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Generation of Superoxide in a Water-Soluble Model System with FeTMPyP[#]

Taku Nakano,* Kanae Ohto, Fumito Okafuji, Yoshihiro Mori, Hiroko Kakuda, Yasumaru Hatanaka, and Noriyoshi Masuoka[1]

Department of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University,
2630 Sugitani, Toyama 930-0194

[1]Department of Biochemistry, Okayama University Graduate School of Medicine and Dentistry,
2-5-1, Shikata-cho, Okayama 700-8558

Received October 28, 2004; E-mail: tnakano@ms.toyama-mpu.ac.jp

In a model system of a water-soluble hemin, tetrakis(4-methylpyridyl)porphinatoiron(III) pentachloride ($FeTMPyPCl_5$), with 3-methyl-2-benzothiazolinone hydrazone (MBTH), superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide, and then hydroxyl radical were generated in a phosphate buffer solution (pH 6.0–7.5) under aerobic conditions. The hemin was degraded in this system. The reactive oxygen species were deduced from inhibition of the degradation by adding SOD and catalase, and one of them was trapped by 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) as a DMPO–hydroxyl radical adduct, which was detected by ESR. The mechanism for activating dioxygen and the degradation of hemin was examined, and it was proposed to be the initial one-electron reduction of hemin by MBTH forming a dioxygen complex and liberating superoxide as one of the routes during the reduction–autoxidation cycle in aqueous media.

The generation of a superoxide anion radical (superoxide[1]) is a well-known process in biological systems.[2] Reactive oxygen species (ROS) were reported to be generated, or hinted, in natural systems including heme-proteins, such as NAD(P)H oxidase in neutrophil and macrophage,[3,4] Cytochrome P450,[5,6] hemoglobin, and myoglobin.[7–11] In systems of hemoglobin and myoglobin, dioxygen coordinates on the heme and then proceeds with two processes: the main and important role is the liberation of dioxygen; the other is the generation of superoxide during autoxidation. There remains a question that the autoxidation shows a peculiar behavior, depending on the partial pressure of dioxygen in hemoglobin cases.[12,13] Recent research has suggested that active oxygens have roles as signal messengers in the body system.[14] The importance of studying activation of oxygen has been acknowledged. A superoxide is formed by the irradiation of light on some kinds of free porphyrins, such as hematoporphyrin; it is now used as a drug for cancer.[15] In a Bleomycin system,[16,17] the formation of hydrogen peroxide was observed. The activation of dioxygen was studied in the case of a heme oxygenase system (HO) which degraded heme; however, neither superoxide nor hydrogen peroxide was observed as being released from the systems.[18]

On the other hand, the generation of superoxide in artificial chemical systems has scarcely been reported with sufficient evidence. It was shown that hemins activated dioxygen in the presence of reducing reagents; Lown and Joshua reported DNA session with hemin-acridine, in which hydroxyl radical was suggested.[19] In studies of models for dioxygen coordination, such as picket-fence heme and capped heme systems, an activation of dioxygen was not reported.[20] Using hydrophobic metal porphyrins in organic solvents containing thiolate with tetraalkylammonium, superoxide was detected by ESR at low temperature.[21,22] During the photo-reduction of water-soluble hemin in a methanolic solution, no sign of the formation of superoxide was observed.[23] In a model oxidation using dioxygen with a reducing reagent in a $MnTPPCl/NaBH_4/O_2$ system,[24,25] the authors argued that the activation of dioxygen was to be a model for P450 and a partial "active oxygen" generation, but the mechanism remained unknown. However, in the system of FeTMPyP and dioxygen the authors did not mention the degradation of hemin or the generation of superoxide from the ferric system.[26] Water-soluble hemins including FeTMPyP were used in studies for modeling SOD;[27,28] in the studies hemins were reversely used as catalysts for the disproportionation of superoxide, but the generation of superoxide was not mentioned. Scavenging superoxide was also reported with MnTMPyP in Mammalian cells to be a model for oxidoreductase.[29] The electrochemical reduction of dioxygen was reported in the presences of FeTMPyP[30] and several cobalt porphyrins.[31] No superoxide formation was apparently reported in these papers. Instead, the authors reported the two-electron reduction of dioxygen to hydrogen peroxide. Using metal porphyrin with Fe, Mn, or Co, superoxide was hinted to be electrochemically generated in organic solvents with tetraethylammonium ion.[32]

Previously, we constructed a model for Cytochrome P450 using $FeTMPyPCl_5$, excess MBTH, and oxidants, and showed a variety of aspects depending on the pH of the solutions.[33] While it showed P450-like activity at pH 1–4 using organic hydroperoxide or hydrogen peroxide, a reduction of the hemin by MBTH occurred at around pH 7 under a nitrogen atmosphere, and the hemin was decomposed rather rapidly when exposed to air. We suspected that a generated ROS degraded the hemin. This would be an example to demonstrate and to control the generation of superoxide in artificial model systems of hemin.

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Experimental

Materials. Fe(III)TMPyPCl$_5$ was prepared as reported in a previous paper.[33] MBTH was purchased from Wako Chemicals. Nitrogen gas was deoxygenated by passing through an Oxisorb column (Messer Griesheim). DMPO in solution was purified by passing through a charcoal column before use. Phosphate-buffered solutions were prepared to be 0.1 mol/dm^3 with an ionic strength of 0.3 mol/dm^3, arranged with potassium chloride. SOD and catalase were purchased from Sigma-Aldrich Co. Other chemicals were of reagent grade.

Apparatus. The spectra were recorded using a Jasco-V530 Spectrometer. ESR signals were recorded using a JEOL JES-TE100 ESR spectrometer at room temperature. The oxygen concentrations in the sample solutions were measured using a Horiba OM-14 DO meter.

Recording Spectral Changes. In typical cases, the following conditions were employed: Fe(III)TMPyPCl$_5$, 1.0×10^{-5} mol/dm^3 (final); MBTH, 1.3×10^{-3} mol/dm^3 (final). The reactions were carried out in 0.1 mol/dm^3 phosphate buffer solutions (3.0 cm^3, pH 6.0–7.5) at 25 °C under nitrogen or a mixture of oxygen and nitrogen gas. The reaction mixtures were stirred during the reaction in quartz cuvettes using glass-coated stirring bars. Spectral changes were recorded at intervals of 5 min. Dissolved dioxygen gas was measured when necessary. The degradation of hemin was recorded at various concentrations of MBTH and dioxygen. The reduction–oxidation changes of the iron porphyrin were measured by recording the absorptions of the Soret bands at 444 (reduced) and 426 nm (oxidized).

Effect of SOD and Catalase. The degradation of hemin was inhibited in the presence of SOD (0.3 mg, 1500 unit/cm^3) or catalase (1.0 mg, 3500 unit/cm^3). SOD, catalase, and both were added into the system, and the spectral changes were recorded. The absorbance of catalase was subtracted when necessary.

ESR Measurement. DMPO as a trapping reagent dissolved under nitrogen was introduced to the reaction mixture in quartz ESR cells. The final concentrations were Fe(III)TMPyPCl$_5$, 1.56×10^{-5} mol/dm^3; MBTH, 6.69×10^{-4} mol/dm^3; and DMPO, 2.80×10^{-1} mol/dm^3 in a 0.2 cm^3 phosphate buffer solution (pH 7.0).

Results

Reduction and Degradation of Hemin. The water-soluble hemin, Fe(III)TMPyP, was reduced by MBTH in the pH region of 6.0–7.5 under anaerobic conditions as shown in Scheme 1. The spectral change of hemin is demonstrated for pH 7.0 in Fig. 1. The Soret bands of reduced and oxidized iron porphyrins were 444 and 426 nm, respectively. The spectrum of the reduced heme was confirmed by reduction using phenylhydrazine in a separate experiment. The spectra indicated the fifth ligand on the Fe(III) species was hydroxide ion, described as Fe(III)TMPyP(OH).[34] When the reaction proceeded under aerobic conditions, hemin was degraded without isosbestic points, as shown in Fig. 2. No clear reduced heme was recorded, but the peak of the Soret band was slightly red-shifted during the breakdown, suggesting the partial existence of reduced hemin, or other species. It was suspected that dioxygen was activated to destroy the hemin. The changes in the Soret band were followed kinetically, but data only for the initial stage were used to simplify the complex systems. The degradation rate was dependent on the concentration of dissolved dioxygen at various pH values; the situation is shown in experiments with various concentrations of dissolved dioxygen as in Fig. 3. It should be noted that the degradation rate increased as the pH increased. The rate was also dependent on MBTH, as shown in Fig. 4, in which the time course of the Soret band at 426 nm is indicated. The dependence of the rate was linear on the concentration of MBTH.

Effects of SOD and Catalase. The inhibitions by SOD and catalase to the degradation indicated that superoxide and hydrogen peroxide were formed during the reduction. The addition of SOD to the reaction mixture made the degradation slower, as shown in Fig. 5. It was demonstrated that the deg-

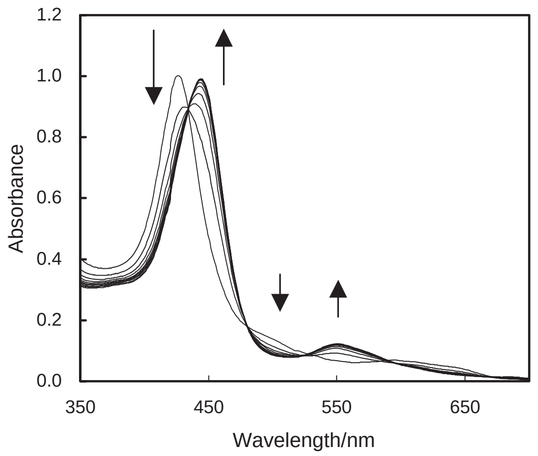
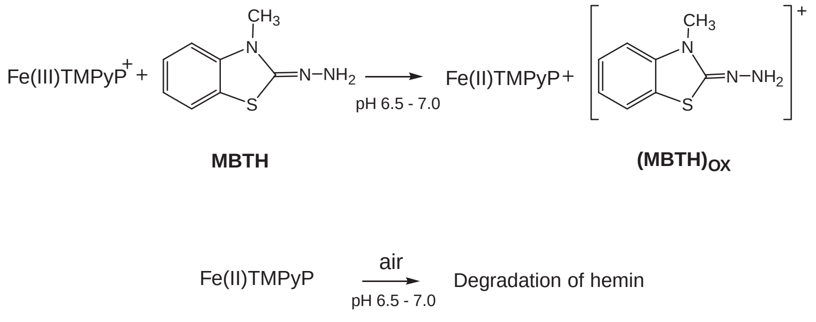


Fig. 1. The spectral change of FeTMPyP(OH) in the phosphate buffer solution (pH 7.0) in the presence of MBTH under anaerobic conditions showing the reduction of hemin. The spectra were recorded at the intervals of 5 min with a scan rate of 400 nm/min.



Scheme 1.

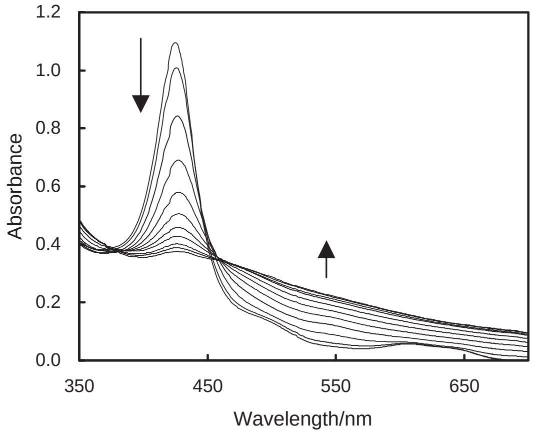


Fig. 2. The degradation of FeTMPyP(OH) in the presence of MBTH in the phosphate buffer solution (pH 7.0) under aerobic conditions. The spectra were recorded at the intervals of 5 min with a scan rate of 400 nm/min. The conditions were similar to those in Fig. 1.

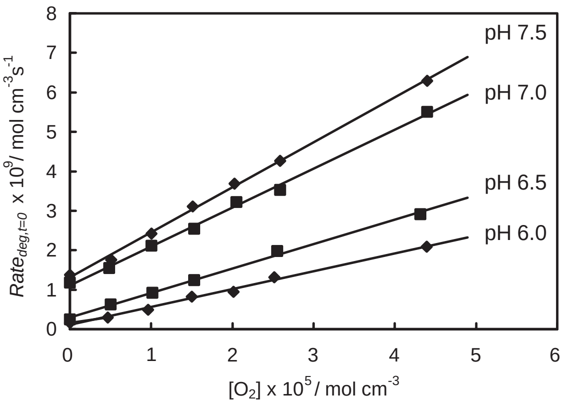


Fig. 3. Degradation rate of hemin depending on the concentration of the dissolved dioxygen in the reaction mixture. Degradation rates of hemin, $Rate_{\mathrm{deg},t=0}$, were obtained from the tangents of the initial decrease rates ($t = 0$) at 426 nm. The conditions were similar to those in Fig. 1.

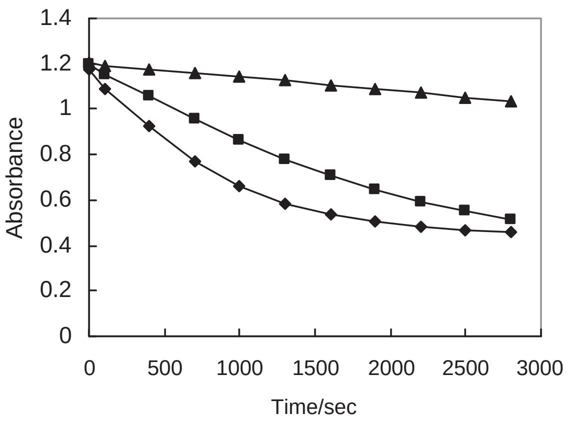


Fig. 4. Degradation of FeTMPyP in air depending on MBTH at pH 7.0. The conditions were similar to those in Fig. 1. Concentration of MBTH, –◆–, 6.9 × 10^{-4}; –■–, 3.5 × 10^{-4}; –▲–, 7.1 × 10^{-5} mol/dm^3.

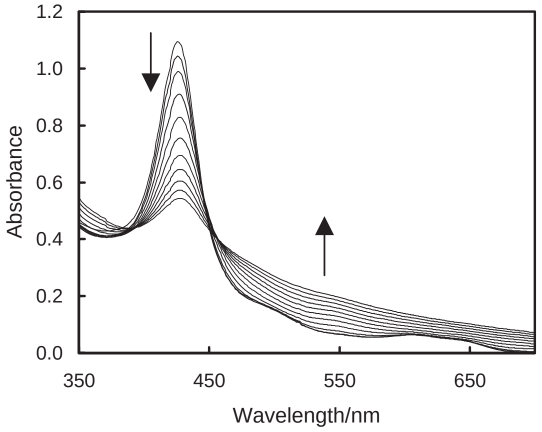


Fig. 5. The prohibition by SOD (500 unit/dm^3) to the degradation of hemin with MBTH and dioxygen. The conditions were similar to those in Fig. 1.

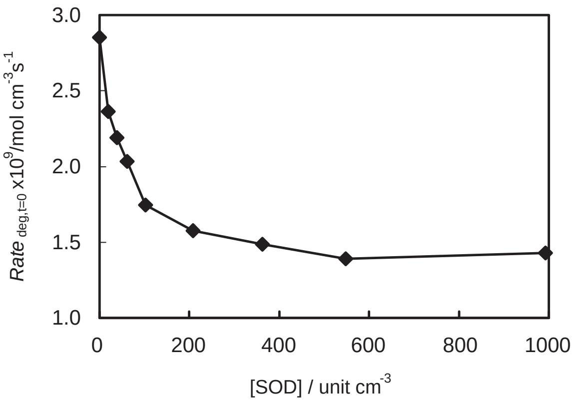


Fig. 6. The prohibition by SOD to the degradation of hemin with MBTH at pH 7.0. Degradation rates, $Rate_{\mathrm{deg},t=0}$, were obtained from the tangents at initial decrease rates ($t = 0$) at 426 nm. The conditions were similar to those in Fig. 5.

radation by superoxide was apparent, as in Fig. 6; in the presence of a sufficient amount (about 500 unit/cm^3) of SOD, the degradation rate reached to the level solely with formed hydrogen peroxide, because SOD disproportionated superoxide before the degradation of hemin. The addition of catalase also affected on the reaction, as shown in Fig. 7, where the degradation was caused not by hydrogen peroxide but by superoxide. When both of SOD and catalase were added, no degradation occurred with a slight red shift of the Soret band and two small peaks around 480 and 550 nm in the visible region, as in Fig. 8. These results gave evidence that hemin was reduced, and superoxide, hydrogen peroxide, and, possibly, hydroxyl radical were produced. It should be stressed that the generated superoxide had reactivity to decompose this particular hemin.

Liberation of Superoxide from Heme–Dioxygen Adduct. Upon exposure on air near the final stage of the reduction process, as indicated in Fig. 9, the spectrum immediately changed to the initial oxidized one, but with less intensity; a computational estimation of the spectra indicated that the resulting species contained 7% of other species at this stage. After this change, the hemin followed the degradation process similarly

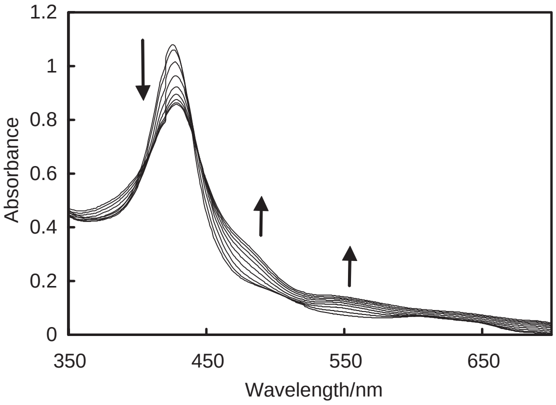


Fig. 7. The prohibition by catalase (3830 unit/cm^3) to the degradation of hemin with MBTH and air. The conditions were similar to those in Fig. 1.

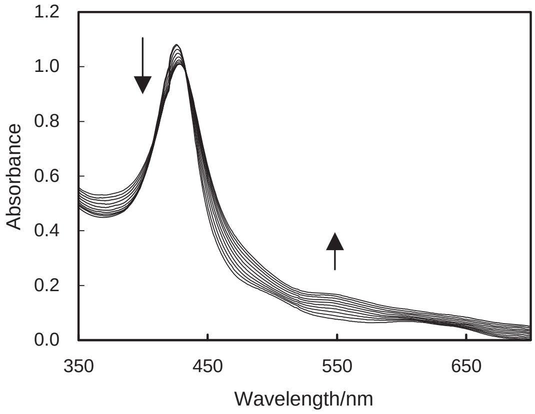


Fig. 8. The prohibition to the degradation by MBTH in the presences of SOD (500 unit/cm^3) and catalase (3830 unit/cm^3). The slight red shift of Soret band and small peaks at around 480 and 550 nm indicated the existence of other species. No degradation was observed. The conditions were similar to those in Fig. 1.

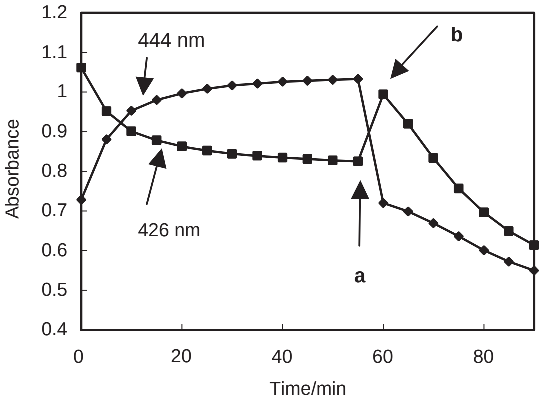


Fig. 9. Exposure to dioxygen near the end of the reduction process. The peak at 426 nm (Soret band) indicated a Fe(III) state and that at 444 nm indicated a Fe(II) state. At the time **a**, air was injected to the solution. At the time **b**, the spectrum indicated the Fe(III) state which recovered more than 90%.

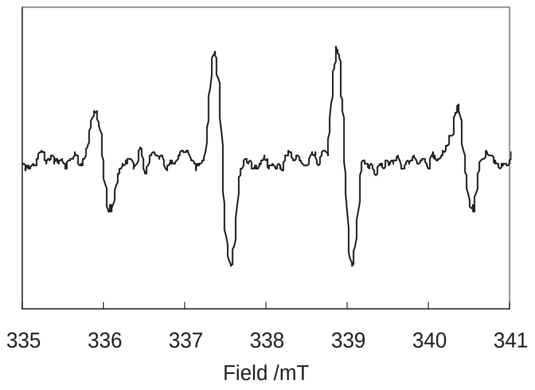


Fig. 10. ESR signal trapping hydroxyl radical in the system using DMPO.

as seen in Fig. 2. It was reasonable to think that the reduced heme reacted with dioxygen to be Fe(II)TMPyP–O_2, which liberated superoxide; the spectrum of the O_2-adduct was not observed during this process. The reverse oxidation to hemin was faster than the degradation of hemin. There were two possible processes. One was that heme was oxidized to be hemin, releasing superoxide; the other passes were without releasing superoxide. The former was only partially and the latter was the main route, suggesting cyclic reactions.

Detection of the Formed ROS by ESR. The formation of ROS in the system was also confirmed by measuring the ESR signals. The generated radical was trapped by DMPO; the eventually confirmed species was hydroxyl radical instead of superoxide. The addition of excess DMPO did not protect hemin from degradation. ESR showed a quartet signal with $a^{\mathrm{N}} = 1.5$ mT and $a^{\mathrm{N}}\beta = 1.5$ mT, which was the same as in the literature,[35] indicating an adduct of DMPO and an OH radical (Fig. 10). The reason might be because the superoxide trapping rate by DMPO was slower than that of forming hydrogen peroxide, and then turning to hydroxyl radical. No other radicals were detected as DMPO adducts by an ESR measurement.

One-Electron Reduction of Hemin. While hemin was reduced, MBTH was oxidized transferring one electron to the hemin; the reduction rate and degradation rate of hemin were dependent on the concentration of MBTH. For checking the oxidation state of oxidized MBTH, DMA was introduced in the reaction mixture under a nitrogen atmosphere, and it was shown that a dye with MBTH and DMA was not formed.[33] Thus, one-electron oxidized MBTH did not react with DMA to form the Indamine dye. In acidic media (pH < 4), the hemin was not reduced,[36] and no effect of dioxygen was observed. Hemin turned to be a high-valent oxoiron intermediate by the addition of hydrogen peroxide, which oxidized MBTH to form a dye.[33,37] In the presence of TBP at pH 7.0 (added as dissolved in methanol), the formation of a blue phenoxyl radical was not observed.[38] These facts indicated that a high-valent oxoiron species was not formed. The oxidation state and the fate of the one-electron oxidized MBTH could not be followed here. We recognized no sign of oxidized MBTH affecting the hemin and generating ROS.

Though NADH was expected to be an electron donor in this particular hemin system, the reduction rate was much slower, and it was difficult to obtain the reproducible results. On the other hand, the addition of ascorbic acid resulted in a partial quick degradation of hemin in air; it was supposed that formed ROS reacted with excess ascorbic acid to be non-reactive, showing the difficulty of control; the dependence on ascorbic acid was not linear. Because a similar degradation occurred with ascorbic acid, no special effect of oxidized MBTH was supposed. The presence of cyanide ion made the reduction and degradation in air slow. Since anions that coordinated on the Fe(III) ion made the reduction slow, resulting in less superoxide, it supported the scheme of the reduction–degradation mechanism.

Discussion

Degradation of Hemin by ROS. Summing up the results, an artificial hemin, Fe(III)TMPyP, was reduced by MBTH under nitrogen and was decomposed in air. Without a reducing reagent, no degradation occurred. A kinetic measurement indicated that hemin was degraded by dioxygen; linearly depending on the concentration, as shown in Fig. 3. It was also dependent on the concentration of MBTH, as shown in Fig. 4. The degradation rate was reduced when SOD and/or catalase was added. It indicated that a reactive oxygen species was produced to decompose hemin. Since SOD, which accelerates the disproportionation of superoxide to hydrogen peroxide and water, inhibited the degradation, it was shown that the generated superoxide decomposed hemin. The hydrogen peroxide generated in the system of FeTMPyP was reported to destroy hemin in electrochemical systems.[36] When air was introduced after the reduction of hemin, more than 90% of the reduced heme immediately turned to the oxidized form, and then degradation started (Fig. 9). It was estimated that one hydrogen peroxide molecule and one dioxygen molecule were needed to open one porphyrin in the model.[39,40] Thus, even if ROS was generated quantitatively, it did not sufficiently decompose hemin, and most of the ROS was consumed in other reactions. The formed ROS did not decompose hemin because the concentrations of ROS were too diluted (10^{-5} mol/dm^3) to react with hemin. ROS might be consumed in reacting with the environment, including excess MBTH. These results suggested that hemin was decomposed by ROS generated during many cycles of steps.

Formation of ROS. Possible reactions for the mechanism were considered, as follow in Fig. 11. The general idea was that the activation of dioxygen in the iron porphyrin system was initiated by reduction of the central iron to which then dioxygen coordinated.[8] The dioxygen coordinating on the Fe(II) ion in the heme goes four ways. The first is the split from iron, as in hemoglobin or myoglobin (reaction (1)). The second is the release of superoxide, leaving trivalent iron back, and the autoxidation of heme (reaction (2)). Eventually superoxide turns to be hydrogen peroxide (reaction (3)). The third is the formation of μ-peroxodimer, which turns to be a high-valent oxoiron(IV) species (reaction (4)) and then μ-oxodimer (reaction (5)). μ-Peroxodimer may turn to be μ-oxodimer, producing hydrogen peroxide (reaction (6)). The last is the formation of high-valent oxoiron(IV) with a π-cation radical on a peripheral porphyrin ring, similar to the intermediate of the P450 systems in reaction (8).

$$\mathrm{Fe(III)PX} \xrightarrow{e^-} \mathrm{Fe(II)P} \overset{O_2}{\rightleftharpoons} \left[\mathrm{Fe(II)P{-}O_2}\right] \qquad (1)$$

$$\left[\mathrm{Fe(II)P{-}O_2}\right] \rightleftharpoons \left[\mathrm{Fe(III)P{-}O_2^{\bullet-}}\right] \xrightarrow{X^-} \mathrm{Fe(III)PX} + \mathrm{O_2^{\bullet-}} \qquad (2)$$

$$2\mathrm{O_2^{\bullet-}} \xrightarrow[\text{(SOD)}]{H^+} \mathrm{H_2O_2} + \mathrm{O_2} \qquad (3)$$

$$\left[\mathrm{Fe(II)P{-}O_2}\right] \xrightarrow{\mathrm{Fe(II)P}} \left[\mathrm{Fe(II)P{-}O_2{-}Fe(II)P}\right] \longrightarrow \left[\mathrm{Fe(III)P{-}{}^{-}O{-}O^{-}{-}Fe(III)P}\right] \longrightarrow 2\mathrm{Fe(IV)PO} \qquad (4)$$

$$\mathrm{Fe(IV)PO} \xrightarrow{\mathrm{Fe(II)P}} \mathrm{Fe(III)P{-}O{-}Fe(III)P} \qquad (5)$$

$$\left[\mathrm{Fe(III)P{-}{}^{-}O{-}O^{-}{-}Fe(III)P}\right] \xrightarrow{H^+} \mathrm{Fe(III)P{-}O{-}Fe(III)P} + 1/2\mathrm{H_2O_2} \qquad (6)$$

$$\mathrm{Fe(III)P{-}O{-}Fe(III)P} \xrightarrow{\mathrm{H_2O}} 2\mathrm{Fe(III)P(OH)} \quad (\text{pH } 7.0) \qquad (7)$$

$$\left[\mathrm{Fe(III)P{-}O_2^{\bullet-}}\right] \xrightarrow{e^-} \mathrm{Fe(III)P{-}O_2^{2-}} \xrightarrow{H^+} \mathrm{P^+Fe(IV){=}O} + \mathrm{H_2O} \qquad (8)$$

Fig. 11. Reaction scheme for hemin and dioxygen. P stands for a porphyrin dianion and X stands for nucleophile, such as $\mathrm{Cl^-}$ or $\mathrm{PO_4^{2-}}$.

For this particular hemin system in aqueous media, the spectra showed the monomer hydroxide at the present pH region,[41] and stable μ-oxodimer formation was observed at pH 10–12, reported in the literature.[42,43] It was kinetically shown that Fe(II)TMPyP was oxidized by dioxygen to be μ-peroxodimer, as in reaction (4).[26] The formed μ-peroxodimer homolitically split to be the high-valent oxoiron(IV) porphyrin, which might decompose hemin. However, because SOD and catalase completely inhibited the degradation, reactive species other than superoxide and hydrogen peroxide were not plausible, and thus the existence of reactive high-valent oxoiron(IV) species was denied. On the other hand, it was reported that the μ-peroxodimer turned to be μ-oxodimer along with hydrogen peroxide (reaction (6)) in aqueous media. Since μ-oxodimer was not stable under the present conditions (pH 7.0), it turned easily to hydroxide (reaction (7)). The process from μ-peroxodimer to μ-oxodimer had not been clearly analyzed. If μ-oxodimer was formed, as in reaction (4), no reactive species was formed. If hydrogen peroxide was formed, as in reaction (6), it should react while affecting the degradation of hemin; these two possibilities in reaction (3) and reaction (6) were not separately estimated at this stage.

Two possible reaction passes, reactions (2,3) and (4–6), would be competitive at around pH 7.0. Because most of the reduced heme (~90%) returned to the initial hemin by autoxidation without degradation in one step, the mechanism through reactions (4) and (5) should be dominant. The partially observed species with a red-shifted Soret band and peaks at around 480 and 550 nm during the reaction was not assigned at this stage. Superoxide generated through reaction (2) would be accumulated during the cycle. The situation is shown in Fig. 12.

Mechanism for the Formation of Superoxide and Hydrogen Peroxide in the FeTMPyP System. The linear dependence of the degradation rate on MBTH indicated that the reduction was rate determining, and that the reduction was a one-electron transfer to hemin from MBTH. An important suggestion concerning hemoglobin systems was that autoxidation should occur accompanying the liberation of superoxide from

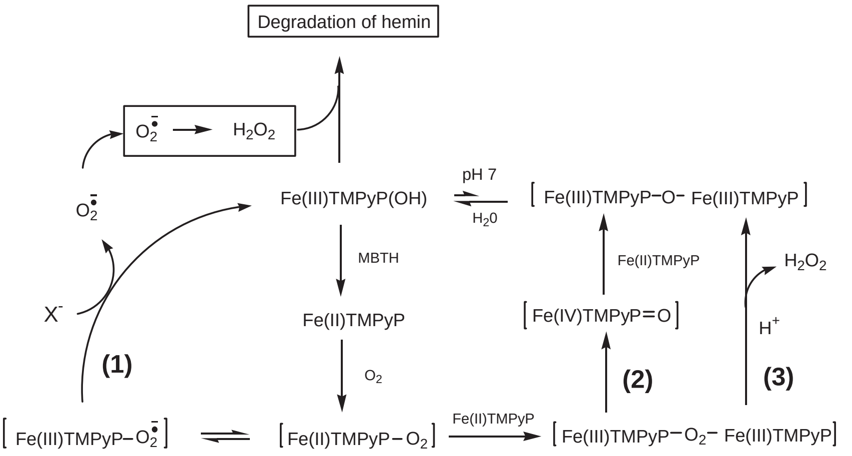


Fig. 12. Proposed reaction scheme for ROS generation and degradation of hemin at pH 6.0–7.5. Route **(1)** is the autoxidation releasing superoxide with nucleophiles. Routes **(2)** and **(3)** are autoxidations without releasing superoxide.

heme–dioxygen complex.[44] Though in many cases of heme proteins, superoxide liberation, as in reaction (2), was reported, stoichiometric and quantitative elucidation had not been achieved. In a generally accepted mechanism, nucleophiles were needed in a hemoglobin study.[7,45] Wallace et al.[10] suggested that nucleophilic anions, such as CN^-, $N_3{}^-$, and SCN^-, caused the release of superoxide from oxyhemoglobin or oxymyoglobin, and stabilized the Fe(III) state in the natural system; in their experiments, no reductant participated in one step, and no μ-oxodimer formation was possible. Though we could not observe the stable dioxygen complex and start from the oxyhemoglobin-like situation here, it was reasonable to acknowledge that phosphate ion and/or chloride ion caused an easier liberation of superoxide. A proton-assisted superoxide formation was suggested in the P450 system.[6,20,45] However, the pH dependence in the present system; as in Fig. 3, indicated that proton-assisted formation was not likely. Most of the dioxygen binding systems were constructed as models in organic solvents in which no effect of anions was expected, and no superoxide formation was suggested.[46,47]

The oxidation state and structure of MBTH was not clear at this stage. In this system, no specific role was recognized, except that of the one-electron reduction of hemin. The "oxidized MBTH" had no reactivity toward DMA to form Indamine Dye. No radical of MBTH was detected by an ESR measurement after the reaction. The radical might be terminated in the environment.

It was indicated that reduction and degradation occurred in reducing systems other than without MBTH. In case of ascorbic acid, a similar degradation of hemin was observed under aerobic conditions. The changes were not linearly dependent on the concentration of ascorbic acid. It was supposed that the generated ROS reacted with excess of ascorbic acid stopped the degradation.

The case of HO was different from the other "heme" proteins; protoheme penetrates into the pocket of the active site in the globin, where it activates dioxygen and destroys itself, using NADH as a reductant. However, in these mechanisms, the liberation of superoxide had not been considered.[18]

The degradation mechanism of FeTMPyP by ROS was still not clear at this point. According to the study about hemin degradation, one hydrogen peroxide and one dioxygen are needed. Since both SOD and catalase prohibited degradation, the present experiment suggested that superoxide could degrade hemin. It was reported that the degradation of heme by hydrogen peroxide occurred when reduced.[39,48] In the present system, there was no evidence to indicate the redox state of hemin to be degraded. The mechanistic study for the degradation of hemin will be reported elsewhere.

Conclusion

The formation of superoxide was confirmed in a system of water-soluble hemin, $Fe(III)TMPyPCl_5$, and MBTH in buffered solution (pH 6.0–7.5). Superoxide subsequently turned to hydrogen peroxide, and then a hydroxyl radical; these species decomposed hemin. SOD and catalase prohibited the degradation of hemin. Superoxide was generated from the heme–dioxygen complex, leaving iron in a Fe(III) state as one of the autoxidation routes of reduced heme. As in aqueous media, with natural heme-proteins generated superoxide being affected by nucleophiles, it was reasonable that in the present system the oxidation of heme–O_2 released superoxide affected by anions in the solution. The present system, indicating the degradation of heme by dioxygen in the presence of a reducing reagent resembles the HO system, but differs in the way of activating dioxygen, because superoxide is not released in the HO system. Thus, the total redox cycle is indicated in Fig. 12. The cycle is the redox cycle between MBTH and dioxygen catalyzed by FeTMPyP(OH). Routes **(2)** and **(3)** are dominant without releasing superoxide and **(1)** is the sub-route to obtain the Fe(III) state, back releasing superoxide. The hemin is partially decomposed by superoxide and partially by hydrogen peroxide during the redox cycle. The formation of ROS would be controlled in the model system for biological systems at a desired location and applied as a supplying system for ROS. Experiments using NADH instead of MBTH will elucidate valuable aspects. Similar, but complicated, reduction and degradation occurred in the case of ascorbic acid, indicating that the present generation of ROS generally occurs in the presence of reducing reagents. Further studies concerning the stoichiometry of the generation of superoxide and its reactivity are underway.

References

Abbreviations: ROS; reactive oxygen species, Fe(III)-TMPyPCl$_5$; tetrakis(4-methylpyridyl)porphinatoiron(III) pentachloride, Fe(II)TMPyPCl$_4$; tetrakis(4-methylpyridyl)porphinatoiron(II) tetrachloride, Fe(III)TMPyP; tetrakis(4-methylpyridyl)-porphinatoiron(III) ion, Fe(II)TMPyP; tetrakis(4-methylpyridyl)-porphinatoiron(II) ion, MBTH; 3-methyl-2-benzothiazolinone hydrazone. DMPO; 5,5-dimethyl-1-pyrroline *N*-oxide, HO; heme oxygenase, SOD; superoxide dismutase, DMA; *N*,*N*-dimethylaniline, TBP; 2,4,6-tri(*t*-butyl)phenol.

1 Superoxide anion radical will be written to be superoxide for short in this paper.

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