60 μmol) and HO-Bt (18.4 mg, 120 μmol). The solution was cooled to −5 °C, and then DCC (13.6 mg, 66 μmol) and DIEA (11.5 μL, 66 μmol) were added. After being stirred at 0 °C for 1 h and at 20 °C overnight, the mixture was concentrated in vacuo, dissolved in DCM (25 mL), filtered, and then washed with water, 5% citric acid, 5% $NaHCO_3$, and brine (each 10 mL at 0 °C). The DCM solution was concentrated and subjected to flash chromatography (2 × 25 cm silica gel; eluent $CHCl_3$/MeOH/AcOH, 18/1/1) to afford a chromatographically pure white solid: yield 110 mg, 65.6%; mp 68–72 °C; $[\alpha]_D$ −8.5 ± 0.3° (*c* = 2, MeOH); amino acid analysis, $Glu_{4.00}Leu_{3.98}Lys_{6.00}$; MS ($^{252}Cf$ fission fragment) *m/e* = 2,793.4 (theoretical 2792.8 for $(M + Na)^+$, Δ = +0.6).

***cyclo*(3-7,10-14,17-21)Boc-[Lys(2Cl-Z)LeuLysGlu(OBzl)LeuLys-(2Cl-Z)Glu]$_2$-OPac; Boc-(1-21)-OPac (20).** Boc-(1-14)-OPac (**18**; 100 mg, 35.6 μmol) was deprotected with TFA (4 mL), and TFA salt **21** was obtained after ether precipitation: yield 88.5 mg, 88%; mp 65–70 °C; R_f(G) 0.92; $[\alpha]_D$ −4.5 ± 0.2° (*c* = 6, DMF). Compound **21** (88.5 mg, 31.5 μmol) was dissolved in DMF (2 mL) together with Boc-(1-7)-OH (**17**; 46 mg, 33 μmol) and HO-Bt (10.1 mg, 66 μmol). At −5 °C, DCC (7.4 mg, 36 μmol) and DIEA (6.27 μL, 36 μmol) were added. After being stirred at 0 °C for 1 h and at 20 °C overnight, the mixture was concentrated in vacuo, diluted with DCM (25 mL), filtered, and then washed with water, 5% citric acid, 5% $NaHCO_3$, and brine (each 10 mL at 0 °C). The peptide solution was concentrated in vacuo and subjected to gel permeation chromatography (3 × 115 cm Sephadex LH-60 eluted with DMF). Fractions containing the major peptide peak were collected and evaporated: yield 35.1 mg, 27.3%; R_f(F) 0.53; amino acid analysis, $Glu_{6.18}Leu_{6.00}Lys_{8.73}$; MS ($^{252}Cf$ fission fragment) *m/e* = 4,070.5 (theoretical 4071.2 for $(M + Na)^+$, Δ = −0.7).

***cyclo*(3-7,10-14,17-21)(LysLeuLysGluLeuLysGlu)$_3$-OH (1-1-1).** Boc-(1-21)-OPac (**20**; 25 mg, 6.1 μmol) was dissolved in 90% AcOH (3 mL), and Zn dust (100 mg) was added to the vigorously stirred mixture. After 1 h, the solution was filtered to remove the Zn and concentrated in vacuo. The residue was dissolved in DCM, filtered again, and the solvent evaporated. This residue was then subjected to flash chromatography (2 × 25 cm silica gel, eluent $CHCl_3$/MeOH/AcOH, 90/10/5). Fractions (7 mL) 20–42 were pooled and concentrated in vacuo: yield 24 mg, 98.8%; R_f(E) 0.55. This intermediate was then completely deprotected as follows. It was dissolved in TFA (1.5 mL) at 0 °C and then thioanisole (282 μL, 2.4 mmol) and TMSOTf (462 μL, 2.4 mmol) were added. After 1 h of stirring at 4 °C, the solution was diluted with ether. The precipitated material was separated by centrifugation and washed with ether. It was then treated for 1 h at 4 °C with a 1 M NH_4F solution (1 mL) adjusted to pH 8 with 5% NH_4OH. This peptide solution was subjected to gel permeation chromatography (0.7 × 18 cm Sephadex G-25 eluted with 0.1 M AcOH). The peptide fractions were pooled and lyophilized. Final purification of the product was carried out by RP-HPLC on a Vydac C-18 column (1.0 × 25 cm) eluted with 0.1% TFA in acetonitrile/water. A linear gradient from 25 to 35% acetonitrile over 15 min, with a flow rate of 4 mL/min, was employed. The product was eluted at 30.5% acetonitrile and lyophilized to give a solid product: yield 2.6 mg of peptide, 16.8%; amino acid analysis, $Glu_{6.00}Leu_{5.82}Lys_{9.00}$; racemization assay 4.8% D-Glu, 1.0% D-Leu, 0.4% D-Lys; MS (^{252}Cf fission fragment) *m/e* = 2,571.5 (theoretical 2571.2 for $(M + Na)^+$, Δ = +0.3).

Circular Dichroism Studies. CD spectra were measured at 25 °C, using an Aviv Model 62ds spectropolarimeter fitted with a fused-silica modulator. Stock peptide concentrations were determined by amino acid analysis after hydrolysis in 6 N HCl at 110 °C for 24 h, using crystalline alanine (Sigma Chemical Co., St. Louis, MO) as an internal standard. Solution spectra were measured in 0.1, 0.5, or 2.0 cm path length cells, as appropriate for the peptide concentration under study, using a time constant of 2.0 s and averaging the data from five scans. Data collected at dynode voltages greater than 450 V were discarded. The CD spectrum of peptide **1-1-1** adsorbed onto siliconized circular quartz slides (22 × 1 mm, Hellma Cells Inc., Jamaica, NY) was determined by the method described previously.[23] Four siliconized slides were immersed for 10 min each in peptide solution (10 μM) in 0.02 M NaH_2PO_4/NaOH buffer, pH 7.5, containing 0.16 M KCl, then rinsed in H_2O, and allowed to air-dry. These slides were then placed in an adapted cell holder, designed to hold the slides vertically in the light path at alternating angles of +5 and −5° from the horizontal direction perpendicular to the light path. Data were collected for eight equally spaced orientations of the slides about the light path, in order to eliminate linear dichroism artefacts,[25] and are presented in Figure 3C as the sum of these spectra after blank subtraction, without further manipulation. This spectrum is thus equivalent in signal intensity to that resulting from a total of 32 coated slides or 64 surfaces.

Acknowledgment. This research was supported by USPHS Program Project HL-18577. We are grateful for the amino acid analyses and racemization assays performed by Mr. Adam A. Profit and the mass spectrometric analyses performed in the laboratory of Dr. Brian Chait.

Reduction of the Oxy Form in Hemoproteins to the Ferryl Form

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Abstract: One-electron reduction of the oxy form in hemoproteins by reducing radicals such as NAD•, CO_2^-, and benzoate electron adduct has been investigated by the pulse radiolysis technique. These radicals reacted with oxymyoglobin to form the stable product, of which spectrum is assigned to the corresponding ferryl form. On the other hand, only benzoate electron adduct reacted with the oxy form of diacetyldeuteroperoxidase to form compound I of the enzyme, though the reaction by other radicals could not be detected. From these results, it was demonstrated that the one-electron reduction state in the oxy form of hemoproteins is the higher oxidation state.

The reduction of a ferrous–dioxygen complex [Fe(II)–O_2] in hemoproteins has received considerable attention, since this reaction appears in the hydroxylation of substrate by cytochrome P-450.[1] The resulting reducing species in P-450 is generally believed to be structurally similar to the high-valent intermediates such as compound I formed during the catalytic cycle of the peroxidase enzymes, though the high-valent intermediate for P-450 has not yet been isolated. This is supported by the fact that P-450 has been shown to catalyze monoxygenation reactions by utilizing oxidizing reagents such as hydroperoxide.[2] In the case of hor-

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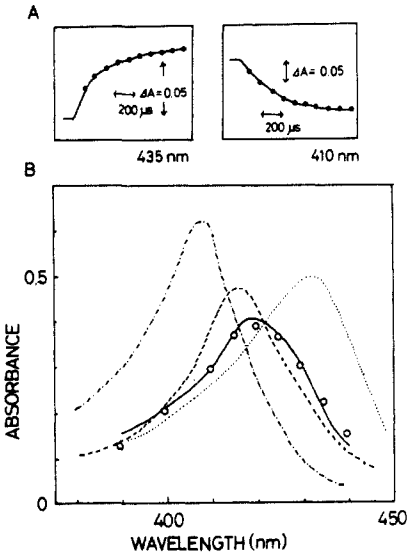

Figure 1. (A) Absorption changes after pulse radiolysis of oxy-Mb (8 μM) in the presence of sodium formate (0.1 M) and N_2O at pH 7.4 (phosphate buffer 10 mM). (B) Comparison of optical absorption spectra of oxy-Mb (---), deoxy-Mb (···), met-Mb, (-·-), ferryl-Mb (—), and the reaction product of CO_2^- with oxy-Mb (○). Experimental points were obtained from the absorbance changes obtained at 1 ms after the pulse.

seradish peroxidase (HRP), Yamazaki has been proposed that compound I is one equivalent reduced state above the oxy form of the enzyme.[3] On the other hand, with heme iron model systems, more convincing data have appeared to show that the high-valent intemediates of an iron–oxo intermediate play a crucial role in hydroxylating alkanes and epoxidizing alkenes.[4] However, the one-electron reduction of the ferrous–dioxygen complex to the higher oxidation states has not yet been observed directly, except for the reaction of the hydrated electron (e_{aq}^-) with the oxy form of hemoproteins.[5] In the case of e_{aq}^-, however, its reaction is nonspecific and some of the e_{aq}^- reacts with the amino acid residues of the protein. The absolute spectrum of the intermediate after pulse radiolysis, therefore, cannot be obtained. In contrast, radicals such as NAD• and CO_2^- are weaker reductants than the e_{aq}^- so that they sometimes have higher selectivity in the reaction.[6]

In this report, we describe the reduction of an oxymyoglobin (oxy-Mb) and an oxy form of HRP to the higher oxidation states by the radicals using a high-dose pulse radiolysis technique. Here, the difference between Mb and HRP is discussed in relation to the mechanism of O–O bond cleavage after the reduction.

Experimental Section

Commercially available sperm whale Mb (Type VI, Sigma) was used without further purification. An oxy form of Mb was prepared as follows. The deoxy-Mb was prepared by the addition of 2–3-fold excess amounts of sodium dithionite to met-Mb, and the deoxy form was passed through a Bio-Rad AG 1-X8 column to eliminate excess sodium dithionite to yield oxy-Mb.

HRP (Sigma) was purified from the crude material by DEAE- and CM-cellulose column chromatography according to the method of Shannon et al.[7] The enzyme used was a main fraction absorbed on the CM-cellulose column. Diacetyldeuteroperoxidase was prepared by recombination of apoperoxidase with diacetyldeuteroheme, followed by DEAE- and CM-cellulose column chromatography by the method of Tamura et al.[8] The oxyperoxidase was prepared by the method of Kobayashi and Hayashi.[5] All other reagents were obtained commercially as the analytical grade. The samples for pulse radiolysis were prepared as described in the previous papers.[5,9]

The pulse radiolysis experiments were performed with an electron linear accelerator of the Institute of Scientific and Industrial Research Osaka University. The pulse width and energy were 7 ns and 27 MeV, respectively. The dose was 19 Krad per 7-ns pulse (beam diameter, ca. 4 mm). Optical absorption spectra were measured with a Shimadzu MPS-2000.

Table I. Rate Constants for Reaction of Various Radicals with Oxymyoglobin

radical	second-order rate constant, M^{-1} s^{-1}	E_0, V
$(C_6H_5COOH)^{\bullet-}$	1.8×10^9	−2.2[10]
CO_2^-	3.0×10^8	−2.0[11]
$(CH_3)_2\dot{C}OH$	2.3×10^8	−1.8[12]
NAD•	1.1×10^8	−0.9[12]
$MV^{\bullet+}$	4.5×10^7	−0.44[14]
O_2^-	ND	−0.27[15]
FMN•	ND	−0.17[16]

Results and Discussion

In the presence of 0.1 M HCO_2^-, CO_2^- is produced via reactions 1 and 2 by pulse radiolysis of N_2O saturated aqueous solution.

$$e_{aq}^- + N_2O + H_2O \rightarrow OH^\bullet + OH^- + N_2 \quad (1)$$

$$OH^\bullet + HCO_2^- \rightarrow CO_2^- + H_2O \quad (2)$$

After pulse radiolysis of oxy-Mb in the presence of HCO_2^- and N_2O, the absorption changes in the Soret region were observed, as shown in Figure 1A. The absorption increased and decreased at 435 and 410 nm, respectively. Under the experimental conditions, 40 μM CO_2^- was generated in 8 μM of oxy-Mb. When met-Mb was irradiated under the same conditions, all the met-Mb in the sample was reduced by CO_2^- to form deoxy-Mb. Similarly, it is considered that all of oxy-Mb in the sample is reduced by using the high-dose pulse radiolysis technique. Figure 1B shows the absorption spectrum of the product obtained in the reaction of CO_2^- with oxy-Mb. The figure also shows the spectra of oxy-Mb, met-Mb, deoxy-Mb, and ferryl-Mb. It is noted that the spectrum of the reaction product is identical to that of ferryl Mb. The second-order rate constant of the reaction at pH 7.4 is calculated to be 3.0×10^8 M^{-1} s^{-1}. The same compound was obtained under the condition of [oxy-Mb] $\gg$ [CO_2^-], where two-electron reduction does not occur. This suggests that ferryl-Mb is not a two-electron reduction product of oxy-Mb.

In the presence of 1 mM NAD^+, methyl viologen (MV^{2+}), or benzoate, one-electron reduction of these compounds was produced by the reaction of e_{aq}^-. These radicals are readily recognized by their characteristic absorption spectra and their high rates of the formation. These radicals also reacted with oxy-Mb to form ferryl-Mb. Table I compares the rate constants for the reaction of various radicals with oxy-Mb. These values were determined under the condition of [Oxy-Mb] $\gg$ [radical]. It is noted that the second-order rate constants increase with the decrease of the redox potential of the radical. That is, the difference in redox potential between oxidant and reductant is a factor controlling the rate of reaction. In contrast, the radicals with redox potential above −0.3 V such as O_2^- (E_{m7} = −0.27 V)[14] and FMN (E_{m7} =

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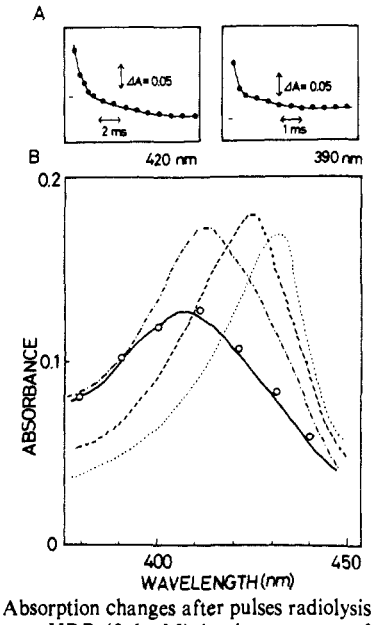


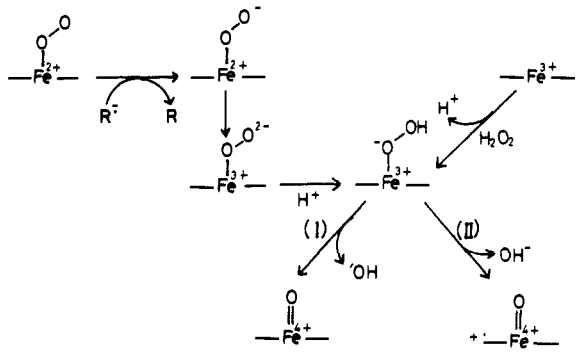
Figure 2. (A) Absorption changes after pulses radiolysis of the oxy form of diacetyldeutero-HRP (2.6 μM) in the presence of 1 mM sodium benzoate at pH 7.4 (phosphate buffer 10 mM). (B) Comparison of optical absorption spectra of compound I (—), compound II (···), the oxy form (- - -), ferric diacetyldeutero-HRP (— · —), and the reaction product of benzoate electron adduct with the oxy form of diacetyldeutero-HRP (o). Experimental points were obtained from the absorbance changes obtained at 3 ms after the pulse.

−0.17 V)[15] were not operative in the reaction. The redox potential of these radicals compares favorably with that of the couple for Fe^{2+}–$O_2 \rightleftharpoons [Fe^{2+}$–$O_2]^-$ in the model system (−0.24 V).[16]

The oxy form of 2,4-diacetyldeuterohemin-substituted HRP was employed in the present experiments, since it is very stable, in contrast to native HRP. In the presence of 1 mM benzoate, e_{aq}^- is converted to the benzoate electron adduct, with an absorption maximum at 450 nm, by pulse radiolysis of the deaerated aqueous solution, whereas OH• is scavenged by benzoate. In pulse radiolysis of the oxy form of diacetyldeutero-HRP, the resulting absorbance at 420 and 390 nm decreased and increased, respectively, after the decay of the benzoate electron adduct (Figure 2A). This suggests that the benzoate electron adduct reacts with the oxy form of diacetyldeutero-HRP. Figure 2B shows the absorption spectrum of the reaction product. The figure also compares the spectra of compound I, compound II, the oxy form, and the ferric form of diacetyldeutero-HRP. It is noted that the spectrum of the product is identical with that of compound I of the enzyme. The second-order rate constant of the reaction was calculated to be 7.5×10^7 M^{-1} s^{-1}. In contrast to oxy-Mb, the reaction of oxy-HRP with other radicals such as NAD and MV^+ could not be detected. This result is not attributable to the difference in the oxidation potential between oxy-HRP and oxy-Mb, since oxy-HRP is more reactive against a number of hydrogen donors, compared with oxy-Mb.[17] We must consider another factor, such as an extent of heme exposure to solvent and a charge group in the vicinity of the electron-transfer site, as discussed in a previous report.[9]

Ferric HRP reacts with H_2O_2 to form compound I, which retains two oxidizing equivalents above the ferric enzyme. The two equivalents are shared between the iron atom as the ferryl

Scheme I



state and the porphyrin as a π cation radical. In the case of Mb, one oxidizing equivalent of H_2O_2 is used to oxidize the ferric iron of Mb to the ferryl form and the fate of one more equivalent is not known. One may ask the question why the porphyrin π-cation-radical species of Mb is not formed. On the other hand, present experiments also show that ferryl-Mb was formed by one-electron reduction of oxy-Mb, in contrast to oxy-HRP. Any such intermediate, which corresponds to a porphyrin π cation radical, could not be detected. At present the difference between Mb and HRP can be interpreted by the following reaction sequence after the reduction (Scheme I). An initial step is the formation of Fe(II)–O_2^-. Then the intramolecular electron transfer from heme iron to the ligand and rapid association of H^+ gives rise to a peroxyiron(III) species, which is equivalent to that obtained by the reaction of H_2O_2 with the ferric heme. Subsequently there are two modes of iron(III)–O–O bond cleavage. A heterolytic cleavage of the O–O bond occurs to form a two oxidizing equivalents above the ferric heme and OH^- (step I). Alternatively, a homolytic cleavage occurs to form a one oxidizing equivalent above the ferric form and OH• (step II). Here, step II (homolytic cleavage) is compatible with the formation of ferryl-Mb. The formation of OH• would result in the detection of free radical in the amino acid residues.[18]

The mechanism of the O–O bond cleavage mediated by iron(III) has been studied extensively by using model systems.[4,19] Groves and Watanabe proposed that two distinct mechanisms can be controlled by the acidic proton.[4] The acidic proton of distal histidine may have an important role in the formation of compound I. This is supported by resonance Raman studies, where the hydrogen bond between the oxo ligand of ferryl-Mb and a nearby amino acid residue of the protein is absent, in contrast to compound II of HRP.[20]

With synthetic heme iron in nonaqueous solvent at low temperature, the reactive intermediates have been isolated spectrophotometrically. The species of $[Fe^{2+}$–$O_2]^-$ has been characterized as a stable product, where its structure was formulated as a high-spin ferric η^2-peroxide complex.[16,20] On the other hand, the peroxoiron(III) complex prior to the formation of the oxo(IV) porphyrin cation radical was observed in the reaction of iron(III) porphyrin with peroxiacids.[4] Any such intermediates, however, could not be detected in the present experiments. This suggests that the reaction of the radicals with the oxyheme is rate-determining in Scheme I. If such intermediates exist, the lifetime of these species is shorter than microseconds in the case of hemoprotein.

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