Enhancement of Peroxygenase Activity of Horse Heart Myoglobin by Modification of Heme-propionate Side Chains

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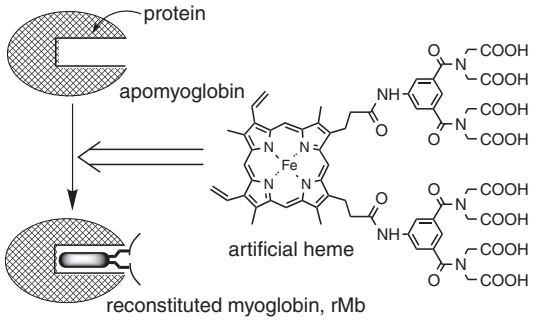
A myoglobin reconstituted with an artificially created heme having two benzene moieties bound at the terminal of two heme-propionates shows a clear enhancement in peroxygenase activity toward thioether and styrene oxidations due to the expanding hydrophobic heme pocket.

Modification of hemoproteins such as myoglobin, heme peroxidases or cytochromes is generally carried out by site-directed mutagenesis.[1] Particularly, myoglobin is one of the best scaffolds to engineer functions into the protein. It is known that the active site modification of myoglobin by replacement of an amino acid at the distal site of the heme pocket with an appropriate one enhances the peroxidase and/or peroxygenase activity.[1–6] However, there have been few examples which demonstrate the construction of an artificial substrate binding site on the protein surface by mutagenesis.[7,8] In contrast, we have focused on a different approach, that is, replacement of native heme with a modified heme, to create a new binding domain in the myoglobin.[9–11] For example, we have recently reported that the modification of two heme-propionate side chains by introducing hydrophobic benzene moieties accelerates the one-electron oxidation of phenol derivatives such as guaiacol in the presence of hydrogen peroxide,[10] whereas native myoglobin, nMb, shows little peroxidase activity. In the next stage, it is of particular interest to study the oxygenation from oxoferryl species by the reconstituted myoglobin having an artificially created substrate binding site. In this communication, we wish to report the conversion of myoglobin into peroxygenase by chemical modification of the heme-propionate side chains.

The modified heme, which has two benzene moieties linked at each terminal carboxylate of the two heme-propionates in the native heme, has been prepared and incorporated into the horse heart apomyoglobin to yield a reconstituted myoglobin, rMb.[12] Based on computer modeling, the benzene moieties may form a hydrophobic pocket on the myoglobin surface. It is expected that a neutral small substrate such as thioanisole or styrene may be of suitable size for the artificial binding site in rMb. Thus, we investigated the peroxygenase activity of the myoglobins toward the substrate oxidation in the presence of H_2O_2. The general procedure for the thioanisole oxidation catalyzed by myoglobins is as follows: A solution of myoglobin (5.0 μM; pH 7.0, 100 mM phosphate buffer) was incubated upon the addition of H_2O_2 (5.0 mM) at 20 °C.[13] The product was mainly the corresponding sulfoxide characterized by GC–MS. The oxidation process was monitored by HPLC after the addition of thioanisole (0–1.5 mM).[14] The catalytic reaction followed the conventional Michaelis-Menten kinetics as shown in Figure 1. From the plots, we determined the kinetic parameters, K_m and k_{cat} as shown in Table 1. In particular, the K_m value of the thioanisole oxidation by rMb is 6-fold smaller than that observed for the nMb, thus, the catalytic efficiency toward the thioanisole oxidation in rMb, which is represented by the k_{cat}/K_m value, is significantly increased compared to that observed in the nMb. In addition, the incorporation ratio of the 18-labeled oxygen atom into the product upon the addition of $H_2{}^{18}O_2$ was 96%, suggesting that the activated oxygen of the oxoferryl species as a reaction intermediate is directly transferred to the substrate in the heme pocket. These findings support the fact that the artificial substrate binding site enhances the peroxygenase activity of myoglobin.

Next, to evaluate the selectivity of the substrate binding



Scheme 1.

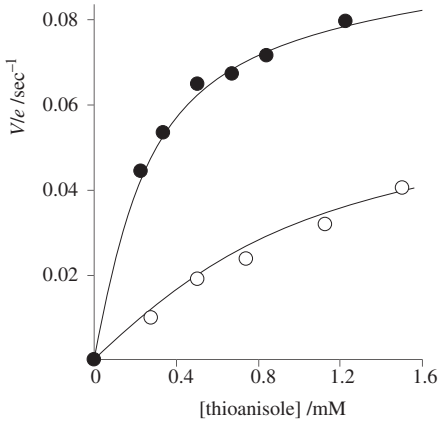


Figure 1. Initial rate, V, of myoglobin-catalyzed oxidation as a function of thioanisole concentration for a fixed amount of myoglobin. Closed and open circles represents the plots for rMb and nMb, respectively. [rMb] = [nMb] = 5.0 μM, [H_2O_2] = 5.0 mM, pH 7.0 (100 mM phosphate buffer) at 20 °C.

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Table 1. Kinetic Parameters and Stereochemistry for the Thioanisole Oxidation Catalyzed by rMb and nMb[a]

Protein	rMb	nMb
K_m	0.28 ± 0.03	1.7 ± 0.2
k_{cat}	0.096 ± 0.010	0.086 ± 0.009
K_m/k_{cat}	0.34	0.050
Initial rate[b] (relative rate)	6.18	1
Incorporation of ^{18}O from $H_2{}^{18}O_2$[c]	96 ± 3	92 ± 3

[a]reaction conditions: [nMb] = [rMb] = 5.0 μM, [thioanisole] = 0–1.5 mM, [H_2O_2] = 5.0 mM, at 20 °C, pH 7.0 (100 mM phosphate buffer). Reaction was monitored by HPLC equipped with YMC Pack ProC4. [b]Turnover frequency per minute during initial stage. [c]Proportionation of 18-labeled oxygen in sulfoxide determined by GC–MS.

site, we compared two substrate oxidations catalyzed by rMb and the native protein. The initial turnover rate of thioanisole oxidation by rMb ($v_{init.} = 1.3\,min^{-1}$) is 6.2-fold faster than that observed for the nMb, whereas the relative rate for the oxidation of the anionic substrate, 4-carboxythioanisole, by rMb is reduced to approximately 60% of that observed for the nMb under the same conditions.[15] This clear difference indicates that the neutral thioanisole can be easily bound into the artificially created heme pocket, whereas the charged substrates such as 4-carboxythioanisole will not easily access the hydrophobic binding site.

Furthermore, it is known that styrene is also one of the suitable substrates to monitor the peroxygenase activity.[4,16,17] The styrene oxidation catalyzed by rMb was monitored by a procedure similar to the thioanisole oxidation. The GC–MS and HPLC analyses support the formation of three products, styrene oxide, phenylacetaldehyde and benzaldehyde, that were identified by comparison with authentic samples, where no decomposition of styrene oxide occurred under the HPLC analytical conditions. The product ratio between styrene oxide and phenylacetaldehyde by rMb is 4.0 : 1.0 which is comparable with that observed by nMb. The initial turnover rate of styrene oxidation catalyzed by rMb ($v_{init.} = 0.28\,min^{-1}$) is more than 10-fold faster than that observed for the nMb.[18] This finding indicates that the modified heme pocket formed by the artificial prosthetic group plays an important role in the acceleration of styrene oxidation in the presence of H_2O_2.

In conclusion, the peroxygenase activity toward hydrophobic thioanisole and styrene oxidation is enhanced by the modification of the heme-propionate side chains.[19–22] This result strongly suggests that further fine-tuning of the artificial moieties at the terminals of the propionates in myoglobin will give us a unique oxidation catalyst with a high substrate specificity and/or stereoselectivity.

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References and Notes

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