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OXIDATION OF NITROXIDE RADICALS BY THE REACTION OF HEMOGLOBIN WITH HYDROGEN PEROXIDE

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The ESR signal of 4-hydroxy-1-oxyl-2,2,6,6-tetramethylpiperidine in hemoglobin solution decreased drastically by the addition of hydrogen peroxide. The results of ion-exchange chromatography and sodium tetraphenylborate on the reaction solution showed an oxidation of the nitroxide radical to cation form. On the basis of the comparison of thin layer-chromatogram with the reaction products of the nitroxide radicals with HCl or Br_2, the formation of 4-hydroxy-1-oxo-2,2,6,6-tetramethylpiperidinium cation was demonstrated. This result was supported by the ^{13}C NMR measurement.

The spin label technique has been widely utilized to investigate the dynamic structures in biological and model membranes (1). As label reagents, the compounds with a nitroxide radical have been used. Nitroxide radicals are, however, readily reduced by leukocytes (2) and reducing agents such as ascorbate (3). The diminished signals are enhanced by the addition of $K_3Fe(CN)_6$ (2). When fatty acid spin-labeled erythrocytes are exposed to H_2O_2, their ESR signals decrease drastically (4). No such a phenomenon is, however, observed in erythrocyte ghost membranes, suggesting the attribution of hemoglobin to the decrease of the signal intensity. It is well known that the reactions of hemoproteins such as hemoglobin and horseradish peroxidase with peroxides oxidize various organic compounds (5-7). We report here that nitroxide radical is oxidized to the corresponding oxoammonium cation by the reaction of hemoglobin with H_2O_2.

Abbreviations: HOTMP, 4-hydroxy-1-oxyl-2,2,6,6-tetramethylpiperidine; TLC, thin-layer chromatography.

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MATERIALS AND METHODS

4-Hydroxy-1-oxyl-2,2,6,6-tetramethylpiperidine (HOTMP) was purchased from Aldrich Chemical Co. Hemoglobin from beef blood was Sigma Chemical Co. Sodium tetraphenylborate and silica gel 70 F_{254} plates were obtained from Wako Pure Chemical Industries. Dowex 1-X8 (200-400 mesh, Cl^- form) and AG 50W-X4 (200-400 mesh, H^+ form) were from Dow Chemical Co. and Bio-Rad, respectively. All other chemicals were of analytical grade.

To 2 ml of 0.9% aqueous NaCl solution containing both HOTMP (53 mg) and hemoglobin (200 mg), 300 µl of H_2O_2 (30%, w/w) was added. The reaction of strong acid with radical was carried out by dissolving HOTMP (50 mg) in 2 ml of 0.5N HCl. These reaction solutions were incubated for several days at 37°C. Acid solution of HOTMP was then neutralized with NaOH. The reaction of HOTMP (40 mg) with Br_2 (0.2 ml) was performed in the 0.9% aqueous NaCl solution (2 ml) at room temperature. HOTMP (50 mg) dissolved in H_2O (2 ml) was reduced with sodium ascorbate (60 mg).

A silica thin-layer chromatogram of these reaction products was developed in butanol/acetic acid/water (4:1:2, v/v/v) and stained with iodine vapor. Ion-exchange resins were washed three times for Dowex 1-X8 at a sequence of 1N HNO_3, H_2O, 1N NaOH, 1M NaCl, and H_2O, and for AG 50W-X4 with 2N HCl, H_2O, 2M NaCl, and H_2O. The reaction solution containing hemoglobin was applied to a AG 50W-X4 (Na^+ form) ion-exchange column (1 x 5 cm). The dark brown band due to hemoglobin was fast eluted with H_2O. Then, the solution eluted with 2N HCl was neutralized with NaOH. A precipitate obtained by adding sodium tetraphenylborate to the neutralized solution was purified using acetone and H_2O, and was dissolved in d_6-dimethyl sulfoxide for ^{13}C NMR measurement.

The ESR spectra were recorded on a JEOL JES FE-1X spectrometer. The ^{13}C NMR spectra were run at 50.1 MHz on a JEOL JNM-FX 200 spectrometer operating in the Fourier transform mode. Samples were inserted into 10 mm tubes and measured at 25°C. The mixture of dioxane/formic acid (1:2, v/v) was used as an external reference and the difference in chemical shifts of both signals was used as a measure of temperature control ($\Delta\delta$ = 98.44 ppm at 25°C). Other NMR experimental conditions are found in the figure legend.

RESULTS AND DISCUSSION

When H_2O_2 was added to HOTMP-hemoglobin solution, the ESR signal intensity of the radicals decreased drastically. The reaction solution was evaporated to remove the excess of H_2O_2 and then H_2O was added to the residue. The ESR signal intensity of the solution did not change by the addition of 5 mM $K_3Fe(CN)_6$ (final concentration), which is often used to oxidize the reductants (2). This result indicates that the decrease of the ESR signal intensity observed here is no reduction of the radicals.

Figure 1 shows the patterns of thin-layer chromatography (TLC) of HOTMP reaction products. The TLC pattern of HOTMP-H_2O_2-hemoglobin system predominantly demonstrated the spots with R_f

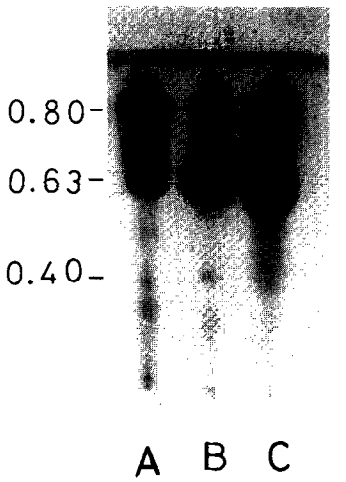

Figure 1. Oxidation of nitroxide radicals by the reaction of hemoglobin with hydrogen peroxide

Thin-layer chromatogram of HOTMP reaction products. HOTMP was treated with H_2O_2-hemoglobin (A), HCl (B), and Br_2 (C). Each sample was spotted on silica gel plate, developed in butanol/acetic acid/water (4:1:2, v/v/v), and stained with iodine vapor.

values of 0.80, 0.63, and 0.40 (Fig. 1A). Similar spots were obtained by the reaction of HOTMP with HCl (Fig. 1B) or Br_2 (Fig. 1C). On the basis of the reduction of HOTMP with ascorbate, two spots (R_f values 0.80, and 0.63) were attributable to the radical and its reductant, respectively. Since the spot with an R_f value of 0.63 was observed in pure HOTMP solution, it is considered that the nitroxide radicals are partially reduced during the development of TLC. The reaction product corresponding to an R_f value of 0.40 was insoluble to nonpolar solvents such as ethyl ether and soluble in polar solvents such as H_2O and CH_3OH. The electrical properties of the product were examined using the ion-exchange chromatography. In the case of cation-exchange resin, the compound with an R_f value of 0.40 was eluted with not H_2O but 2N HCl. On the other hand, the compound was readily driven through an anion-exchange column with H_2O. Sodium tetraphenylborate is widely used as a reagent to detect cation molecules (8). Addition of sodium tetraphenylborate to each solution indicated

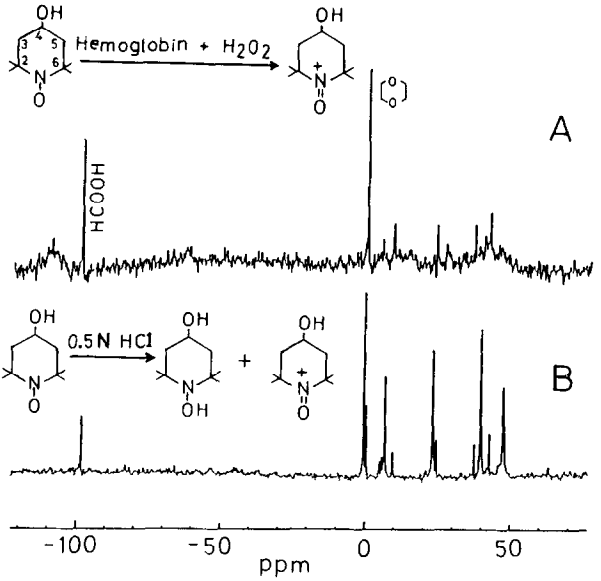

Figure 2. Oxidation of nitroxide radicals by the reaction of hemoglobin with hydrogen peroxide

Proton-decoupled ^{13}C NMR spectra at 50.1 MHz of HOTMP in H_2O_2-hemoglobin (A), and HCl (B) solutions. The reaction solutions were prepared as described under MATERIALS AND METHODS. The measurement conditions were as follows: spectral width 10 KHz, data points 16K, pulse width 15 μs (90°), pulse repetition time 8 sec, numbers of pulses 3800 (A), and 3200 (B). The mixture of dioxane/formic acid (1:2, v/v) was used as an external reference.

in Fig. 1 resulted in the formation of a precipitate. Concerning the spot with an R_f value of 0.40, the TLC pattern of the supernatant showed the faint spot compared with that of the original solution. Oxoammonium salts are formed by the reaction of nitroxide radicals with strong acids or Br_2 (9). These results suggest that the spot with an R_f value of 0.40 may be corresponding to the oxoammonium salt.

Figure 2 shows the ^{13}C NMR spectra of HOTMP in H_2O_2-hemoglobin and HCl solutions. The NMR signal was not observed in the HOTMP-free solutions, indicating that the signals arise from the reaction products of HOTMP. The ^{13}C NMR signal intensity of the supernatant by the addition of sodium tetraphenylborate to the solution, in which hemoglobin is removed using a cation-exchange

Table I. ^{13}C NMR chemical shifts* of HOTMP in H_2O_2-hemoglobin (A), and HCl (B) solutions.**

Carbons	^{13}C chemical shifts (ppm)		
	A	B	
C-2,6	9.09 (s)	9.09 (s)	-0.90 (s)
C-3,5	23.98 (t)	24.03 (t)	22.81 (t)
C-4	5.37 (d)	5.40 (d)	6.44 (d)

* Positive chemical shifts are upfield from dioxane (external reference). Results of off-resonance decoupling are given in parentheses (s=singlet; d=doublet; t=triplet).
** pH values of solutions A, and B are 5.2.

column, diminished significantly and the corresponding signals were observed in the precipitates.

The ^{13}C NMR spectrum of HOTMP in H_2O_2-hemoglobin solution is different from that in HCl. The spectrum of the latter demonstrated the existence of two kinds of the reaction products, as indicated in TLC. The values of the ^{13}C chemical shifts of the two species were consistent with those of HOTMP in H_2O_2-hemoglobin and ascorbate solutions. On the basis of the results of ^{13}C chemical shifts and off-resonance decoupling, each carbon atom was assigned as shown in Table 1. These results indicate that HOTMP is oxidized by H_2O_2-hemoglobin and 4-hydroxy-1-oxo-2,2,6,6-tetramethylpiperidinium cation is formed.

Organic compounds are oxidized by hemoprotein-peroxide system. Nitroalkanes are oxidized by the reaction of horseradish peroxidase with H_2O_2 (6). In the H_2O_2-hemoglobin system, phenoxy radicals are obtained from phenols (5). These products may be mediated by the intermediates (5, 10) composed of hemoproteins and peroxides. We expect that the detailed study on the formation of

the oxoammonium cation in our system provides very valuable information on the reaction mechanisms of hemoproteins with peroxides.

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