cyclopropanation Using Engineered Myoglobin-Based Catalysts**

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***Abstract:*** *Using rational design, an engineered myoglobin-based catalyst capable of catalyzing the cyclopropanation of aryl-substituted olefins with catalytic proficiency (up to 46800 turnovers) and excellent diastereo- and enantioselectivity (98–99.9%) was developed. This transformation could be carried out in the presence of up to 20 g $L^{-1}$ olefin substrate with no loss in diastereo- and/or enantioselectivity. Mutagenesis and mechanistic studies support a cyclopropanation mechanism mediated by an electrophilic, heme-bound carbene species and a model is provided to rationalize the stereopreference of the protein catalyst. This work shows that myoglobin constitutes a promising and robust scaffold for the development of biocatalysts with carbene-transfer reactivity.*

Expanding the scope of engineered and artificial biocatalysts beyond the realm of chemical transformations catalyzed by natural enzymes lies at the forefront of the biocatalysis field.[1] Olefin cyclopropanation is a particularly valuable transformation owing to the occurrence of cyclopropyl moieties in many bioactive natural and synthetic compounds. Furthermore, cyclopropanes constitute versatile intermediates for a variety of synthetically useful ring-opening transformations.[2] A well-established chemical approach to olefin cyclopropanation involves transition-metal-catalyzed decomposition of diazo reagents followed by metallocarbenoid insertion into C=C bonds.[3] A wide range of transition-metal complexes have demonstrated utility in this respect, with the use of chiral ligands enabling these reactions to proceed in an asymmetric manner.[3] Despite this progress, achieving high levels of both diastereo- and enantioselectivity, also in combination with high catalytic activity, has remained a significant challenge in these processes, particularly in the context of intermolecular cyclopropanation reactions in the presence of acceptor-only carbene donors.[4]

Pioneering studies by Callot, Kodadek, and Woo demonstrated the ability of metalloporphyrins to promote olefin cyclopropanation in the presence of diazoacetates.[5] More recently, Arnold and co-workers reported that a similar reactivity is exhibited by $P450_{BM3}$, with engineered variants of this P450 enzyme catalyzing the cyclopropanation of styrene in the presence of ethyl diazoacetate (EDA) with good *Z* diastereoselectivity (up to 84% *de*) and good to high enantioselectivity (90–99% $ee_Z$).[1f,6] Our group recently discovered that, along with other heme-containing proteins, myoglobin is able to activate arylsulfonyl azides in intramolecular nitrene C–H insertion reactions,[1g,7] suggesting that this hemoprotein could also be useful for promoting mechanistically related carbene-transfer processes. Herein we report the rational design of engineered myoglobin-based catalysts which can support the cyclopropanation of a variety of aryl-substituted olefins with catalytic proficiency as well as excellent *E* diasteroselectivity and enantioselectivity.

The oxygen-binding metalloprotein myoglobin contains a heme (iron-protoporphyrin IX) cofactor coordinated at the proximal side through a histidine residue. Because of its small size (17 kDa) and robustness toward mutagenesis and other structural modifications,[8] we selected this protein as a potentially promising scaffold for developing biocatalysts to promote non-native transformations such as nitrene-[1g,7] and carbene-transfer reactions. In initial studies, we tested the ability of sperm whale myoglobin (Mb) to catalyze the cyclopropanation of styrene (**1a**) in the presence of EDA (**2**) as the carbene source. Under reducing and anaerobic conditions, Mb was found to effectively promote this reaction, supporting about 180 turnovers and leading to (*E*)-ethyl 2-phenylcyclopropanecarboxylate (**3a** and **3b**) as the major products (86% *de*; Table 1). Notably, this cyclopropanation activity compares well with that reported for the $P450_{BM3}$-based variants in vitro (200–360 total turnovers)[1f] under similar reactions conditions (0.02 mol% protein, 3:1 styrene/EDA), while exhibiting complementary diastereoselectivity. Despite its promising activity, wild-type Mb showed no asymmetric induction in the cyclopropanation reaction, thus leading to a racemic mixture for both the *Z* and *E* product as observed for free hemin (Table 1).

Control experiments showed that the absence of reductant (dithionite) or the presence of air resulted in no cyclopropanation product, thus indicating that ferrous myoglobin is the catalytically active species and that $O_2$ is deleterious to this reactivity, likely because of the competition with the diazo reagent for binding to the heme. Based on these results and previous studies with metalloporphyrin catalysts,[4d,5b,c,9] we hypothesized the Mb-catalyzed cyclopropanation reaction to involve a heme-bound carbene intermediate formed upon reaction of EDA with the protein in its reduced, ferrous state (Figure 1b). End-on[4d,5c,9b,10] attack of the styrene molecule to this heme-carbenoid species would then lead to the cyclopropanation product. While the *E* selectivity of the Mb-catalyzed reaction clearly indicated

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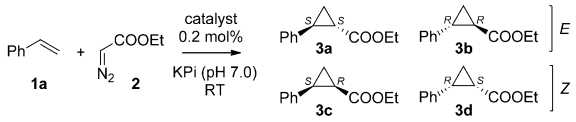
***Table 1:*** Activity and selectivity of wild-type myglobin and its variants toward styrene cyclopropanation with ethyl diazoacetate.[a]




| Catalyst | Conv. [%][b] | TON | $de_E$ [%] | $ee_E$ [%][c] | $ee_Z$ [%][c] |
|---|---|---|---|---|---|
| Hemin | 29 | 145 | 74 | 0 | 0 |
| Mb | 36 | 180 | 86 | 6 | 0 |
| Mb(L29A) | 38 | 190 | 82 | −1 | 1 |
| Mb(H64V) | 73 | 365 | 92 | 2 | −1 |
| Mb(V68A) | 56 | 280 | 96 | 68 | −1 |
| Mb(V68F) | 52 | 260 | 98 | >99.9 | 26 |
| Mb(F43V) | 41 | 205 | 88 | 44 | 15 |
| Mb(F43W) | 45 | 225 | 88 | 34 | 3 |
| Mb(F43V,V68A) | 91 | 455 | 36 | 67 | 71 |
| Mb(F43V,V68F) | >99 | 500 | 78 | >99.9 | 13 |
| Mb(H64V,V68A) | >99 | 500 | 99.9 | >99.9 | −6 |
| Mb(H64V,V68A) | >99[d] | 10000[d] | 99.9 | 99.9 | −6 |
| Mb(H64V,V68A) | 47[e] | 46800[e] | 99.9 | 99.9 | −6 |

[a] Reactions conditions: 20 μM Mb (or hemin), 30 mM styrene, 10 mM EDA, 10 mM dithionite, 16 h. [b] The conversion is based on GC analysis and relative to the limiting reagent. [c] *trans*=1*S*,2*S* and *cis*=1*R*,2*S* as determined by GC analysis using a chiral stationary phase. [d] Reaction conditions: 20 μM protein, 0.2 M styrene, 0.4 M EDA, 10 mM dithionite, 1 h. [e] Reaction conditions: 2 μM protein, 0.2 M styrene, 0.4 M EDA, 10 mM dithionite, 16 h.

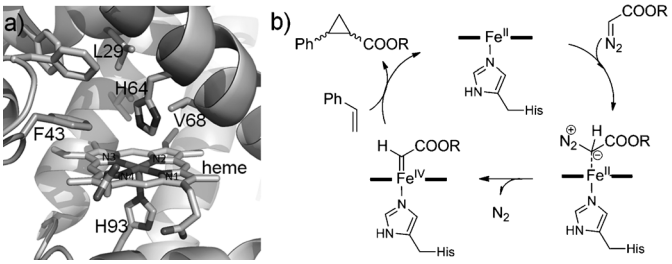



***Figure 1.*** a) Active site of sperm whale myoglobin (pdb 1A6K). The residues targeted for mutagenesis are Phe43, His64, and Val68. b) Proposed mechanism for myoglobin-catalyzed styrene cyclopropanation with diazo esters.

that a *trans* heme-carbene/styrene arrangement is preferred, the lack of enantioselectivity also suggested that the native Mb scaffold is unable to dictate facial selectivity for the styrene approach to the heme-carbene intermediate. Accordingly, we reasoned that mutation of the amino-acid residues lying at the periphery of the porphyrin cofactor could provide a means to improve the diastereo- and enantioselectivity of this catalyst, possibly by imposing only one modality of attack of the styrene molecule on the heme-carbene group.

Upon inspection of the sperm whale Mb crystal structure,[11] residues Phe43, His64, and Val68, were selected as promising targets for mutagenesis because of their close proximity to the distal face of the heme (Figure 1a). Specifically, a Mb variant where the distal histidine (His64) is mutated to Val, Mb(H64V), was considered as this mutation was previously found to increase the C–H amination activity of this protein on bulky arylsulfonyl azides.[7] On the other hand, positions 43 or 68 were substituted with amino acids carrying a larger [i.e., Mb(F43W), Mb(V68F)] or smaller apolar side chain [i.e., Mb(F43V), Mb(V68A)], in an attempt to affect the catalyst selectivity in the cyclopropanation reaction by varying the steric bulk on either side of the heme (Figure 1a). Finally, a Mb(L29A) variant, which contains a mutation at a remote position not expected to directly interact with the heme-bound carbene during catalysis, was used as a negative control.

Analysis of these Mb variants revealed an important effect of the active-site mutations on the activity and/or selectivity of the hemoprotein toward styrene cyclopropanation with EDA (Table 1). In particular, the H64V mutation resulted in a twofold increase in the turnover number (TON), the highest among this set of single mutants, while having marginal effect on diastereo- and enantioselectivity. Conversely, all the mutations at the level of Phe43 and Val68 dramatically improved the enantioselectivity of the Mb variant as compared to wild-type Mb, thus resulting in formation of the 1*S*,2*S*-stereoisomer **3a** with $ee_E$ values ranging from 44 to 99.9%. The V68 substitutions also resulted in an appreciable increase in both catalytic activity (TON) and *E* diastereoselectivity. In contrast, the L29A mutation had essentially no effect on either the cyclopropanation activity or diastero- and enantioselectivity of the protein. Thus, in accord with our design strategy, the H64V mutation was particularly effective in enhancing Mb-dependent cyclopropanation activity, whereas the mutations at the level of V68 and F43 were beneficial toward tuning its diastereo- and enantioselectivity. To combine the beneficial effects of these mutations, a series of Mb double mutants were prepared (Table 1). Gratifyingly, variant Mb(H64V,V68A) was found to exhibit high activity as well as excellent *E* diastereoselectivity (>99.9%) and 1*S*,2*S* enantioselectivity (>99.9%), and it was thus selected for further investigations.

Mb(H64V,V68A)-catalyzed cyclopropanation was determined to follow Michaelis–Menten kinetics, with estimated $K_M$ values of about 2 mM and 5 mM for styrene and EDA, respectively (see Figure S1 in the Supporting Information). To further optimize this transformation, the impact of the olefin/diazoester ratio on the efficiency of the reaction was first examined. These experiments revealed an increase in TON as the EDA to styrene ratio was raised from 1:5 to 6:1, with significant amounts (37% of total products) of dimerization byproducts (diethyl maleate and fumarate) accumulating only in the presence of a large (sixfold) excess of the diazo compound (see Figure S2). Overall, a twofold excess of EDA over styrene was found to be optimal for maximizing cyclopropanation turnovers while minimizing dimerization (<1% of total products). Notably, by using this reagent ratio and a catalyst loading of 0.01 mol%, quantitative conversion of the olefin could be achieved in the presence of up to 0.2 M styrene (20 $g\,L^{-1}$) within one hour (see Table S2). Furthermore, despite the reaction being biphasic at this reagent concentration, excellent levels of diastereo- and enantioselectivity (99.9% *ee*, 99.9% *de*) were maintained, thus indicating that the Mb variant is stable under these reaction conditions (release of hemin from the protein would indeed lead to racemization). Quantitative conversion of the olefin in

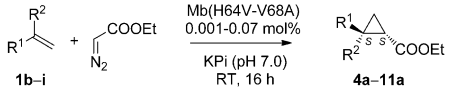
  

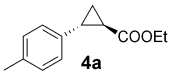
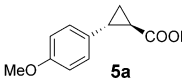
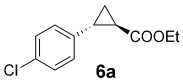
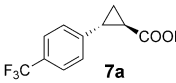
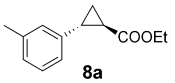
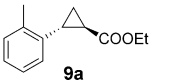
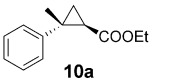
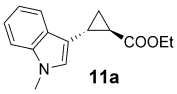
these reactions also suggested that the TONs supported by the Mb catalyst are in excess of 10000. To examine this aspect, reactions at high substrate loading (0.2 M styrene, 0.4 M EDA) were repeated using decreasing amounts of the hemoprotein (20 to 1 μM; see Table S2). At a catalyst loading of 0.001 mol%, Mb(H64V,V68A) was found to support about 30000 turnovers after 1 hour and over 46000 total turnovers after overnight incubation with styrene and EDA (Table 1). Notably, high total turnover numbers (TTNs) were maintained using stoichiometric amounts of reductant relative to the Mb catalyst (TTN = 10600). Time-course experiments also revealed that Mb(H64V,V68A)-catalyzed cyclopropanation proceeds very rapidly, with an initial rate of 1000 turnovers $min^{-1}$ over the first 10 minutes and an average rate of 500 turnovers $min^{-1}$ over the first hour of the reaction. Overall, the catalytic efficiency of this engineered Mb rivals that of some of the most active transition-metal catalysts reported to date for similar transformations (TTN of 11–98000),[4d,12] while offering greater diastereo- and stereocontrol (compared to 75–94% *de* and 83–98% *ee*).[4d,12] Furthermore, unlike the latter, slow addition of the diazo reagent was not required in the Mb-catalyzed reactions to minimize the undesired dimerization reaction.

To examine the substrate scope of the Mb variant, a variety of styrene derivatives and other olefin substrates were subjected to Mb(H64V,V68A)-catalyzed cyclopropanation in the presence of EDA. Using a catalyst loading of 0.07 mol%, efficient cyclopropanation of *para*- (**1b–e**), *meta*- (**1f**), and *ortho*-substituted (**1g**) styrenes could be achieved with yields ranging from 69 to 92% (Table 2). Importantly, excellent levels of *E* diastereoselectivity and, when measurable, of 1*S*,2*S* enantioselectivity were observed in each case, thus highlighting the broad scope of the Mb-based catalyst in terms of activity and selectivity across the substituted styrene derivatives. At lower catalyst loadings (0.001 mol%), Mb(H64V,V68A) was found to support TTNs ranging from 7760 to 14500 on these substrates. Analysis of reactions with α-methylstyrene (**1h**) and *trans*-β-methylstyrene showed efficient and highly diastereo- and enantioselective cyclopropanation only in the case of the former, thus suggesting that β substitutions on the alkene group are not tolerated by the Mb catalyst. Among alternative alkene substrates, *N*-methyl-3-vinyl-indole (**1i**) could be converted into the corresponding cyclopropanation product with high selectivity, although the efficiency of this reaction was compromised by the instability of this substrate in water. In contrast, no appreciable cyclopropanation activity was observed in the presence of 1-hexene or *trans*-penta-1,3-diene, thus evidencing the chemoselective reactivity of the Mb-based catalyst toward aryl-substituted olefins versus aliphatic ones.

To gain insights into the mechanism of the Mb-catalyzed cyclopropanation, the relative rates (i.e., $k_X/k_H$ ratios) for cyclopropanation of *para*-substituted styrenes (*p*-$XC_6H_4CH{=}CH_2$, **1b–e**) versus styrene were estimated from competition experiments in the presence of Mb(H64V,V68A) as the catalyst and methanol (20%) as a cosolvent. Electron-donating substituents were found to accelerate the cyclopropanation reaction, while electron-withdrawing substituents lead to reduced rates, a phenomenon consistent with

***Table 2:*** Substrate scope for Mb(H64V,V68A)-catalyzed cyclopropanation.




| Substrate | Product | Conv. [%] (TON)[a] | TTN[b] | *de* [%] | *ee* [%] |
|---|---|---|---|---|---|
| **1b** |  **4a** | 77 (1150) | 7760 | 99.6 | n.a.[c] |
| **1c** |  **5a** | 89 (1330) | 11670 | 99.9 | n.a.[c] |
| **1d** |  **6a** | 92 (1380) | 12280 | 99.8 | 99.9 |
| **1e** |  **7a** | 69 (1035) | 8660 | 99.9 | 99.9 |
| **1f** |  **8a** | 73 (1095) | 14500 | 99.8 | 99.9 |
| **1g** |  **9a** | 85 (1275) | 11700 | 99.4 | 99.9 |
| **1h** |  **10a** | 86 (1290) | 8510 | 97.2 | 96 |
| **1i** |  **11a** | 10 (150) | 1790 | 97.4 | n.a.[c] |

[a] Reactions conditions: 20 μM Mb, 30 mM styrene, 60 mM EDA. Yield is based on GC conversion. [b] Reactions conditions: 2 μM Mb, 200 mM styrene, 400 mM EDA. [c] Enantiomers could not be resolved. n.a. = not available.

cyclopropanations operated by electrophilic metal carbene intermediates.[4d,5c,9b,12a,13] Furthermore, a plot of the $\log(k_X/k_H)$ values against the Hammett constants $\sigma^+$ for the corresponding *para* substituents yielded a reasonably good ($R^2 = 0.79$) linear correlation with a small negative $\rho^+$ of $-0.34 \pm 0.07$ (see Figure S3). This value is comparable to that measured for similar reactions with iron porphyrin catalysts ($\rho^+ = -0.41$[9b]) and it is suggestive of a partial positive charge build-up at the benzylic carbon atom in the transition state. Unlike the latter, however, no significant secondary isotope effect [$k_H/k_D = 0.96 \pm 0.02$ (see Figure S4) compared to 0.87][5c] was observed for the Mb(H64V,V68A)-catalyzed cyclopropanation of styrene versus [$D_8$]styrene. Thus, while these results point at subtle mechanistic differences between the two systems, the Hammett analyses support a general mechanism for the Mb-catalyzed cyclopropanation involving an electrophilic heme-carbene species analogous to that proposed for iron porphyrin catalysts.[5c,9b]

To rationalize the activity and selectivity enhancements brought about by the mutations in Mb(H64V,V68A), a model

of this protein was generated based on the available structure of the closely related Mb(H64V) variant.[14] Inspection of the model revealed a wider opening leading the distal cavity because of the H64V mutation (see Figure S5 a,b), which is likely to increase the accessibility of the heme center to the diazoester and olefin substrate. The V68A mutation, on the other hand, expands the size of the distal cavity above the nitrogen atom N2 of the heme group (Figure S5 c,d). In the crystal structure of Fe-(porphyrin)-carbene complexes,[9b] the carbene moiety is roughly aligned (15–20° deviation) with the diagonal N–Fe–N bonds of the porphyrin ring. Assuming a similar geometry is adopted by the heme-bound carbene, four orientations of this group are possible, that is, two projecting the ester moiety toward the protein core (i.e., above heme atoms N2 or N3; Figure 1) and two projecting it toward the solvent-exposed face of the heme cofactor (i.e., above heme atoms N1 or N4). Among the possible arrangements for an end-on attack of styrene to this intermediate (see Figure S6), the one featuring the carbene ester group above heme N2 and the styrene phenyl group extending toward the solvent (geometry **A**, Figure 2; see Figure S6) is consistent with the experimentally observed *E* and 1*S*,2*S* selectivity of the catalyst and appears sterically feasible. While leading to the same cyclopropane stereoisomer, geometry **B** (Figure 2 and S6) imposes steric clashes between the styrene ring and Phe43. Thus, the V68A mutation could favor **A** by better accommodating the carbene ester group in proximity of N2, thereby explaining the dramatic effect of this mutation toward improving 1*S*,2*S* enantioselectivity (6→99.9% *ee*). This scenario would also explain the remarkable tolerance of Mb(H64V,V68A)-induced selectivity to variations on the aryl group of the olefin (which is solvent-exposed in **A**) but not at the β-position, because of steric clashes between the β substituent and the carbene ester group and/or heme porphyrin ring. Another implication of this model is that an increase in steric bulk at the level of the alkyl ester group or of the α-carbon atom in the diazo reagent is expected to cause a decrease in diastereo- and enantioselectivity, as these changes would disfavor **A** over other geometries (Figure S6). In agreement with these predictions, Mb(H64V,V68A)-catalyzed styrene cyclopropanation with *tert*-butyl diazoacetate (**12**) or ethyl diazopropanoate (**13**) yielded the corresponding 1*S*,2*S* cyclopropane products (**14a, 15a**) with lower diastereoselectivity (82% *de* and 74% *de*, respectively) and drastically reduced enantioselectivity (58% *ee* and 1% *ee*, respectively). Thus, while further studies are clearly necessary to fully substantiate it, the proposed model can justify the stereochemical outcome of the Mb(H64V,V68A)-catalyzed cyclopropanation reactions and qualitatively predict the effect of structural modifications at the level of the diazo reagent.

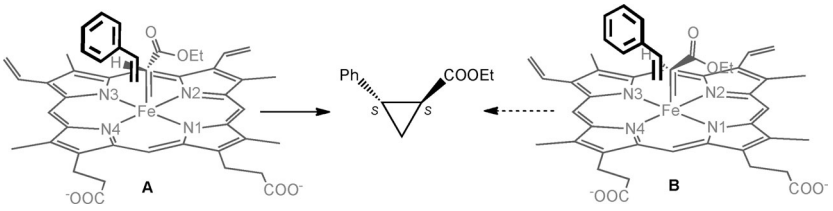



***Figure 2.*** Proposed geometries for styrene approach to the putative heme-carbene complex leading to the (1*S*,2*S*)-ethyl 2-phenylcyclopropanecarboxylate stereoisomer. The orientation of the heme rings is the same as in Figure 1.

In summary, this work demonstrates that myoglobin constitutes a versatile and robust scaffold for the development of highly active and selective olefin cyclopropanation catalysts. By rational design, an engineered Mb variant capable of catalyzing the cyclopropanation of a variety of aryl-substituted olefins with an unprecedented combination of catalytic proficiency (10–46800 TON) and excellent *E* diastereo- and enantioselectivity (>99%) was obtained. The practical utility of this biocatalyst is further highlighted by its ability to operate at high reagent concentration (i.e., 0.2–0.4 M) and in presence of organic cosolvents (e.g., 20% MeOH). Mutagenesis and Hammett analyses support the intermediacy of an heme-bound electrophilic carbene species in these reactions, analogous, albeit not identical, to that operating in cyclopropanation reactions catalyzed by iron porphyrins in organic solvents. Importantly, the much greater reactivity and selectivity offered by the Mb catalyst as compared to free hemin highlights the key role of the protein matrix in modulating the catalytic efficiency and stereochemical outcome of the reaction. Finally, a model was presented for rationalizing the selectivity of the Mb-based catalyst which could be useful for further tuning this scaffold in order to access other cyclopropane stereoisomers. Based on the present results, we anticipate that myoglobin-derived catalysts can prove useful for a variety of other synthetically valuable carbene-transfer reactions.

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**Keywords:** biocatalysis · carbenes · iron · protein engineering · small-ring systems

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