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Porphyrinoid Chemistry in Hemoprotein Matrix: Detection and Reactivities of Iron(IV)-Oxo Species of Porphycene Incorporated into Horseradish Peroxidase

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The porphyrin high-valent iron complex is one of the current research targets with respect to investigations of the reactive intermediates in the catalytic cycles of heme-containing oxygenases.[1] Normally, such highly oxidized states generated in model complexes for the hemoproteins have been treated in organic solvents without moisture and dioxygen at low temperature because of their unstable properties.[1,2] However, in the heme pocket of horseradish peroxidase (HRP) containing protohemin IX, **1**, the iron(IV)-oxo porphyrin π-cation radical (so-called "Compound I") and its one-electron-reduced form (iron(IV)-oxo called "Compound II") are observable in water at room temperature.[3] Therefore, it is likely that the protein matrix of HRP is an appropriate tool for investigating the highly oxidized species of the heme derivatives.

Iron porphycene is a structural isomer of iron porphyrin, in which two bipyrroles are linked to two ethylene bridges.[4] Its physicochemical properties including the redox and spectroscopic behaviors have been collected,[5] whereas very limited reports on the iron(IV)-oxo states of porphycene are available.[6] We have recently prepared an artificially created prosthetic group **2** (Chart 1) using the porphycene framework and inserted it into apomyoglobin to evaluate the reactivities of the iron porphycene in the myoglobin matrix.[7] On the basis of that study, the peroxidase activity of the reconstituted protein with **2** was slightly enhanced, compared to the native myoglobin;[7c] however, it was still hard to detect the intermediates upon the addition of H_2O_2 to the reconstituted protein. In this paper, we report the first evidence of the formation of the iron(IV)-oxo porphycene π-cation radical of **2** in the HRP matrix[8] and disclose the peroxidase activities of the reconstituted HRP with **2**, rHRP(**2**).

Iron porphycene **2** was smoothly inserted into apoHRP under neutral conditions.[9] The UV−vis spectrum of the ferric rHRP(**2**) shows two characteristic bands at 382 and 621 nm with a shoulder around 550 nm (spectrum A in Figure 1a). This feature is similar to that of the myoglobin reconstituted with **2**,[7] suggesting that the iron atom of **2** is coordinated by a histidine residue of rMb(**2**). The EPR spectrum of rHRP(**2**) at 5 K mainly shows low-spin signals ($S = 1/2$), although the native HRP (nHRP) exhibits a high-spin character ($S = 5/2$) (Figure S1). The low-spin character of rMb(**2**) stems from the large ligand field splitting due to the low symmetry in the porphycene framework.[10]

We first carried out the reaction of rHRP(**2**) with $H_2O_2/K_4[Fe(CN)_6]$ or H_2O_2/guaiacol (2-methoxyphenol), followed by a UV−vis spectral analysis (Figure 1). Upon the addition of 1 equiv of H_2O_2 to the ferric rHRP(**2**), the absorbances at 382 and 623 nm decreased with slight red shifts to give spectrum B in the figure. The addition of 1 equiv of $K_4[Fe(CN)_6]$ (a one-electron reductant) to the solution produced spectrum C, and the further addition of $K_4[Fe(CN)_6]$ gave the original spectrum of the ferric rHRP(**2**). Very similar spectral changes were also observed in the reaction with guaiacol. Spectrum B changed to the original spectrum A directly when thioanisole was added. Furthermore, we observed the appearance and disappearance of the band around 800 nm after the addition of H_2O_2 and $K_4[Fe(CN)_6]$, respectively (Figure 1b and c), indicative of the transient formation of the porphycene ring π-radical.[8,10] On the basis of a series of spectral changes, the first species formed by H_2O_2 can be identified as the iron(IV)-oxo porphycene π-cation radical, and the second intermediate was assigned to the iron(IV)-oxo species.

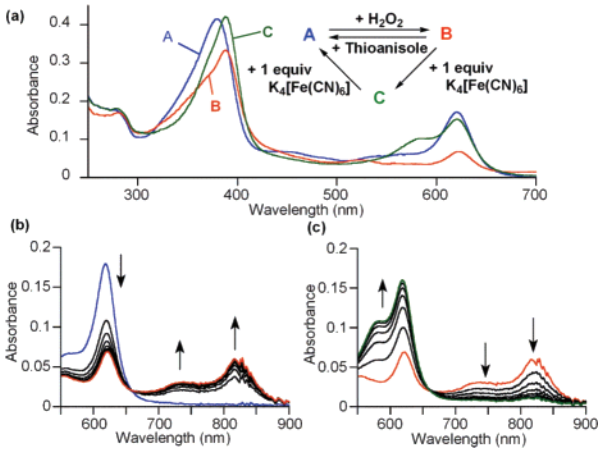

Figure 1. (a) UV−vis spectra of the intermediates in the reaction of rHRP(**2**) with $H_2O_2/K_4[Fe(CN)_6]$ in 50 mM sodium phosphate buffer (pH = 7.0) at 10 °C. Spectrum A: ferric rHRP(**2**) (5.7 μM). Spectrum B: after the addition of 1.1 equiv of H_2O_2. Spectrum C: after the addition of 1 equiv of $K_4[Fe(CN)_6]$ to B. (b) Spectral changes every 4 ms over 40 ms in the near-infrared region for the reaction of rHRP(**2**) with H_2O_2. (c) Spectral changes every 10 ms over 100 ms in the near-infrared region after the first addition of 1 equiv of $K_4[Fe(CN)_6]$.

Chart 1. Structures of Protohemin IX **1** and Iron Porphycene **2**

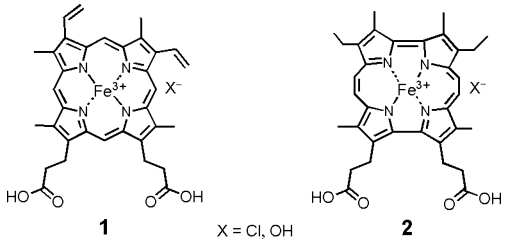

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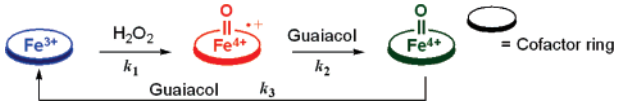
Table 1. Turnover Number (TN) of Catalytic Oxidations and Rate Constants of Each Process Involved in Guaiacol Oxidations[a,b]

protein	guaiacol oxidation[c,d] (TN min^{-1})	thioanisole oxidation[d,e] (TN min^{-1})	k_1[f,g] ($M^{-1}s^{-1}$)	k_2[f,h] ($M^{-1}s^{-1}$)	k_3[f,i] ($M^{-1}s^{-1}$)
nHRP	2400 ± 100	1.4 ± 0.1	$(1.0 \pm 0.1) \times 10^7$	$(4.4 \pm 0.5) \times 10^6$	$(2.2 \pm 0.3) \times 10^5$
rHRP(**2**)	2300 ± 100	17 ± 2	$(1.2 \pm 0.1) \times 10^6$	$>10^8$[j]	$(9.0 \pm 1.0) \times 10^5$

[a] In 50 mM sodium phosphate buffer (pH = 7.0). [b] The data were taken as the averages of 3-run experiments. [c] [HRP] = 0.1 μM, $[guaiacol]_0$ = 300 μM, $[H_2O_2]_0$ = 4 mM. [d] At 25 °C. [e] [HRP] = 4.7 μM, $[thioanisole]_0$ = 4.4 mM, $[H_2O_2]_0$ = 4 mM. [f] At 10 °C. [g] Rate constant of iron (IV)-oxo π-radical formation. [h] Rate constant of iron(IV)-oxo formation. [i] Rate constant of regeneration of a ferric state. [j] Too fast to precisely determine because 40% of the reaction was completed within the dead-time of the stopped-flow apparatus.

Scheme 1. Reaction of HRP with H_2O_2/Guaiacol



Next, the peroxidase activity of rHRP(**2**) was investigated. Both rHRP(**2**) and nHRP show a similar turnover number for the oxidation of guaiacol, whereas the superiority of rHRP(**2**) was exhibited for the thioanisole oxidation (Table 1).

In order to discuss the reaction mechanism, the kinetic analyses for each process of the guaiacol oxidation were carried out according to Scheme 1.[12] The spectral changes after mixing a ferric protein with excess H_2O_2 were followed by simple one-phase kinetics (Figures S2−S4). On the other hand, when the iron(IV)-oxo porphycene π-cation radical was mixed with excess guaiacol,[13] two-phase spectral changes were observed (Figures S5 and S6). The first and the second stages were attributed to the formation of the iron(IV)-oxo and the regeneration of the ferric state from the iron(IV)-oxo, respectively. The determined rate constants are described in Table 1. Interestingly, the value of k_1 for rHRP(**2**) was found to be 10-fold smaller than that of nHRP, whereas k_2 and k_3 for rHRP(**2**) are larger than the corresponding values of the nHRP. Therefore, we can conclude that the ferric state of rHRP(**2**) is *less reactive* toward H_2O_2, whereas the ferryl species are *more reactive* during oxidations, compared to nHRP.

The low reactivity of ferric rHRP(**2**) probably originates from its low-spin character.[14] The larger values of k_2 and k_3 for rHRP(**2**) suggest the more positive redox potentials of the oxo-ferryl species, compared to nHRP. It is known that the redox potentials of these species for nHRP can be determined by the oxidation of the ferric species by K_2IrCl_6 (+892 mV vs SHE).[15] However, the iridium complex was found to be insufficient for the oxidation of ferric rHRP(**2**), indicating that the redox potentials of the ferryl species for rHRP(**2**) are more positive than those for nHRP. The reactivity of iron(IV)-oxo porphycene π-cation radical significantly contributes to the catalytic activity for the thioanisole oxidation.[16] Therefore, the high catalytic activity of rHRP(**2**) for the thioanisole oxidation is consistent with the kinetic analysis. On the other hand, the overall rate of guaiacol oxidation would be determined by the delicate balance of the reaction rates during each step, leading to the similar catalytic activities between the two proteins.

In conclusion, we successfully detected iron(IV)-oxo porphycene π-cation radical above 0 °C in water using the HRP matrix. The kinetic study indicates that the reactivities of both intermediate species are clearly higher than those observed for the native heme. This finding will provide a valuable insight into understanding high-valent metal complex chemistry of a series of tetrapyrrole ligands.

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Supporting Information Available: Detailed procedure of the preparation of rHRP(**2**), EPR spectra, GC−MS data, and the kinetic measurements and analyses for guaiacol oxidation are described. This material is available free of charge via the Internet at http://pubs.acs.org.

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