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C(sp^3)–H Bond Hydroxylation Catalyzed by Myoglobin Reconstituted with Manganese Porphycene

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Supporting Information

ABSTRACT: Myoglobin reconstituted with manganese porphycene was prepared in an effort to generate a new biocatalyst and was characterized by spectroscopic techniques. The X-ray crystal structure of the reconstituted protein reveals that the artificial cofactor is located in the intrinsic heme-binding site with weak ligation by His93. Interestingly, the reconstituted protein catalyzes the H_2O_2-dependent hydroxylation of ethylbenzene to yield 1-phenylethanol as a single product with a turnover number of 13 at 25 °C and pH 8.5. Native myoglobin and other modified myoglobins do not catalyze C–H hydroxylation of alkanes. Isotope effect experiments yield KIE values of 2.4 and 6.1 for ethylbenzene and toluene, respectively. Kinetic data, log k_{obs} versus BDE(C(sp^3)–H) for ethylbenzene, toluene, and cyclohexane, indicate a linear relationship with a negative slope. These findings clearly indicate that the reaction occurs via a rate-determining step that involves hydrogen-atom abstraction by a Mn(O) species and a subsequent rebound hydroxylation process which is similar to the reaction mechanism of cytochrome P450.

Myoglobin, an oxygen storage protein, has a heme *b* cofactor which is also used by heme-dependent enzymes such as horseradish peroxidase and cytochrome P450s.[1] However, myoglobin has very weak peroxidase and monooxygenase activities and does not catalyze alkane hydroxylation via a C–H bond activation upon the addition of H_2O_2 or peracids as oxygen donors. Therefore, molecular engineering of myoglobin for enhancement of enzymatic activities is a challenging study. Advances in this area are expected to lead to generation of new biocatalysts and to improve our understanding of the catalytic mechanisms of native enzymes. Methods used to modify hemoproteins include site-directed mutagenesis of amino acid residues in the heme pocket[2] and replacement of native heme with an artificial cofactor.[3] We have recently enhanced the peroxidase activity of myoglobin by the latter method using artificial cofactors including an iron porphyrinoid[4] and a propionate-modified heme.[5] Furthermore, a hemoprotein reconstituted with a manganese porphyrinoid is expected to provide a good candidate for modulating the function of myoglobin, because manganese porphyrins and porphyrinoids such as manganese corrole and corrolazine have been studied as catalysts of C–H bond activation.[6] For example, 9,10-dihydroanthracene, cyclohexane-1,4-diene, and their derivatives are often employed as substrates for Mn^V(O) porphyrinoids, whereas the number of C–H bond activations for a simple alkane with a large bond dissociation energy (BDE) value for the C(sp^3)–H bond is quite limited. However, there have been no examples of clear enhancement of the enzymatic activity of hemoproteins as a result of manganese porphyrinoid substitution.[7] Here, we report the first example of C(sp^3)–H bond hydroxylation catalyzed by myoglobin reconstituted with manganese porphycene as shown in Figure 1.

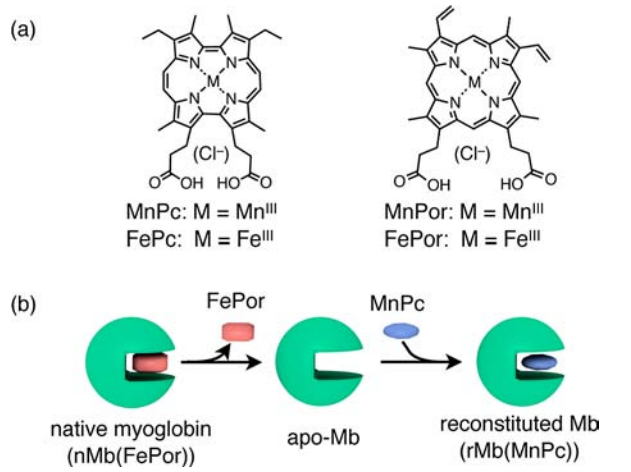

Figure 1. (a) Molecular structures of the metal porphycenes and porphyrins. (b) Schematic representation for the preparation of rMb(MnPc).

Porphycene, a constitutional porphyrin isomer, has remarkably different physicochemical properties than those of porphyrin.[8] We inserted a manganese ion into the previously reported porphycene (Pc) macrocycle and hydrolyzed the propionate diester moieties to yield the manganese(III) complex, MnPc. This cofactor was then incorporated into the apomyoglobin obtained by removal of heme from horse heart myoglobin. The reconstituted protein, rMb(MnPc), was purified and characterized by ESI-MS and UV–vis spectroscopic methods. The detected mass number was assigned as a multiply ionized species of rMb(MnPc) (found m/z = 1953.4, calculated m/z (z = +9) = 1953.3). This indicates that MnPc makes a stable complex with the apoprotein. The UV–vis spectrum showed the red shift in the Q-region (λ_{max} = 568 and

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631 nm) due to imidazole ligation of histidine compared to free MnPc (Figure S1).

The crystal structure of rMb(MnPc) was further obtained at 2.2 Å resolution and revealed His93-ligation to Mn with a bond length of 2.58 Å in the heme pocket (Figure 2a). The

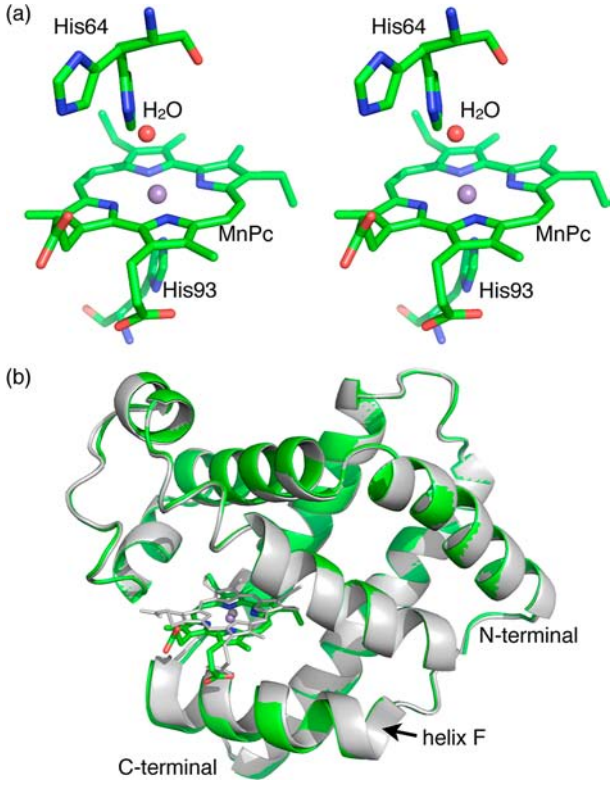

Figure 2. (a) Stereoview of the crystal structure of rMb(MnPc) around MnPc (this study, PDB ID: 3WI8). The bonds to Mn are not shown for clarity. The distal His64 residue has two conformers. (b) Superimposition of rMb(MnPc) (green) and rMb(MnPor) (gray, PDB ID: 2O58).

interaction between two propionate side chains and surrounding amino acid residues was different from that of native myoglobin (nMb(FePor)) and myoglobin reconstituted with a protoporphyrin IX manganese complex (rMb(MnPor)), but generally similar to that of myoglobin reconstituted with iron porphycene, rMb(FePc) (Figure S2).[9] The polypeptide structures of rMb(MnPc) and rMb(MnPor)[10] are superimposable with an RMSD (root-mean-square deviation) value of 0.37 Å for Cα atoms. It is apparent that helix F moves to the opposite side of the cofactor. The other helices are positioned similar to those of nMb(FePor) (Figure 2b). The coordination bond between Mn and His93 in rMb(MnPc) is approximately 0.36 Å longer than that of rMb(MnPor) (Table S2), and the temperature factors (*B*-factors) of the helix F and MnPc are notably higher (Figure S3). These results suggest that MnPc is relatively loosely bound to apoMb.[11] On the distal side of the heme pocket, the His64 residue clearly adopts two different conformations. The occupancy of the coordinated water molecule to Mn is small (roughly 0.5), and the O−Mn bond of rMb(MnPc) is longer with a distance of 2.79 Å. According to this crystal structure, the presence of MnPc in the heme pocket will provide both six- and five-coordinate species.

Myoglobin-catalyzed hydroxylation of ethylbenzene was carried out upon addition of H_2O_2 at pH 8.5 and 25 °C. Figure 3 shows that rMb(MnPc) promotes hydroxylation and provides only 1-phenylethanol as a product with a total

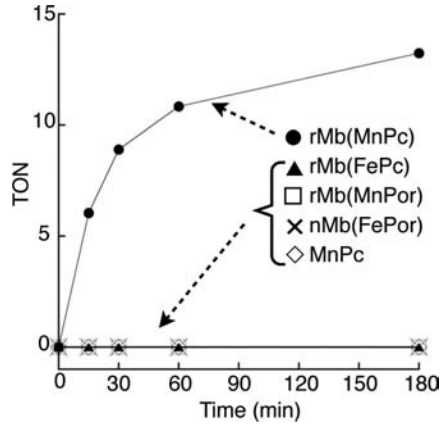

Figure 3. Time course plots of catalytically produced 1-phenylethanol by Mbs and MnPc. Conditions: [protein or MnPc] = 20 μM, [H_2O_2] = 10 mM, [ethylbenzene] = 8 mM, in 100 mM potassium phosphate buffer, pH 8.5, containing 8% CH_3CN at 25 °C. In the case of MnPc, DMSO (1% v/v) was added to the solution for complete dissolution of the complex.

turnover number (TON) of 13 and initial turnover frequency (TOF) of 33 h^{-1}.[12] In addition, no byproducts (such as phenol or ketone derivatives) were detected in this reaction. Furthermore, the protein matrix plays an important role in the hydroxylation because free MnPc does not produce any products under the same conditions. It is noteworthy that other myoglobins do not have ethylbenzene hydroxylation activity. Table 1 summarizes H_2O_2-dependent hydroxylation and

Table 1. Hydroxylation and Sulfoxidation Activities of Myoglobins[a]

	ethylbenzene[b]		thioanisole[c]	
substrate	TON[d]	TOF (h^{-1})[e]	TON[d]	TOF (h^{-1})[f]
protein				
rMb(MnPc)	13	33	61	31
rMb(FePc)	0	0	157	142
rMb(MnPor)	0	0	0	0
nMb(FePor)	0	0	89	61

[a]Reactions were monitored in 100 mM potassium phosphate buffer at 25 °C. [b]Conditions: [protein] = 20 μM, [H_2O_2] = 10 mM, [ethylbenzene] = 8.0 mM in the buffer, pH 8.5, containing 8% CH_3CN. [c]Conditions: [protein] = 8 μM, [H_2O_2] = 10 mM, [thioanisole] = 2.0 mM in the buffer, pH 7.0, containing 1% CH_3CN. [d]Turnover number after completion of the reaction. [e]Turnover frequency at 5 min after starting the reaction. [f]Turnover frequency at 30 min after starting the reaction. TON or TOF = 0 indicates that the product was not detected by GC analysis.

sulfoxidation promoted by myoglobins. Interestingly, the activity of rMb(MnPc) toward thioanisole sulfoxidation is much lower than that of nMb(FePor), whereas the TON and TOF values of rMb(FePc) for the sulfoxidation are significantly higher than those for other myoglobins. These results indicate that high oxidation potential is not necessary for alkane hydroxylation[13] and appears rather to provide a disadvantage in the case of a myoglobin-based system.[14] On the other hand, rMb(MnPor) has no hydroxylation or sulfoxidation activity due to poor reactivity with H_2O_2.[7a] For rMb(MnPc), the formation

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of the compound I-like species, a two electron-oxidized $Mn^{V}(O)$ species,[6] was preliminarily identified in a reaction with cumene hydroperoxide, which is a useful reagent to evaluate the O−O bond cleavage in peroxide. The ratio of two products, cumyl alcohol and acetophenone, which are derived from heterolytically and homolytically peroxide-cleaved products, respectively, is determined to be 7.0 at pH 7.0. This value is 2.5-fold larger than that of nMb(FePor), and generally similar to the value obtained for rMb(FePc).[4a,15] Given this finding, it appears that the addition of H_2O_2 to rMb(MnPc) predominantly generates a compound I-like intermediate in the catalytic cycle.

Next, to determine whether the mechanism promoted by rMb(MnPc) includes the C−H activation, deuterated ethylbenzene, C_8D_{10}, was employed as a substrate and the initial rate constants for the hydroxylation of C_8H_{10} and C_8D_{10}, k_H and k_D, respectively, were evaluated. The obtained kinetic isotope effect, KIE (k_H/k_D), determined at 25 °C was 2.4 (Figure S4). This value is lower than the theoretical value (ca. 7), but the difference in kinetics between the normal substrate and the deuterated substrate is clearly observed.[16] Furthermore, the initial rates of the hydroxylations of ethylbenzene, toluene, and cyclohexane were logarithmically plotted against BDE(C(sp^3)−H) for each substrate, and a linear relationship was identified with a negative slope as shown in Figure 4a.[17,18] These findings

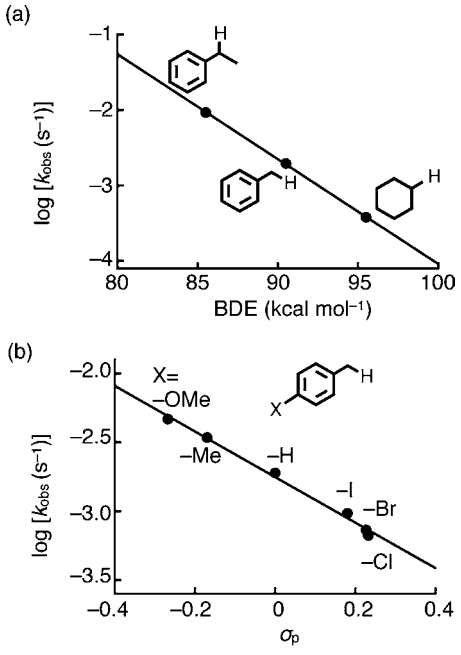

Figure 4. (a) Plots of log k_{obs} for the catalytic hydroxylation of ethylbenzene, toluene, and cyclohexane against each bond dissociation energy value (BDE).[18] (b) Plots of log k_{obs} for the catalytic hydroxylation of *para*-substituted toluene against the Hammett constant, σ_p.[19] The values for k_{obs}, initial rate, were obtained as TOF in the reaction at 5 min.

provide support for our proposal that hydrogen-atom abstraction from the alkane C−H bond by the compound I-like species of rMb(MnPc) is the rate-determining step. In addition, the substituent effect of toluene derivatives is evaluated using *para*-methoxy-, methyl-, iodo-, bromo-, and chloro-toluenes. The Hammett plot of σ_p values for each substrate shows the linear relationship with the ρ value of −1.65 (Figure 4b). The negative value implies that the transition state for C−H activation partially includes the electron transfer process.[19] Moreover, the isotope-labeling study using $H_2{}^{18}O_2$ yields the ^{18}O-labeled alcohol quantitatively. This effectively rules out the oxygen atom transfer from a water molecule or dissolved O_2 and indicates that the OH-rebound mechanism occurs after the H-abstraction from the substrate alkane C−H bond by the $Mn^{V}(O)$ species of rMb(MnPc). Taken together, all of these findings demonstrate that the catalytic hydroxylation reaction proceeds through the cytochrome P450-like C−H bond activation process as follows: $Mn^{V}(O)Pc + R{-}H \rightarrow Mn^{IV}(OH)Pc + R\cdot \rightarrow Mn^{III}Pc + R{-}OH$.[20]

In conclusion, myoglobin reconstituted with manganese porphycene catalyzes H_2O_2-dependent alkane hydroxylation in the heme pocket. This is remarkable because native myoglobin does not have hydroxylation activity. One of the reasons proposed for the lack of such activity is that myoglobin has no definite substrate-binding site such as the binding sites present in the P450s. Watanabe and co-workers have reported the hydroxylation of Trp43 which is close to heme within the heme pocket of the F43W/H64L myoglobin mutant.[21] In contrast, our work presented here first demonstrates that the catalytic activity of myoglobin toward substrate hydroxylation can be dramatically improved only by the insertion of MnPc into apomyoglobin. This finding obviously indicates that a $Mn^{V}(O)$ porphycene complex is a unique intermediate capable of promoting C(sp^3)−H bond activation followed by H-abstraction and OH-rebound to give a corresponding alcohol. The myoglobin protein matrix is essential for this reaction, and it is of interest that the C−H bond activation mechanism is similar to that of cytochrome P450s. Further mechanistic study and optimization of the catalytic reaction conditions are now in progress.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures, experimental details, and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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