



Strategies for the expression and characterization of artificial myoglobin-based carbene transferases

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Abstract

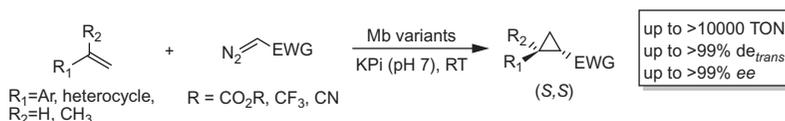
Myoglobin has recently emerged as a versatile metalloprotein scaffold for the design of efficient and selective biocatalysts for abiological carbene transfer reactions, including asymmetric cyclopropanation reactions. Over the past few years, our group has explored several strategies to modulate the carbene transfer reactivity of myoglobin-based catalysts, including the substitution of the native heme cofactor and conserved histidine axial ligand with non-native porphyrinoid ligands and alternative natural and unnatural amino acids as the metal-coordinating ligands, respectively. Herein, we report protocols for the generation and reconstitution *in vitro* and *in vivo* of myoglobin-based artificial carbene transferases incorporating non-native iron-porphyrinoid cofactors, also in combination with unnatural amino acids as the proximal ligand. These strategies are effective for imparting these myoglobin-based cyclopropanation biocatalysts with altered and improved function, including tolerance to aerobic conditions and improved reactivity toward electrondeficient olefins.



1. Introduction

Cyclopropanes are key structural motifs present in many pharmacologically active natural products and drug molecules (Chen, Powner, & Richard, 2012; Talele, 2016). The cyclopropane ring provides a rigid carbocycle which can contain up to three stereogenic centers and thus give rise to eight possible stereoisomers. Because of the unique conformational properties of cyclopropane ring and well-defined spatial orientation of substituents appended to it, the incorporation of cyclopropane rings into bioactive molecules can contribute favorable properties such as high potency, high receptor selectivity, and cell permeability (Barnes-Seeman et al., 2013; Chen et al., 2012; Gagnon, Duplessis, & Fader, 2010; Pietruszka, 2003). Substituted cyclopropanes are also valuable synthetic intermediates for ring-opening reactions (Cavitt, Phun, & France, 2014). While cyclopropanes have been traditionally synthesized *via* transition metal-catalyzed cyclopropanation reactions involving alkenes and carbene donor reagents such as diazo compounds, recent advances have made possible the synthesis of cyclopropanes by enzymatic means, thus providing an attractive and environmentally friendly strategy to obtain these valuable compounds (Brandenberg, Fasan, & Arnold, 2017). Specifically, our group and others have recently reported the successful application of engineered hemoproteins and other metalloenzymes for ‘abiological’ carbene transfer reactions, including olefin cyclopropanation reactions (Brandenberg et al., 2017). In particular, our group has demonstrated the versatility of myoglobin as a scaffold for the design of efficient and selective biocatalysts for the

asymmetric cyclopropanation of olefins in the presence of a variety of diazo compounds, including α -diazo acetates, 2-diazo-trifluoroethane, and diazoacetonitrile to yield the corresponding cyclopropane products in high yields and with high degrees of diastereo- and enantioselectivity (Scheme 1) (Bajaj, Sreenilayam, Tyagi, & Fasan, 2016; Bordeaux, Tyagi, & Fasan, 2015; Chandgude & Fasan, 2018; Tinoco, Steck, Tyagi, & Fasan, 2017; Vargas, Khade, Zhang, & Fasan, 2019). These myoglobin-based ‘cyclopropanases’ have been applied for the stereoselective synthesis of the chiral cyclopropane core of various commercial drugs at the multi gram scale (Bajaj et al., 2016). In addition, engineered myoglobins have proven useful for realizing a variety of other (asymmetric) carbene transfer reactions, including aldehyde olefination, Doyle-Kirmse reactions, and Y–H insertion reactions (Y=N, S, Si) (Moore, Steck, Bajaj, & Fasan, 2018; Sreenilayam, Moore, Steck, & Fasan, 2017a, 2017b; Steck, Sreenilayam, & Fasan, 2020; Tyagi & Fasan, 2016; Tyagi, Sreenilayam, Bajaj, Tinoco, & Fasan, 2016). Protein engineering of the myoglobin scaffold, particularly through mutagenesis of the amino acid residues located around the heme cofactor binding site (Fig. 1), has been shown to alter and enhance the catalytic activity, substrate scope, and stereoselectivity of Mb-based carbene transferases in the aforementioned reactions.



Scheme 1 Myoglobin-catalyzed cyclopropanation reactions.

As a complementary strategy toward tuning the carbene transferase activity of this metalloprotein, our group has also investigated the substitution of the native heme cofactor and conserved histidine axial ligand with non-native porphyrinoid ligands and alternative amino acids as the metal-coordinating ligands, including non-canonical amino acids (ncAAs). This chapter will describe methods and protocols for the preparation and characterization of cofactor-reconfigured myoglobins as artificial metalloenzymes for carbene transfer reactions. As shown by various studies, these structural modifications have proven useful for imparting myoglobin with novel and unique catalytic activities in the context of carbene transfer reactions, including tolerance to aerobic conditions, altered chemoselectivity, and expanded reaction scope (Carminati & Fasan, 2019; Moore et al., 2018; Sreenilayam et al., 2017a, 2017b).

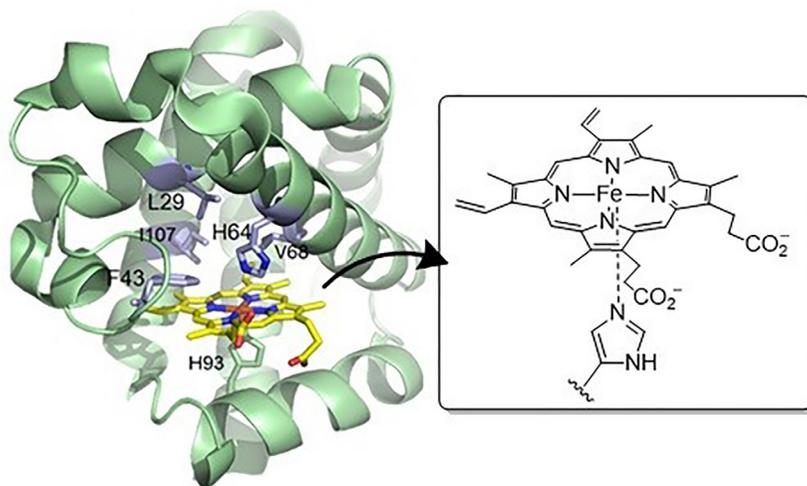


Fig. 1 Wild-type sperm whale myoglobin and heme b structure. The residues located in the distal pocket of the protein are shown as stick models and labeled.

2. Design of cofactor reconfigured myoglobin-based carbene transferases

Myoglobin is a small (16 kDa), globular protein involved in binding and storing oxygen in the muscular tissue (Ordway and Garry, 2004). In myoglobin, the heme cofactor, iron protoporphyrin IX, is embedded in the protein structure through coordination by a conserved ‘proximal’ histidine residues (His93 in sperm whale myoglobin) (Springer, Sligar, Olson, & Phillips, 1994). While myoglobin has not catalytic function, the carbene transferase activity of this hemoprotein derives from its ability to react with diazo compounds to form an iron porphyrin carbene (IPC) complex which is considered to be the reactive species mediating the cyclopropanation of olefins and other carbene transfer reaction (Bordeaux et al., 2015; Tyagi et al., 2016; Tyagi & Fasan, 2016; Wei, Tinoco, Steck, Fasan, & Zhang, 2018). Mechanistic studies on myoglobin-catalyzed cyclopropanations support a mechanism in which initial activation of the diazo compound by means of the ferrous form of the protein results in the formation of an iron porphyrin carbene species. In the case of olefin cyclopropanation with α -diazo esters, this electrophilic carbene species was found to react with the olefin substrate *via* a concerted and asynchronous insertion into the C=C bond (Wei et al., 2018). Experimental and computational studies have

also shed light into the role of the protein scaffold in myoglobin-catalyzed cyclopropanation reactions (Tinoco et al., 2019; Wei et al., 2018). The cofactor and histidine axial ligand (His93), coordinated directly to the metal, determine the formation of the iron porphyrin carbene species and influence its electrophilic character (Tinoco et al., 2019, Wei et al., 2018). The nature of the amino acid residues in the ‘active’ site (positions 29, 43, 64, 68 and 107 in sperm whale myoglobin; Fig. 1) play a critical role in influencing and controlling the stereoselectivity of the cyclopropanation reaction, *e.g.*, by dictating the orientation of the heme-bound carbene within the distal pocket and facial selectivity of olefin attack to this species (Chandgude, Ren, & Fasan, 2019; Tinoco et al., 2019; Vargas et al., 2019).

Because of their critical role in the formation of the IPC intermediate, we envisioned that modification of the heme cofactor and metal-coordinating proximal ligand could have a profound effect on the carbene transferase properties of myoglobin, including extending the functional capabilities of these biocatalysts beyond those exhibited by engineered myoglobins with a native histidine-ligated heme configuration. Accordingly, over the past few years, our group has investigated the effects of replacing the metal, porphyrin ligand, and metal-coordinating axial ligand on the activity and stereoselectivity of Mb-based biocatalysts as carbene transferases. Engineered myoglobins containing non-native metals, including Co, Mn, Ir, Rh, and Ru, were prepared by incorporating different metalloprotoporphyrin IX derivatives into apomoglobin and resulted in functional carbene transferase featuring novel or altered catalytic activity and chemoselectivity (Moore & Fasan, 2019; Moore et al., 2018; Sreenilayam et al., 2017a). For example, manganese- and cobalt-containing myoglobins were found to catalyze the intermolecular carbene C—H insertion in phthalan, a reaction inaccessible using the native iron-based myoglobin (Sreenilayam et al., 2017a, 2017b), whereas a serine-ligated Co-based myoglobin was found to feature a unique chemoselectivity profile, enabling the chemoselective cyclopropanation of olefins in the presence of more reactive functional groups such as amines and silanes (Moore et al., 2018). On the other hand, substitution of the native heme cofactor with a non-native iron-chlorin e6 (Fe(Ce6) cofactor enabled the development of an efficient and stereoselective cyclopropanase capable of operating under aerobic conditions (Sreenilayam et al., 2017a, 2017b). Compared to protoporphyrin IX (ppIX), chlorin e6 (Ce6) contains a partially saturated pyrrole ring and three adjacent carboxylic groups connected to the tetrapyrrole structure, which contribute to make this porphyrinoid ligand more electrondeficient than ppIX. Since that the presence of electron-withdrawing groups in heme

cofactors can reduce their affinity for oxygen (Shibata et al., 2010), the acquired oxygen tolerance of the designer Fe(Ce6)-based carbene transferase can be at least in part attributed to the ability of the non-native cofactor to eliminate or diminish the inhibitory effect of oxygen during the carbene transfer (*i.e.*, cyclopropanation) reaction (Sreenilayam et al., 2017a, 2017b). In another work, myoglobin variants carrying non-native axial ligands, including non-proteinogenic amino acids such as β -(3-thienyl)-alanine (3ThA), 3-(3'-pyridyl)-alanine (3PyA) and *p*-aminophenylalanine (pAmF), were found to provide stable and functional carbene transferases, with a His93Asp mutation conferring enhanced activity under non-reducing conditions (Moore & Fasan, 2019). Similar strategies toward the development of myoglobin-based carbene transferases with altered cofactors have been undertaken by other groups (Hayashi et al., 2012, 2018; Key, Dydio, Clark, & Hartwig 2016; Oohora et al., 2017; Pott et al., 2018; Wolf, Vargas, & Lehnert, 2017). Recently, we have also demonstrated that modification of the cofactor environment in myoglobin-based carbene transferases can prove useful toward expanding their reaction scope by altering the nature of the iron-porphyrin carbene species and mechanism of myoglobin-catalyzed cyclopropanation (Carminati & Fasan, 2019; Sreenilayam et al., 2017a, 2017b). With the goal of increasing the electrophilicity of the ICP intermediate and thus increasing the reactivity of Mb-based cyclopropanases toward less reactive olefins, the hemin cofactor was substituted for a non-native iron 2,4-diacetyl deuteroporphyrin IX (Fe(DADP)) cofactor. Compared to ppIX, 2,4-diacetyl deuteroporphyrin IX contains two acetyl groups conjugated to the pyrrolic skeleton instead of the vinyl groups, making this porphyrin ligand more electron deficient compared to ppIX. Upon incorporation into apomyoglobin, this effect translated in an increase in the redox potential ($E^0_{\text{Fe}^{3+}/\text{Fe}^{2+}}$) of the metalloprotein (Carminati & Fasan, 2019) as expected from a stabilization of its ferrous form over the ferric form (Bhagi-Damodaran, Petrik, Marshall, Robinson, & Lu, 2014). This modification was further combined with a substitution of the axial histidine ligand (His93) with the genetically encodable unnatural amino acid *N*-methyl-histidine (NMH), which was previously reported to increase the redox potential ($E^0_{\text{Fe}^{3+}/\text{Fe}^{2+}}$) of myoglobin, possibly due to the loss of the hydrogen bond with the serine residue in position 92 (Hayashi et al., 2018). Importantly, this dual cofactor/axial ligand substitution resulted in an artificial carbene transferase with greatly enhanced activity toward the cyclopropanation of electrondeficient olefins (Carminati & Fasan, 2019). In addition, this cofactor/proximal ligand configuration

imparted radical reactivity to the carbene transferases, favoring a radical-type cyclopropanation mechanism as opposed to non-radical concerted cyclopropanation mechanism exhibited by engineered myoglobins featuring a native histidine-ligated heme (Carminati & Fasan, 2019; Tinoco et al., 2019; Wei et al., 2018).

3. Synthesis of iron porphyrinoid complexes

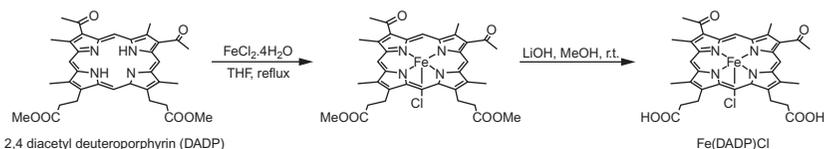
2,4-Diacetyl deuteroporphyrin IX (DADP) and chlorin e6 (Ce6) were selected as new porphyrinoid ligands for the preparation of cofactor-substituted myoglobin-based carbene transferases. The synthesis of Fe(DADP) and Fe(Ce6) complexes is described below.

3.1 Chemicals

1. Iron chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$)
2. 2,4-Diacetyl deuteroporphyrin IX (DADP) (*see* Note 1)
3. chlorine e6 (Ce6) (*see* Note 1)
4. L-Ascorbic acid
5. Lithium hydroxyde (LiOH)
6. Organic solvents: acetone, tetrahydrofurane and methanol

3.2 Synthesis of iron 2,4-diacetyl deuteroporphyrin IX chloride complex

The iron 2,4-diacetyl deuteroporphyrin IX chloride complex can be synthesized *via* metalation of 2,4-diacetyl deuteroporphyrin IX dimethyl ester in the presence of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$. This complex, which is obtained in nearly quantitative yield, gives the desired Fe(DADP) complex after hydrolysis of the ester groups (Scheme 2).



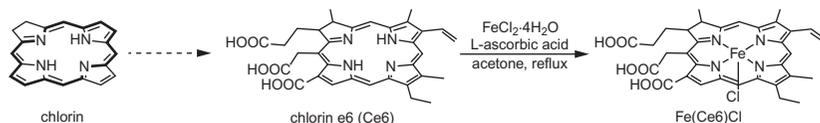
Scheme 2 Synthesis of iron 2,4-diacetyl deuteroporphyrin chloride complex (Fe(DADP)Cl).

3.2.1 Protocol

1. Dissolve 50 mg 2,4-diacetyldeuteroporphyrin IX dimethyl ester and 160 mg $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ into 20 mL anhydrous THF in a dry 50 mL round bottom flask equipped with a stir bar and an argon balloon.
2. Place reflux condenser on the flask and heat reaction to 70°C for 24 h.
3. Let reaction cool back down to room temperature and evaporate the solvent under reduced pressure in a rotary evaporator.
4. Recover the crude with CH_2Cl_2 (10 mL). Wash the organic fraction with 10 mL water for three times and dry the organic solution over MgSO_4 .
5. Filter the solution and evaporate the solvent to obtain the iron 2,4-diacetyldeuteroporphyrin IX dimethyl ester ($\text{Fe}(\text{DADP})\text{Cl}$) (54 mg, 95% yield).
6. Dissolve 54 mg iron 2,4-diacetyldeuteroporphyrin IX dimethyl ester in a solution of 5.0 mL THF, 2.0 mL MeOH and 2.0 mL H_2O in a 25 mL round-bottom flask with a stir bar.
7. Add 43 mg lithium hydroxide and stir the reaction at 25°C for 2 h.
8. Filter the reaction to obtain the product as a dark solid (36 mg, 70% yield).

3.3 Synthesis of iron chlorine e6 chloride complex

The iron chlorin e6 chloride complex, $\text{Fe}(\text{Ce6})\text{Cl}$, can be synthesized *via* metalation of metal-free chlorin e6 in the presence of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ and L-ascorbic acid in acetone. The iron complex, which is obtained in quantitative yield as dark green solid, was characterized by UV-vis analysis showing a Soret band around 395 nm (Scheme 3).



Scheme 3 Synthesis of iron chlorin e6 chloride complex ($\text{Fe}(\text{Ce6})\text{Cl}$).

3.3.1 Protocol

1. Filter the reaction to obtain the product as a dark solid (36 mg, 70% yield).
2. Dissolve 100 mg chlorin e6 in 100 mL acetone in a dry 500 mL round-bottom flask equipped with a stir bar and an argon balloon.
3. Dissolve 360 mg (+)-ascorbic acid in 100 mL acetone and add to the chlorin e6 solution.
4. Add 100 mg $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ into the reaction mixture.

5. Cover the flask with aluminum foil and place the flask under a reflux condenser. Heat the reaction mixture to 60 °C for 4 h.
6. Let the reaction mixture cool down to room temperature and carry out a solvent extraction using 100 mL CH₂Cl₂.
7. Wash the organic fraction with 100 mL of a brine solution for three times, then with 100 mL of 0.01 M HCl solution, and then with 200 mL H₂O. After separation of the organic layer using a separatory funnel, dry the organic solution over MgSO₄.
8. Filter the solution and evaporate the solvent to obtain the iron chlorin 6 chloride complex (Fe(Ce6)Cl) (113 mg, 95% yield) as a dark green powder.

3.4 UV–vis characterization of Fe(DADP)Cl and Fe(Ce6)Cl

The electronic absorption spectrum of metalloporphyrin complexes is characterized by the presence of an intense absorption band around 380–450 nm, commonly referred to as Soret band, and a series of weaker absorption bands around 600–750 nm, commonly referred to as Q bands. Different metalloporphyrin complexes exhibit Soret and Q bands with characteristic λ_{\max} and extinction coefficients (ϵ) which can be used for both characterization of these complexes and quantitation. UV–vis characterization of Fe(DADP)Cl and Fe(Ce6)Cl complexes was carried out as described below.

1. Using an analytical balance, weigh approximately 6 mg of the metalloporphyrin complex.
2. First solution: dissolve 6 mg of iron complex in 25 mL CH₂Cl₂ in a graduated flask to obtain an approximately 10⁻⁴ M solution of the complex.
3. Second solution: dilute 1 mL of 10⁻⁴ M solution in 100 mL CH₂Cl₂ in a graduated flask to obtain an approximately 10⁻⁶ M solution.
4. Fill a quartz cuvette with 1 mL of 10⁻⁴ M solution. Record a UV–vis spectrum of the complex from 300 nm to 750 nm to detect the Q bands. Repeat the same process using 1 mL of the 10⁻⁶ M solution to detect the Soret band.
5. Apply the Beer's law equation ($A = \epsilon (M^{-1} \text{ cm}^{-1}) \times L \text{ cm} \times [\text{concentration}] (M)$) to determine the extinction coefficients of the complexes.
6. Repeat steps 1–5 two more times for statistically significant measurement of ϵ values. Fe(Ce6)Cl UV–vis (CH₂Cl₂): $\lambda_{\max}(\epsilon) = 395 \text{ nm} (33840)$, 603 nm (5900), 684 nm (7700). Fe(DADP)Cl UV–vis (CH₂Cl₂): $\lambda_{\max}(\epsilon) = 415 \text{ nm} (43101)$, 513 nm (6613), 540 nm (6271), 644 nm (3192).

3.5 Notes

1. Chlorin e6 (Ce6) and 2,4-diacetyldeuteroporphyrin IX dimethyl ester are commercially available and they were purchased from Santa Cruz Biotechnology and Frontier Scientific Inc. respectively.



4. Incorporation of non-native cofactors into myoglobin

The incorporation of the Fe(DADP)Cl and Fe(Ce6)Cl complexes into myoglobin can be achieved *via* reconstitution of the protein lacking the heme cofactor (*i.e.*, apomyoglobin) with the non-native cofactors either *in vitro* or *in vivo*. Conventionally, reconstitution of apomyoglobin with non-native cofactors have been accomplished by chemical removal of hemin *via* extraction with 2-butanone, followed by reconstitution of the denatured apomyoglobin with the cofactor of interest and extensive dialysis of the mixture to promote refolding of the holoprotein (Hayashi et al., 2002; Teale, 1959). This approach, however, is laborious and time consuming and it may result in protein complexes that are heterogenous in nature, due to partial refolding of the cofactor-substituted protein. As an alternative strategy, apomyoglobin can be expressed recombinantly in *E. coli* cultures grown in iron-free media, which prevents the biosynthesis of hemin within the cell and thus its incorporation into apomyoglobin to give the holoprotein. According to this approach, apomyoglobin is expressed in *E. coli* under iron-free conditions and then purified, *e.g.*, using a polyhistidine tag and Ni-affinity chromatography. Purified apomyoglobin can be then reconstituted with the desired cofactor *in vitro*, followed by removal of excess metalloporphyrin through dialysis (Key et al., 2016). While this protocol is technically simpler than Teale's butanone extraction method, the expression yield of apomyoglobin expressed under iron-free conditions is typically lower than that of holoprotein expressed in conventional growth media (*e.g.*, Luria-Bertani medium). In addition, apomyoglobin is unstable and prone to precipitation, which can further reduce the final yield of the desired cofactor-substituted myoglobin. As a more convenient alternative to this protocol, *in vitro* reconstitution of apomyoglobin with the non-native cofactor of interest can be achieved directly during cell lysis, which bypasses the need for purification and isolation of poorly stable apo form of this protein (Carminati & Fasan, 2019; Sreenilayam et al., 2017a, 2017b). This is particularly important in the context of engineered variants of myoglobins, whose apo forms may be further destabilized by the presence of (active site)

mutations. This *in vitro* reconstitution protocol, which is described in more detail below, is an adaption of a procedure described by Watanabe and coworkers for the cofactor substitution in heme-containing enzymes (Kawakami, Shoji, & Watanabe, 2012).

Our group has also implemented efficient protocols for the recombinant expression of cofactor-substituted myoglobins *in vivo*, in which the non-native cofactor is incorporated into apomyoglobin directly during protein expression in *E. coli* (Bordeaux, Singh, & Fasan, 2014; Sreenilayam et al., 2017a, 2017b). This procedure involves the simple addition of the non-native cofactor to bacterial cells expressing apomyoglobins. In this case, cellular uptake of the non-native cofactor is achieved using a heterologous outer membrane heme transporter (ChuA), which is co-expressed in the bacterial host along with the desired hemoprotein (Varnado & Goodwin, 2004). For certain metalloporphyrin cofactors (e.g., Co-ppIX), we also found that, in addition to ChuA, the co-expression of chaperones such as GroEL/ES helped increase the expression yield of cofactor-substituted myoglobin, likely though preventing or reducing unfolding of the apomyoglobin precursor (Bordeaux et al., 2014). Overall, this *in vivo* reconstitution method represents a more straightforward and convenient approach for the preparation of cofactor-substituted myoglobins compared to the *in vitro* reconstitution methods mentioned above. In addition, it enables the application of these artificial metalloenzymes in whole-cell biocatalysis and biotransformations (Sreenilayam et al., 2017b). Although the ChuA transporter is rather promiscuous in terms of substrates, this method is dependent upon the ability of ChuA (or that of endogenous heme transporters in *E. coli*) to mediate the cellular uptake of the desired metalloporphyrin cofactors and so it may not be viable for all metalloporphyrin complexes. Altogether, the aforementioned *in vitro* and *in vivo* reconstitution methods represent two complementary strategies useful for the generation of cofactor-substituted myoglobin variants for carbene transfer reactions or other applications.

4.1 Chemicals

1. Fe(Ce6)Cl in DMF (30 mg/mL).
2. Fe(DADP)Cl in DMF (30 mg/mL).
3. *N*-3-methyl-L-histidine (NMH).
4. Antibiotics: ampicillin (AMP), chloramphenicol (CAM).
5. Isopropyl- β -D-1-thiogalactopyranoside (IPTG).

6. Arabinose.
7. Luria-Bertani broth (LB): 10 g of tryptone, 10 g NaCl, 5 g yeast extract in 1 L of deionized H₂O.
8. M9-CAGL medium: 200 mL of M9 salts (5×), 10 mL of casamino acids (20%, m/v), 10 mL of glycol, 1 mL of MgSO₄ (2 M), and 100 μL of CaCl₂ (1 M) and 779 mL of deionized H₂O.
9. M9 (5×) salt: 15 g of Na₂HPO₄, 7.5 g of K₂HPO₄, 2.5 g of NaCl, 5 g of NH₄Cl in 1 L of deionized H₂O.
10. Ni NTA Buffers: lysis buffer (50 mM KPi, 250 mM NaCl, 10 mM histidine, pH 8.0), wash buffer: (50 mM KPi, 250 mM NaCl, 20 mM histidine, pH 8.0), elution buffer (50 mM KPi, 250 mM NaCl, 250 mM histidine, pH 7.0).
11. Pyridine solution: 1.75 mL of pyridine, 0.75 mL of NaOH 1 M, 32 mg of K₃[Fe(CN)₆].

4.2 Protocols for *in vitro* reconstitution of Mb variants incorporating the non-native cofactors [Fe(Ce6)] and [Fe(DADP)]

This protocol describes a procedure for the *in vitro* incorporation of the non-native cofactor [Fe(Ce6)] or [Fe(DADP)] into the sperm whale myoglobin variant Mb(H64V,V68A). In this protocol, the Mb(H64V,V68A) protein containing a C-terminal His tag is expressed from a pET22b-based plasmid vector in which the gene of the protein is placed under the control of an IPTG-inducible T7 promoter (Bordeaux et al., 2015). This plasmid requires the use of lysogenic DE3 strains of *E. coli* such as BL21(DE3) or C41(DE3). Alternative expression plasmids and *E. coli* strains can be used for the same purpose.

1. Add 1.0 μL of pET22_Mb(H64V,V68A) plasmid to 50 μL aliquot of chemically competent *E. coli* C41(DE3) cells. Mix gently the DNA with the cells and incubate in ice for 15 min.
2. Incubate the cells at 42 °C for 45 s. Remove and place in ice for 5 min.
3. Add 350 μL of LB media to the cells and place at 37 °C for 1 h under shaking.
4. Spread 100 μL of cells on LB-Agar plates containing ampicillin (100 mg/L) and incubate the plate at 37 °C overnight.
5. Inoculate a single colony from the plate into 5 mL of LB medium containing ampicillin (100 mg/L) and incubate at 37 °C overnight with shaking at 200 rpm.

6. Pellet the overnight culture by centrifugation at 4000 rpm for 5 min and discard the supernatant.
7. Suspend the cell pellets in 1 mL ddH₂O and transfer to 1 L of M9-CAGL media which was prepared as follow: in a 1 L sterile flask add 779 mL of deionized H₂O, 200 mL of M9 salts (5×), 10 mL of casamino acids (20%, m/v), 10 mL of glycol, 1 mL of MgSO₄ (2 M), and 100 μL of CaCl₂ (1 M). Add 1 mL ampicillin (100 mg/mL) (*see* Note 1).
8. Incubate the cell culture at 37 °C with shaking at 180 rpm until optical density at 600 nm (OD₆₀₀) reaches 1.4.
9. Add IPTG to a final concentration of 0.5 mM. Place the culture in an incubator at 20 °C with shaking at 180 rpm for 20–24 h.
10. Harvest the cells by centrifugation at 4000 rpm for 20 min. Resuspend the cell pellet in 20 mL of Ni NTA Lysis Buffer and add Fe(Ce6)Cl or Fe(DADP)Cl at a final concentration of 30 mg/L. Place the cell suspension solution in ice for 5 min.
11. Lyse the cells by sonication and centrifuge the cell lysate at 14000 rpm for 30 min at 4 °C (*see* Note 2 and 3).
12. Purify the protein by Ni-NTA chromatography using the following protocol: transfer the clarified lysate to a Ni-NTA column (Column volume: ~5 mL) pre-equilibrated with Ni-NTA Lysis Buffer. Wash the resin with 50 mL of Ni-NTA Lysis Buffer and then with 50 mL of Ni-NTA Wash Buffer. Elute the protein with Ni-NTA Elution Buffer.
13. Combine the fractions containing the protein and buffer exchange the protein solution into potassium phosphate buffer (KPi, 50 mM, pH 7) using a 10 kDa Centricon filter or through dialysis.
14. Determine the protein concentration *via* UV–vis spectroscopy (*vide infra*).
15. Filter sterilize the protein solution using a 0.22 μm filter and store the protein at 4 °C.

4.3 Protocol for *in vivo* reconstitution of Mb variants incorporating the [Fe(Ce6)] and [Fe(DADP)] non-native cofactors

This protocol describes a procedure for the recombinant expression of [Fe(Ce6)] and [Fe(DADP)]-containing myoglobins. As in the previous protocol, histagged Mb(H64V,V68A) variant is expressed from a pET22b-based plasmid vector using a DE3 lysogenic *E. coli* strain (Bordeaux *et al.*,

2015). The heme transporter ChuA and chaperone proteins GroES/EL are co-expressed using a second pACYC-based plasmid (pGroES/EL-ChuA), in which the ChuA gene is under the control of an IPTG-inducible T7 promoter and the GroES/EL genes are under the control of an arabinose-inducible araBAD promoter.

1. Add 1.0 μL of pET22_Mb(H64V,V68A) and 1.0 μL of GroES/EL-ChuA plasmid to 50 μL aliquot of competent *E. coli* C41(DE3) cells. Mix gently the DNA with the cells and incubate in ice for 15 min.
2. Incubate the cells at 42 °C for 45 s. Remove and place in ice for 5 min.
3. Add 350 μL of LB media to the cells and incubate at 37 °C for 1 h with shaking.
4. Spread 100 μL of cells on LB-Agar plates containing ampicillin (100 mg/L) and chloramphenicol (34 mg/L). Incubate the plate at 37 °C overnight.
5. Inoculate a single colony from the plate into 5 mL of LB medium containing ampicillin (100 mg/L) and chloramphenicol (34 mg/L) and incubate at 37 °C overnight with shaking at 200 rpm.
6. Pellet the overnight culture by centrifugation at 4000 rpm for 5 min and discard the supernatant.
7. Resuspend the cell pellet in 1 mL ddH₂O and use this solution to inoculate 1 L of M9-CAGL medium prepared as follow: in a 1 L flask add 779 mL of deionized H₂O, 200 mL of M9 salts (5 \times), 10 mL of casamino acids (20%, m/v), 10 mL of glycol, 1 mL of MgSO₄ (2 M), and 100 μL of CaCl₂ (1 M). Add 1 mL ampicillin (100 mg/mL) and 1 mL chloramphenicol (34 mg/mL) (*see* Note 1).
8. Incubate the cells at 37 °C with shaking at 180 rpm until OD₆₀₀ reaches 1.4.
9. Condense cells by centrifugation at 4000 rpm for 20 min and resuspend in 200 mL of fresh M9-CAGL medium.
10. Add Fe(Ce6)Cl (or Fe(DADP)Cl) and IPTG to a final concentration of 30 mg/L and 0.5 mM, respectively. Place the culture in an incubator at 20 °C with shaking at 180 rpm for 20–24 h.
11. Harvest the cells by centrifugation at 4000 rpm for 20 min. Resuspend cell pellets in 20 mL of Ni NTA Lysis Buffer and put in ice. Lyse the cells by sonication and centrifuge the cell lysate at 14000 rpm for 30 min at 4 °C (*see* Note 2 and 3).
12. Purify the protein by Ni-NTA chromatography using the following protocol: transfer the clarified lysate to a Ni-NTA column (Column volume: \sim 5 mL) equilibrated with Ni-NTA Lysis Buffer. Wash the

resin with 50 mL of Ni-NTA Lysis Buffer and then with 50 mL of Ni-NTA Wash Buffer. Elute the protein with Ni-NTA Elution Buffer.

13. Combine the fractions containing the protein and buffer exchange the protein solution into potassium phosphate buffer (KPi, 50 mM, pH 7) using a 10 kDa Centricon filter or through dialysis.
14. Determine the protein concentration *via* UV-vis spectroscopy (*vide infra*).
15. Filter sterilize the protein solution using a 0.22 μm filter and store the protein at 4 $^{\circ}\text{C}$.

4.4 Protocol for recombinant expression of Mb(H64V,V68A,H93NMH)[Fe(DADP)]

This protocol describes a procedure for the recombinant expression of the cofactor-reconfigured variant Mb(H64V,V68A,H93NMH)[Fe(DADP)], in which hemin is substituted for Fe(DADP) and the proximal axial histidine (His93) is substituted for the non-canonical amino acid *N*-methyl-histidine (NMH). NMH is genetically incorporated into protein using the amber stop codon (TAG) suppression technology (Santoro, Wang, Herberich, King, & Schultz, 2002) and an orthogonal engineered pyrrolysyl-tRNA synthetase/tRNA_{CUA} pair (Xiao et al., 2014). Accordingly, an amber codon (TAG) was engineered in position 93 of Mb(H64V,V68A) *via* site-directed mutagenesis, generating the plasmid pET22_Mb(H64V,V68A,H93TAG). The engineered pyrrolysyl-tRNA synthetase/tRNA_{CUA} pair for the genetic incorporation of NMH are encoded by a pEVOL-based vector (plasmid pEVOL-PylRS(NMH)).

1. Add 1.0 μL of pET22_Mb(H64V,V68A,H93TAG) plasmid and 1.0 μL of pEVOL-PylRS(NMH) plasmid to a 50 μL aliquot of chemically competent *E. coli* BL21(DE3) cells. Mix gently the DNA with the cells and incubate in ice for 15 min.
2. Place the cells at 42 $^{\circ}\text{C}$ for 45 s. Remove and place in ice for 5 min.
3. Add 350 μL of LB media to the cells and incubate at 37 $^{\circ}\text{C}$ for 1 h.
4. Spread 100 μL of cells on LB-Agar plates containing ampicillin (100 mg/L) and chloramphenicol (34 mg/L). Incubate the plate at 37 $^{\circ}\text{C}$ for 16 h.
5. Inoculate a single colony from the plate into 5 mL of LB medium containing ampicillin (100 mg/L) and chloramphenicol (34 mg/L) and incubate at 37 $^{\circ}\text{C}$ overnight with shaking at 200 rpm.

6. Pellet the overnight culture by centrifugation at 4000 rpm for 5 min and discard the supernatant.
7. Resuspend the cell pellets in 1 mL ddH₂O and transfer to 1 L of M9-CAGL media which was prepared as follow: in a 1 L flask add 779 mL of deionized H₂O, 200 mL of M9 salts (5 ×), 10 mL of casamino acids (20%, m/v), 10 mL of glycerol, 1 mL of MgSO₄ (2 M), and 100 μL of CaCl₂ (1 M). Add 1 mL ampicillin (100 mg/mL) and 1 mL chloramphenicol (34 mg/mL) (*see* Note 1).
8. Grow the cell culture at 37 °C with shaking at 180 rpm until OD₆₀₀ reaches 0.6.
9. Centrifuge the cell culture at 4000 rpm for 20 min and resuspend the cell pellet in 200 mL of fresh M9-CAGL.
10. Add *N*-3-methyl-L-histidine (NMH) and arabinose to the cell culture to a final concentration of 5 mM and 6 mM, respectively, and incubate the culture at 24 °C for 20 min with shaking at 180 rpm.
11. Add IPTG to a final concentration of 0.5 mM. Place the culture in an incubator at 20 °C with shaking at 180 rpm for 20–24 h.
12. Harvest the cells by centrifugation at 4000 rpm for 20 min. Resuspend cell pellets in 20 mL of Ni NTA Lysis Buffer and add Fe(DADP)Cl at a final concentration of 30 mg/L and place the cell suspension in ice.
13. Lyse the cells by sonication and clarify the cell lysate by centrifugation at 14000 rpm for 30 min at 4 °C (*see* Note 2 and 3).
14. Purify the protein by Ni-NTA chromatography using the following protocol: transfer the clarified lysate to a Ni-NTA column equilibrated with Ni-NTA Lysis Buffer. Wash the resin with 50 mL of Ni-NTA Lysis Buffer and then with 50 mL of Ni-NTA Wash Buffer. Elute the protein with Ni-NTA Elution Buffer.
15. Combine the fractions containing the protein and buffer exchange the protein solution into potassium phosphate buffer (KPi, 50 mM, pH 7) using a 10 kDa Centricon filter or through dialysis.
16. Determine the protein concentration *via* UV-vis spectroscopy (*vide infra*).
17. Filter sterilize the protein solution using a 0.22 μm filter and store the protein at 4 °C.

4.5 Spectroscopic characterization of cofactor-substituted myoglobin variants

Protein concentrations in their ferrous form are determined *via* UV-vis spectroscopy using the extinction coefficients of $\epsilon_{433} = 196 \text{ mM}^{-1} \text{ cm}^{-1}$

for Mb(H64V,V68A)[Fe(Ce6)], $\epsilon_{546} = 3451 \text{ M}^{-1} \text{ cm}^{-1}$ for Mb(H64V, V68A)[Fe(DADP)] and $\epsilon_{430} = 85,246 \text{ M}^{-1} \text{ cm}^{-1}$ for Mb(H64V,V68A, H93NMH)[Fe(DADP)], which were determined using the pyridine hemochrome assay. (Berry & Trumpower, 1987). UV-vis and CD spectra of the proteins are collected for further characterization.

4.5.1 Pyridine binding assay

The extinction coefficients for the cofactor-substituted myoglobins can be determined using the pyridine-binding assay protocol as follow:

1. Mix vigorously 1.75 mL of pyridine, 0.75 mL of 1 M NaOH and 32 mg of $\text{K}_3[\text{Fe}(\text{CN})_6]$ in an Eppendorf and centrifugate at 14000 rpm for 30 s. Discard the aqueous base excess.
2. Fill a cuvette with 0.75 mL of protein in KPi buffer (50 mM, pH 7) and add 0.25 mL of the pyridine solution.
3. Add few grains (less than 2 mg) of sodium dithionite and mix gently.
4. Record the UV-vis spectra from 750 nm to 300 nm.
5. Determine the protein concentration from the absorbance of the cofactor using extinction coefficients of $\epsilon_{395} = 33.8 \text{ mM}^{-1} \text{ cm}^{-1}$ for Fe(Ce6) and $\epsilon_{415} = 43.1 \text{ mM}^{-1} \text{ cm}^{-1}$ for Fe(DADP).

4.5.2 Measurement of UV-vis spectra

1. Prepare a 1 mL solution of Mb variant in KPi buffer (50 mM, pH 7) (approx. 5 μM) in a quartz cuvette.
2. For a spectrum of the protein in ferric form, record a UV-vis spectrum of the protein solution from 300 to 500 nm. The Soret band for ferric Mb(H64V, V68A)[Fe(Ce6)], Mb(H64V,V68A)[Fe(DADP)] and Mb(H64V,V68A, H93NMH)[Fe(DADP)] appears at 413 nm, at 415 nm and 412 nm, respectively.
3. For a spectrum of the protein in ferrous form, add few grains (less than 2 mg) of sodium dithionite to the protein solution and mix gently. Record the UV-vis spectrum of the protein solution from 300 to 500 nm. The Soret band for ferrous Mb(H64V,V68A)[Fe(Ce6)], Mb(H64V,V68A) [Fe(DADP)] and Mb(H64V,V68A,H93NMH)[Fe(DADP)] appears at 433, at 434 and 430 nm, respectively.
4. For a spectrum of the CO-bound form of the protein, after addition of sodium dithionite as in step 3, gently bubble CO into the protein solution using a needle for 1 min. Record a UV-vis spectrum of the protein solution from 300 to 500 nm. The Soret band for CO-bound form of Mb(H64V,V68A)[Fe(Ce6)], Mb(H64V,V68A)[Fe(DADP)] and Mb(H64V,V68A,H93NMH)[Fe(DADP)] appears at 429, at 428 and 422 nm, respectively (Fig. 2).

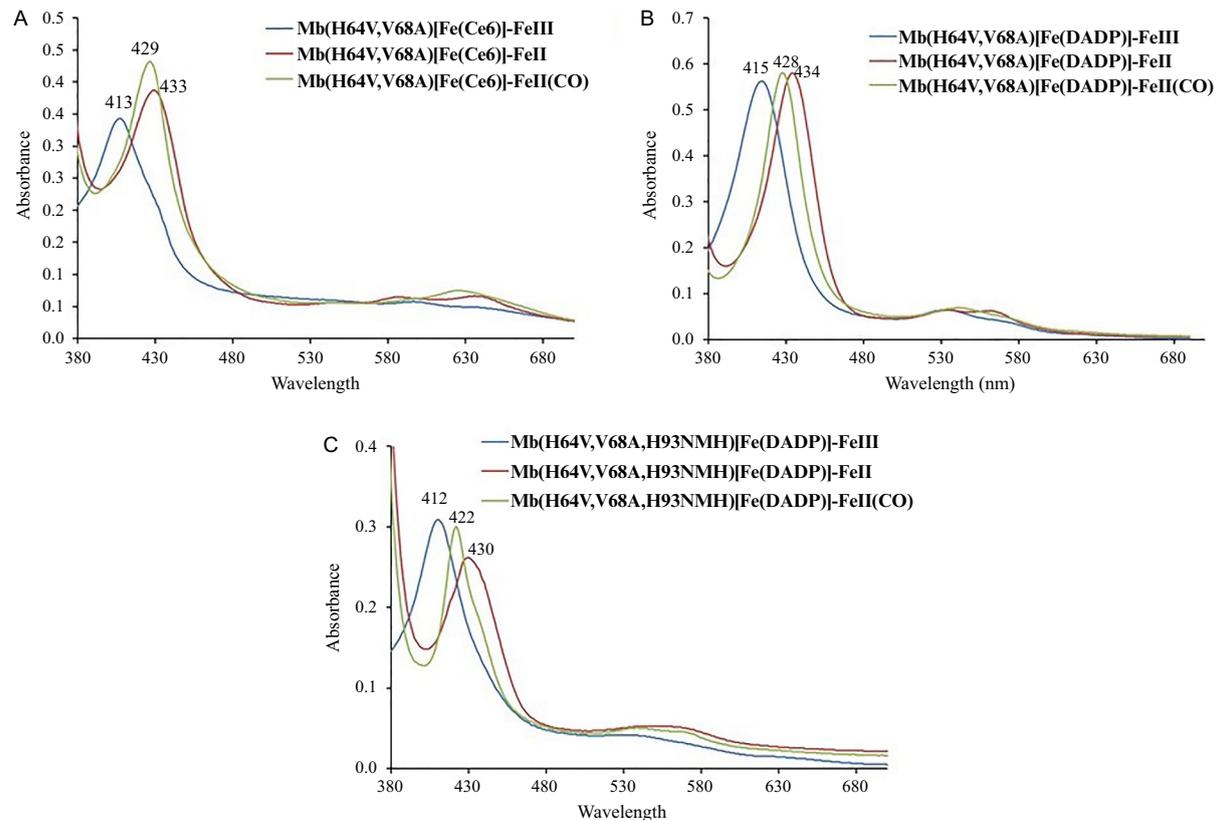


Fig. 2 (A) Absorption spectra for ferric, ferrous and CO-bound form of Mb(H64V,V68A)[Fe(Ce6)]. (B) Absorption spectra for ferric, ferrous and CO-bound form of Mb(H64V,V68A)[Fe(DADP)]. (C) Absorption spectra for ferric, ferrous and CO-bound form of Mb(H64V,V68A,H93NMH)[Fe(DADP)].

4.5.3 Circular dichroism

4.5.3.1 For collecting CD spectra of the Mb variants

1. Prepare a 1 mL solution of Mb variant in KPi buffer (50 mM, pH 7) (approx. 3 μ M) in a quartz cuvette
2. Record a UV CD spectra of the protein solution from 250 to 190 nm, at 20 °C, at a scan rate of 50 nm/min with a bandwidth of 1 nm and an averaging time of 10 s per measurement

4.5.3.2 For a visible CD spectra

1. Prepare a 1 mL solution of Mb variant in KPi buffer (50 mM, pH 7) (approx. 10 μ M for Fe(Ce6)-variant and 25 μ M for Fe(DADP)-variants) in a quartz cuvette.
2. Add few grains (less than 2 mg) of sodium dithionite to the protein solution and mix gently.
3. Gently bubble CO into the protein solution using a needle for 1 min.
4. Record a UV CD spectra of the protein solution from 500 to 300 nm, at 20 °C, at a scan rate of 50 nm/min with a bandwidth of 1 nm and an averaging time of 10 s per measurement (Fig. 3).

4.6 Notes

1. All reagents for the M9-CAGL media must be prepared freshly and autoclaved or filter sterilized separately before combining.
2. Typical method for cell sonication: amplitude 70, pulse-on time 1 s, pulse-off time 4 s, total process time 2 min. The temperature is maintained below 25 °C.
3. It is recommended to keep the lysate and the purified protein at 4 °C to prevent protein degradation



5. Catalytic activity

The cyclopropanation activity of the artificial carbene transferases Mb(H64V,V68A,H93NMH)[Fe(Ce6)], Mb(H64V,V68A)[Fe(DADP)], and Mb(H64V,V68A,H93NMH)[Fe(DADP)], was tested in olefin cyclopropanation reactions with ethyl diazoacetate.

5.1 Cyclopropanation activity of Mb(H64V,V68A)[Fe(Ce6)]

The carbene transferase activity of Mb-based catalysts is significantly reduced under aerobic conditions, which is attributed to the competitive binding of oxygen to the metal center. The substitution of the native heme cofactor in

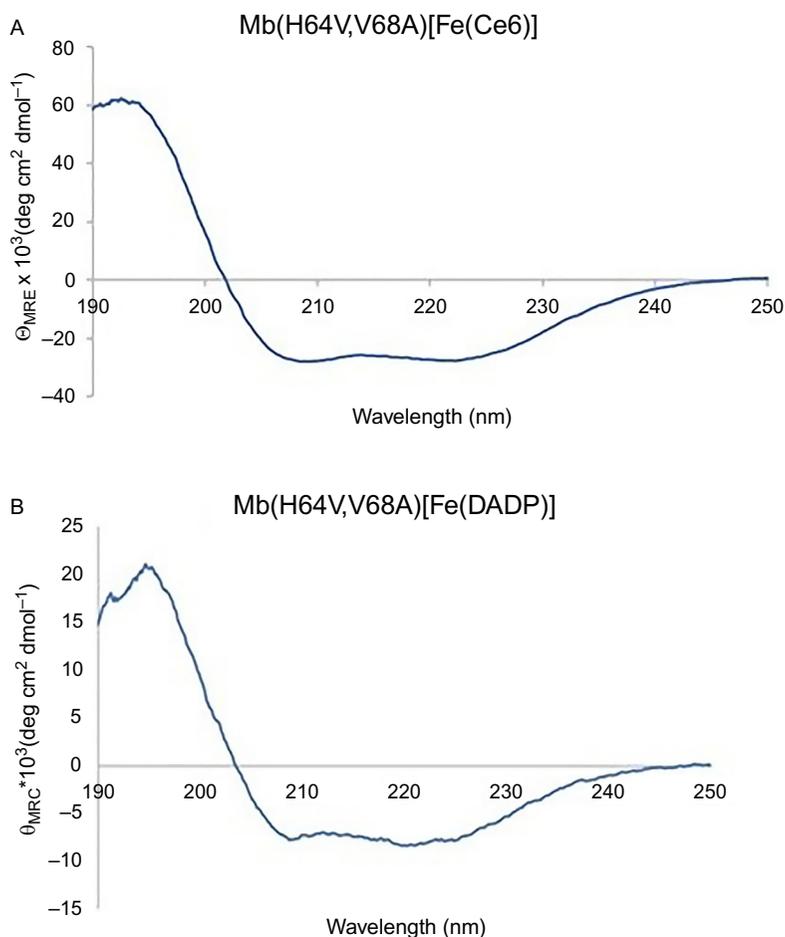
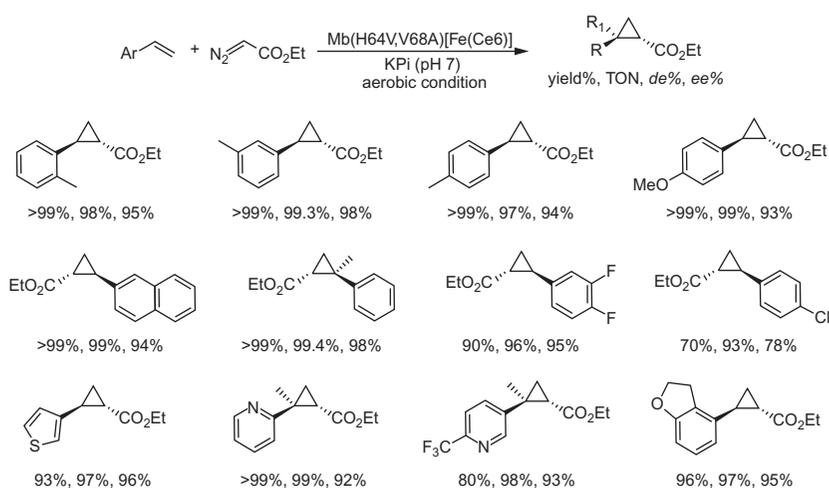


Fig. 3 Near-UV Circular Dichroism spectrum of (A) Mb(H64V,V68A)[Fe(Ce6)] and (B) Mb(H64V,V68A)[Fe(DADP)].

engineered myoglobin variants with the Fe(Ce6) complex reduces their susceptibility to oxygen inhibition, as indicated by the high catalytic activity (TON) and stereoselectivity of Mb(H64V,V68A)[Fe(Ce6)] in catalyzing olefin cyclopropanation reactions under aerobic conditions (Sreenilayam et al., 2017b). Michaelis–Menten kinetic studies confirmed the superior performance of Mb(H64V,V68A)[Fe(Ce6)] under aerobic conditions in comparison to Mb(H64V,V68A), showing a k_{cat} of 2840 min⁻¹ for the iron chlorin-containing variant compared to 545 min⁻¹ for the myoglobin variant containing the native cofactor.

The oxygen-tolerant biocatalyst Mb(H64V,V68A)[Fe(Ce6)] efficiently catalyzes the cyclopropanation of several styrenyl and vinylarene derivatives. Electron-donating and electron-withdrawing groups in *ortho*, *meta*-, and *para*-position of styrene derivatives are well tolerated resulting in the corresponding cyclopropane products in high yields (>99%) and high diastereo- and enantiomeric excess (up to 99% *de* and 99% *ee*) (Sreenilayam et al., 2017b). Pyridinyl, thiophenyl, and benzofuranyl substrates are also cyclopropanated efficiently, further demonstrating the broad substrate scope of the Mb(H64V,V68A)[Fe(Ce6)] catalyst (Scheme 4).



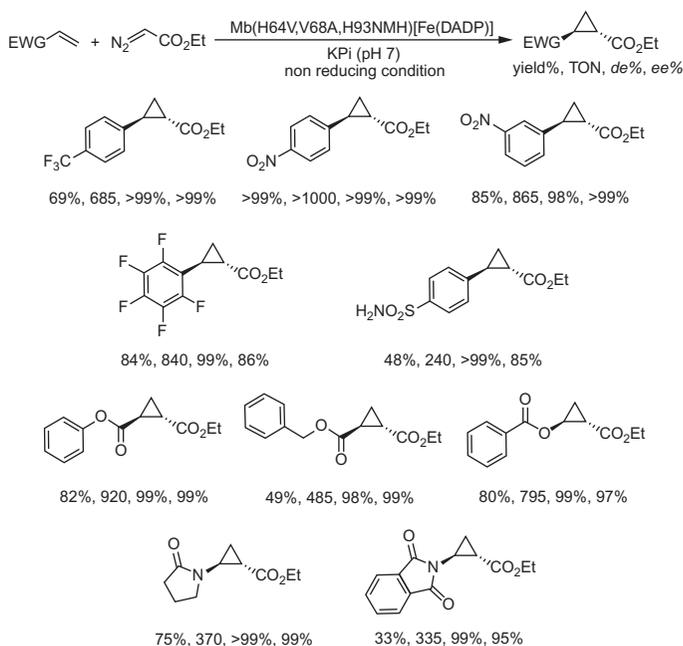
Scheme 4 Mb(H64V,V68A)[Fe(Ce6)]-catalyzed cyclopropanation reaction under aerobic condition.

Furthermore, using the aforementioned *in vivo* protocol for the recombinant expression of Mb(H64V,V68A)[Fe(Ce6)], the biocatalytic cyclopropanation reaction can be performed using whole cells expressing the Mb variant. In a preparative-scale whole-cell reaction, 0.1 g of styrene was converted into the corresponding cyclopropane in high yield and stereoselectivity (93% yield, 96% *de* and 90% *ee*) (Sreenilayam et al., 2017b). The substitution of Fe(ppIX) with Fe(Ce6) can thus enhance the oxygen-tolerance of myoglobin-based cyclopropanation catalysts and the possibility to utilize the Mb(H64V,V68A)[Fe(Ce6)] in whole cells further support its utility and scalability for the asymmetric synthesis of cyclopropanes.

5.2 Cyclopropanation activity of Fe(DADP)-containing myoglobin variants

The metal-catalyzed cyclopropanation of electrondeficient olefins with diazo compounds is a notoriously challenging transformation due to the electrophilic character of most metallocarbenoid species implicated chemo- and biocatalytic carbene transfer reactions. Substitution of the heme cofactor with Fe(DADP) in a Mb-based cyclopropanase was designed to enhance its reactivity toward these challenging substrates (Carminati & Fasan, 2019). Consistent with the electrodeficient nature of the DADP ligand compared to ppIX, Mb(H64V,V68A)[Fe(DADP)] exhibits a more positive redox potential compared to Mb(H64V,V68A) ($E_{\text{Fe}^{3+}/\text{Fe}^{2+}}^0 = 54 \pm 1 \text{ mV}$ *vs.* $59 \pm 1 \text{ mV}$). This cofactor substitution was then combined with the axial ligand substitution H93NMH, which was also meant to increase the reduction potential of the metalloprotein. These combined modifications dramatically increases the redox potential of the protein, resulting in $E_{\text{Fe}^{3+}/\text{Fe}^{2+}}^0$ value of $146 \pm 3 \text{ mV}$ for Mb(H64V, V68A,H93NMH)[Fe(DADP)] and indicating a synergistic role of the electron deficient porphyrin ligand and noncanonical axial ligand toward increasing the redox potential of the metalloprotein.

This synergistic effect of NMH-ligated Fe(DADP) cofactor configuration translates in a greatly enhanced catalytic activity of Mb(H64V, V68A,H93NMH)[Fe(DADP)] toward the asymmetric cyclopropanation of electron-deficient alkenes, while maintaining high activity toward electronrich olefins (Scheme 5). In addition to a variety of styrene derivatives, Mb(H64V,V68A,H93NMH)[Fe(DADP)] is able to cyclopropanate with high yield and stereoselectivity challenging substrates such as pentafluoro-, 4-trifluoromethyl-, 4-nitro-styrenes, phenyl and benzyl acrylates, and vinyl benzoate which are either poorly reactive or unreactive substrates for heme-based myoglobins (Scheme 5). Furthermore, mechanistic studies revealed that the Mb(H64V,V68A,H93NMH)[Fe(DADP)]-catalyzed cyclopropanation proceeds *via* a stepwise radical mechanism mediated by a radical metallocarbene intermediate as opposed to a concerted, non-radical mechanism mediated by an electrophilic metallocarbene intermediate exhibited by Mb(H64V,V68A) (Tinoco et al., 2019; Wei et al., 2018).



Scheme 5 Mb(H64V,V68A,H93NMH)[Fe(DADP)]-catalyzed cyclopropanation reaction of electron deficient alkenes.

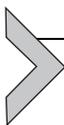
The expanded reaction scope of Mb(H64V,V68A,H93NMH)[Fe(DADP)] toward the cyclopropanation of electrondeficient alkenes highlights the utility of cofactor redesign as a strategy for tuning the catalytic activity of myoglobin-based carbene transferases.

5.3 Protocol for cyclopropanation reaction

A general protocol is provided here for a cyclopropanation reaction under anaerobic and reducing conditions. Reactions were carried out at a 400 μL scale using 10 μM myoglobin variant, 10 mM styrene, 20 mM EDA and 10 mM sodium dithionite.

1. Add 40 μL of sodium dithionite (from a 0.1 M stock solution in buffer) in potassium phosphate buffer (50 mM, pH 7.0) by gently bubbling argon into the mixture for 3 min in a sealed vial equipped with a stirring bar.

2. Gently degas a solution of the myoglobin variant in potassium phosphate buffer (50 mM, pH 7.0) by bubbling argon into the mixture for 3 min in a sealed vial.
 3. Mix the two solution together using a cannula.
 4. Stir the reaction at 25 °C for 2 min.
 5. Add 10 μ L of styrene (from a 0.4 M stock solution in ethanol) with a gas-tight syringe and stir the reaction for 1 min.
 6. Add 10 μ L of EDA (from a 0.8 M stock solution in ethanol) with a gas-tight syringe
 7. Stir the reaction for the desired amount of time (*e.g.*, 0.5–16 h) at 25 °C.
 8. For product analysis, add an internal standard to the reaction mixture, extract the solution with dichloromethane and analyze the organic extract by gas chromatography as described in (Bordeaux et al., 2015).
- Aerobic reactions can be carried out without degassing the solution with argon and in open reaction vessels. Reactions under non-reducing condition can be performed in a similar manner without adding sodium dithionite to the potassium phosphate buffer solution (50 mM, pH 7.0).



6. Summary

Engineered myoglobins have emerged as powerful biocatalysts for asymmetric olefin cyclopropanation reactions. Complementing protein engineering, rational redesign of the cofactor environment through the incorporation of non-native cofactors and/or axial ligands provides a promising strategy to drastically change the reactivity of this metalloprotein as a carbene transfer catalyst. This approach has proven useful to enhance the reactivity of Mb-based cyclopropanases under aerobic and/or non-reducing conditions (Carminati & Fasan, 2019; Sreenilayam et al., 2017b) and to extend their scope to reactions inaccessible with native heme-containing counterparts, such as the cyclopropanation of electrodefficient olefins (Carminati & Fasan, 2019) and the chemoselective cyclopropanation of olefins in the presence of unprotected Y—H bonds, thereby expanding the utility of these biocatalysts for synthetic applications.

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