The Reaction of Hemoproteins with Hypochlorous Acid

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We have studied absorption spectral changes and heme modifications in the reaction of horseradish peroxidase (HRP), myoglobin (Mb), or bovine liver catalase (BLC) with hypochlorous acid (HOCl). Under acidic condition, HOCl is easily released from an Fe^{III}–HOCl complex. In contrast, the O–Cl bond cleavage in an Fe^{III}–OCl intermediate occurs under neutral and alkaline conditions. The heterolysis proceeds in HRP and BLC, but the homolysis in Mb seems to be associated with the generation of meso-chlorinated heme adduct.

An important function of the heme enzyme like myeloperoxidase (MPO) is the oxidation of halide with hydrogen peroxide to produce hypohalous acids (HOX, X = Cl and Br) for defense against infection.[1] The crystal structure of MPO reveals that the heme iron is in the six-coordinated high-spin state with His-336 as the proximal histidine and a water molecule as the sixth ligand.[2] More interestingly, 1- and 5-methyl groups of the heme prosthetic group form ester linkages with Glu-242 and Asp-94, respectively, and 2-vinyl side chain is covalently modified with Met-243 to form a vinyl–sulfonium bond (See Supporting Information Figure S1).[11] The linkages appear to play important roles in controlling the redox potential of the catalytic intermediate, maintaining a solvent network in the active site, and protecting heme from undesired modifications by its own reaction product (i.e., hypohalous acids).

In order to study the modification of prosthetic group in the reaction of hemoproteins with hypochlorous acid (HOCl) further more, we have decided to utilize horseradish peroxidase (HRP), myoglobin (Mb), and bovine liver catalase (BLC).[3] Those hemoproteins share the same prosthetic group that is heme b; however, HRP, Mb, and BLC bear imidazolate, imidazole, and tyrosinate as the proximal ligands, respectively. Consequently, the extent of electron donation to the heme iron by the proximal ligands differs among the proteins. In this study, the reaction with hypochlorous acid was performed under acidic, neutral, or alkaline condition, and the modification of prosthetic group in the hemoproteins was investigated by high-pressure liquid chromatography (HPLC) as well as mass spectrometry (MS).

The absorption spectrum of HRP exhibits the Soret band at 402 nm when the heme iron is in the ferric state (i.e., iron(III)).[4] The addition of hypochlorous acid to HRP does not cause any spectral changes at pH 5 (Figure 1a). The equilibrium between the ferric heme iron and its hypochlorous acid complex (i.e., $Fe^{III} + HOCl \rightleftarrows Fe^{III}$–HOCl) seems to be shifted to the iron(III) state under the condition. In contrast, at pH 7 and 9, we observed the decrease in the Soret together with the increase in the visible bands upon the mixing of HRP and hypochlorite (i.e., ^{-}OCl) (Figure 1b). The spectrum is typical for a ferryl porphyrin radical cation (i.e., Fe^{IV}=O $Por^{\bullet+}$, Por = porphyrin), which is considered as a two-electron oxidation product of the ferric state.[4] The ability for the species to perform an oxidation

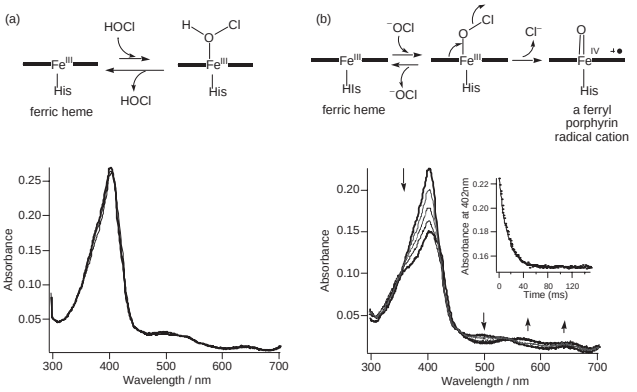

Figure 1. Absorption spectral changes for the reaction of HRP with hypochlorous acid at (a) pH 5 in 0.025 M sodium citrate buffer and (b) pH 9 in 0.05 M $Na_2B_4O_7$, 0.2 M H_3BO_3, and 0.05 M NaCl buffer. The spectra at 0, 7, 15, 30, and 50 ms after mixing are shown. The ferric state of HRP (thick solid line) is transformed into a ferryl porphyrin radical cation (dotted line) at pH 9. The inset indicates the changes in absorbance at 402 nm.

reaction was confirmed by the production of phenoxy radical in the solution containing HRP, hypochlorite, and guaiacol.[5] The results suggest that the heterolytic cleavage of the O–Cl bond in an Fe^{III}–OCl intermediate preferentially occurs under neutral and alkaline conditions to degrade hypochlorous acid and forms a ferryl porphyrin radical cation.

Next, we have examined if HRP reacts with H_2O_2 and Cl^{-} to generate hypochlorous acid and modify the prosthetic group. The heme adducts were extracted after the reaction, loaded on an ODS column, and the retention times as well as the values of molecular ions of modified heme adducts were compared with those in previous reports.[6–9] When the incubation was performed at pH 5, two polar heme adducts (peaks 1 and 2 in Figure 2a) were eluted before heme b (peak 3 in Figure 2) was washed out from the column. The molecular mass of the new adducts indicated the addition of one chlorine atom and one hydroxy group to heme b (Figure S2).[11] Thus, hypochlorous acid seems to transform the vinyl side chain of heme into chlorohydrin (i.e., –CH=CH_2 → –CHOH–CH_2Cl). It is interesting to note that the monochlorinated heme adduct, which was presumably generated by the substitution of the *meso*-hydrogen with chlorine, was detected in the reaction mixture (peak 4 Figure 2a). The electrophilic addition of HOBr to the vinyl group was also observed in the reaction of HRP with H_2O_2 and Br^{-} under acidic condition (Figure 2b). Furthermore, HRP bearing the modified heme adducts was found to exhibit only 10% of peroxidase activity with respect to the native enzyme. The result supports an idea that the active site of a hypochlorous-acid-producing hemoenzyme needs to be protected from undesired chemical modifications. On the contrary, at pH 7 and 9, the vinyl group modifications were not observed. These seem to

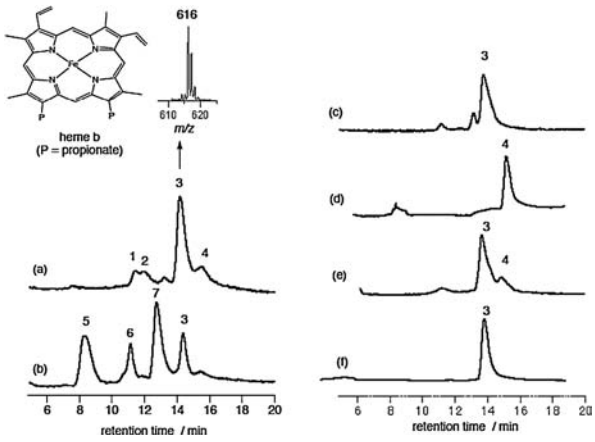

Figure 2. HPLC traces of the prosthetic group extracted from the solution containing (a) HRP with H_2O_2 and Cl^-, (b) HRP with H_2O_2 and Br^-, (c) BLC with hypochlorous acid, (d) Mb with hypochlorous acid, (e) HRP with hypochlorous acid, and (f) Mb. Inset; the structure and the mass spectrum of heme b corresponding to peak 3. The molecular ion of heme b was observed at m/z 616. Four types of modified heme adducts, peaks 1 & 2 (m/z 668, heme b + OH + Cl), peak 4 (m/z 650, heme b − H + Cl), peak 5 (m/z 808, heme b + 2OH + 2Br), and peaks 6 & 7 (m/z 712, heme b + OH + Br), were detected in the reaction mixtures (Figure S2).[11] The reactions (a) and (b) were performed in 0.025 M sodium citrate at pH 5. For (c), (d), and (e), 100 equiv excess of hypochlorous acid with respect to the protein was mixed with BLC, Mb, and HRP, respectively, in 0.05 M $Na_2B_4O_7$, 0.2 M H_3BO_3, and 0.05 M NaCl at pH 7. Essentially the same results were obtained at pH 9. The extraction of heme b from Mb solution affords HPLC trace (f).

be reasonable observations because hypochlorous acid is degraded on the heme iron under neutral and alkaline conditions (Figure 1b).

The chlorination of *meso*-carbon could proceed via the electrophilic substitution of aromatic hydrogen atom by HOCl (Scheme S1a)[11] or the homolytic cleavage of O–Cl bond in an Fe^{III}–OCl intermediate (Scheme S1b).[11] If the Fe^{III}–OCl intermediate is responsible for the reaction, the meso-chlorination should be facilitated in a hemoprotein with a neutral ligand because electron donation to the heme iron is not strong enough to promote the heterolysis. The hypothesis was examined by mixing hypochlorous acid with Mb, HRP, and BLC, which respectively have imidazole, imidazolate, and tyrosinate as the proximal ligand. As expected, heme b is recovered from BLC treated with hypochlorous acid (Figure 2c), but the meso-chlorinated adduct was formed mainly in the heme pocket of Mb (Figure 2d). A partial conversion of heme b to the meso-chlorinated adduct was observed in HRP (Figure 2e). Rapid-scanning stopped-flow experiments suggest that BLC reacts with hypochlorite to generate a ferryl porphyrin radical cation via the heterolytic cleavage of O–Cl bond in the Fe^{III}–OCl intermediate (Figure S3a).[10,11] In contrast, a ferryl species (i.e., Fe^{IV}=O), which is produced by the homolysis of O–Cl bond, is observed by mixing Mb and hypochlorite (Figure S3b).[11]

In summary, we report herein that (1) the Fe^{III}–HOCl intermediate readily releases HOCl under acidic condition, (2) the Fe^{III}–OCl intermediate of HRP and BLC is transformed into a ferryl porphyrin radical cation via a heterolytic cleavage of O–Cl bond under neutral and alkaline condition, and (3) the homolytic cleavage of O–Cl bond in the Fe^{III}–OCl intermediate preferentially occurs in Mb to generate the meso-chlorinated adduct probably because the electron donation of proximal ligand is weaker than that of HRP and BLC. The results suggest the involvement of Fe^{III}–OCl intermediate in the meso-chlorination reaction. These imply that MPO, a hypochlorous-acid-producing enzyme, utilizes an anionic imidazolate ligand for the heme iron and performs the reaction under acidic condition to decrease the formation of meso-chlorinated heme adduct.

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11 Supporting Information is available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/.

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