Oxidation of nitroxide radicals by an iron-hydrogen peroxide-amino acid system

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The oxidation of nitroxide radicals by the reaction of hemoglobin with hydrogen peroxide occurred using both $FeSO_4$ and amino acids instead of hemoglobin. Tyrosine, phenylalanine, tryptophan, methionine and cysteine in globin were connected with the formation of the oxoammonium cation. Of these amino acids, tyrosine was especially effective in its oxidation. It was found that both the binding of nitroxide radicals to ferric iron and the activation of amino acids by hydroxyl radicals were important in the radical oxidation.

Nitroxide radical oxidation Amino acid Fenton reaction Hydroxyl radical ESR ^{13}C-NMR

1. INTRODUCTION

Peroxides in the presence of hemoproteins can oxidize various organic compounds. Epinephrine and catecholamines are oxidized to semiquinone radicals by the horseradish peroxidase-H_2O_2 system [1,2]. Phenoxy radicals are obtained from phenols using both methemoglobin (MetHb) and H_2O_2 [3]. Reaction of horseradish peroxidase with H_2O_2 produces intermediate complexes such as compound I and compound II [1,2]. In the MetHb-H_2O_2 system, the existence of a similar intermediate complex was proposed [3]. However, its properties have not been exactly elucidated. We have found that nitroxide radicals in Hb solution are readily oxidized to the corresponding oxoammonium cations by the addition of H_2O_2 [4]. This work describes that the oxidation of nitroxide radicals occurs by using both nonheme iron, $FeSO_4$, and some defined amino acids in globin instead of Hb.

2. MATERIALS AND METHODS

4-Hydroxy-1-oxyl-2,2,6,6-tetramethylpiperidine (HOTMP) and 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) were from Aldrich Chemical Co. 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) and xanthine oxidase (butter milk, grade 1) were from Sigma and all amino acids from Nakarai Chemicals. Sodium tetraphenylborate and silica gel 70 F_{254} plates were from Wako. AG 50W-X4 (200–400 mesh, H^+ form) was from Bio-Rad. All other chemicals were of analytical grade.

Human Hb was purified as in [5]. MetHb (1 mM) was prepared using 5 mM $K_3Fe(CN)_6$. A mixture of H_2O_2 (9 mM) and MetHb (0.2 mM) with and without NaN_3 (12 mM) was incubated for 5 min at 37°C. A change in the oxidized state of iron of MetHb was examined by measuring the ESR spectra at −196°C. The ESR spectra of TEMPO (0.3 mM) added to the mixture were measured at 25°C. The preparation of globin was carried out by the methyl ethyl ketone method [6]. To globin (3 mg/ml) solution containing TEMPO (0.3 mM), H_2O_2 (9 mM) was added with and without 3 mM $FeSO_4$. In NMR measurements, the samples containing 0.15 M HOTMP, 0.17 M amino acid, 5 mM $FeSO_4$ and 0.88 M H_2O_2 in 0.5 N HCl were incubated for 3 h at 37°C. As a scavenger of hydroxyl radicals, DMPO was used at 1.5 M. Silica thin-layer chromatography and ion-exchange chromatography of these reaction products were carried out as described [4]. Active ox-

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ygen species such as hydroxyl radical, superoxide anion and singlet oxygen were generated using Fenton's reagent, xanthine-xanthine oxidase and H_2O_2-NaOCl, respectively. 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. The difference in chemical shifts of dioxane and formic acid as an external reference was used as a measure of temperature control.

3. RESULTS AND DISCUSSION

When H_2O_2 was added to MetHb solution with and without TEMPO, the ESR signal of TEMPO dramatically disappeared and the signal due to non-heme iron at $g = 4.3$ appeared (fig.1). By the addition of NaN_3 to MetHb solution, the decrease in signal intensity of TEMPO and the appearance of non-heme iron were suppressed. The same results were also obtained using OxyHb instead of MetHb. Recently, authors in [7] have reported that the hydroxy radical is readily generated by a reaction of Hb with H_2O_2 and Hb behaves as a Fenton reagent. Therefore, the production of non-heme iron from Hb might be connected with the decrease in signal intensity of TEMPO. To clarify the role of Hb, globin was isolated from Hb. The ESR spectrum of TEMPO in globin solution did not change on adding H_2O_2. When $FeSO_4$ as non-heme iron was used, there was no marked change in the ESR spectrum of TEMPO by the reaction of $FeSO_4$ with H_2O_2, which is well known as a Fenton reaction. When Fenton's reagent was added to

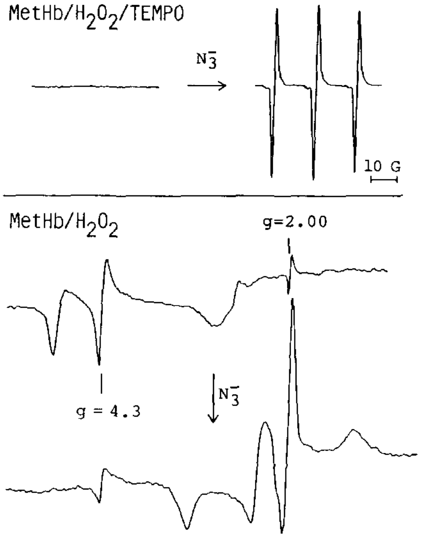

Fig.1. ESR spectra of TEMPO (25°C) and MetHb (−196°C) in the MetHb-H_2O_2 reaction in the presence and absence of NaN_3. The spectrometer settings for TEMPO and MetHb were 100 kHz modulation amplitude, 1 and 6.3 G; microwave power, 1 and 10 mW; scan range, 200 and 5000 G; scan speed, 8 and 4 min, respectively.

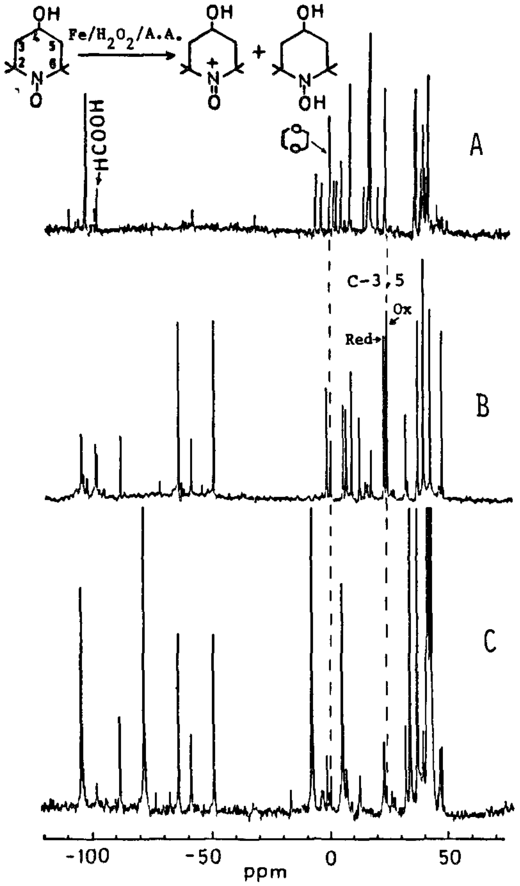

Fig.2. Proton-decoupled ^{13}C-NMR spectra at 50.1 MHz of HOTMP in $FeSO_4$-H_2O_2-cysteine (A), -tyrosine (B) and -tyrosine-DMPO (C) systems. The measurement conditions were as follows: spectral width 10 kHz, data points 16 K, pulse width 15 μs (90°), pulse repetition time 8 s, number of pulses 2770 (A), 5900 (B) and 1000 (C). Samples were inserted into 10-mm tubes and measured at 25°C. A.A., amino acid.

globin solution, the signal intensity of TEMPO decreased drastically, indicating the requirement of both globin and iron for chemical reaction of the radicals.

The change in ESR spectra observed using both $FeSO_4$ and amino acids in globin instead of Hb was examined by measuring the ^{13}C-NMR spectra. Fig.2 illustrates the ^{13}C-NMR spectra of HOTMP added to the $FeSO_4$-H_2O_2-cysteine (A), -tyrosine (B) and -tyrosine-DMPO (C) systems under acid conditions. On the basis of the ^{13}C chemical shifts of HOTMP in 0.5 N HCl and Hb-H_2O_2 solution [4], it was found that the spectra revealed the reduction and oxidation products of HOTMP. The formation of the 4-hydroxy-1-oxo-2,2,6,6-tetramethylpiperidinium cation, which is an oxidant of HOTMP, was confirmed by means of ion-exchange chromatography, thin-layer chromatography and sodium tetraphenylborate, as in [4]. The ^{13}C-NMR spectra of HOTMP obtained using 3 aromatic amino acids, i.e. Tyr, Phe and Trp, were almost the same although the signal intensities of reduced and oxidized forms in each reaction solution were different. On the other hand, the spectra observed in sulfur-containing amino acids such as cysteine and methionine were very complicated due to the appearance of many unidentified signals. In the case of cysteine, no reduced form of HOTMP was observed. Using other amino acids, no oxidation of nitroxide radicals occurred. Namely, only the reduced form of HOTMP due to acid conditions was observed. The ability to produce the oxoammonium cation was compared using 5 amino acids (table 1). Tyrosine was especially effective. As a scavenger of hydroxyl radicals, DMPO was added to the reaction system. The production of oxoammonium cation was significantly inhibited (fig.2C). Amino acid radicals are generated by hydroxyl radicals [8]. Tyrosine cross-linking and tyrosine radicals due to the reaction of myoglobin with H_2O_2 have been reported [9,10]. Therefore, it is expected that the activation of the amino acids by hydroxyl radicals is important in the formation of oxoammonium cation.

Table 1

Effect of amino acids on oxidation of nitroxide radicals

Amino acid	Relative efficiency of oxidation
Tyr	1.0
Cys	0.79 ± 0.01
Phe	0.66 ± 0.03
Trp	0.45 ± 0.01
Met	0.29 ± 0.10

Oxidation of HOTMP was performed using the $FeSO_4$-H_2O_2-amino acid system. The formation of oxoammonium cation was estimated from the ratio of peak heights of HOTMP oxidant (relative to C-3, 5 carbons) and dioxane as external reference. The efficiency of the oxidation by amino acids was normalized by that of tyrosine. Values are means ± SD for at least 2 experiments

A titanous chloride-H_2O_2 system also produces hydroxyl radicals [8]. However, no oxidation of nitroxide radicals occurred using Ti^{3+} instead of Fe^{2+}. Fig.3 shows the ESR spectral changes of TEMPO added to the Fe^{2+}-H_2O_2 and Ti^{3+}-H_2O_2 systems under acid conditions. The ESR signal intensity of TEMPO decreased on its binding to oxidized ferric iron, whereas it remained almost constant in the Ti^{3+}-H_2O_2 system. The ESR signal due to non-heme ferric iron at $g = 4.3$ decreased on in-

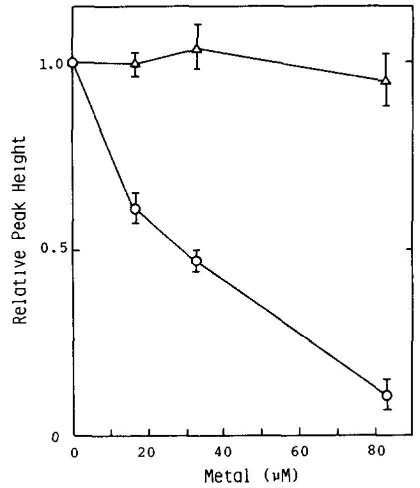

Fig.3. Binding of TEMPO to metal in $FeSO_4$-H_2O_2 (○) and $TiCl_3$-H_2O_2 (△) systems. The assay was performed at 37°C for 5 min using 0.3 mM TEMPO, 0.9 mM H_2O_2 and various concentrations of metals under acid conditions (pH 2.4). The ESR spectra of TEMPO were recorded as indicated in the legend to fig.1 at 25°C. The results are the means ± SE of at least 2 experiments.

teraction with TEMPO, indicating electron transfer from TEMPO to ferric iron.

In the biological reaction of iron compounds with oxidants or reductants, the generation of active oxygen species such as singlet oxygen [11], hydroxyl radical [12] and superoxide anion [13] is expected. No oxidized form of nitroxide radicals was observed in NMR measurements of HOTMP added into the solution generating active oxygen, indicating that nitroxide radicals are stable to these active oxygen species.

The oxidation of nitroxide radicals by the Hb-H_2O_2 system may be partially explained in terms of the Fe^{2+}-H_2O_2-amino acid system. We propose that the transfer of electrons through iron from nitroxide radicals to amino acids activated by hydroxyl radicals is very important in the radical oxidation.

REFERENCES

[1] Kalyanaraman, B., Felix, C.C. and Sealy, R.C. (1984) J. Biol. Chem. 259, 354–358.
[2] Kalyanaraman, B., Felix, C.C. and Sealy, R.C. (1984) J. Biol. Chem. 259, 7584–7589.
[3] Shiga, T. and Imaizumi, K. (1973) Arch. Biochem. Biophys. 154, 540–547.
[4] Yamaguchi, T., Nakano, T. and Kimoto, E. (1984) Biochem. Biophys. Res. Commun. 120, 534–539.
[5] Drabkin, D.L. (1946) J. Biol. Chem. 164, 703–723.
[6] Ascoli, F., Fanelli, M.R.R. and Antonini, E. (1981) Methods Enzymol. 76, 72–94.
[7] Sadrzadeh, S.M.H., Graf, E., Panter, S.S., Hallaway, P.E. and Eaton, J.W. (1984) J. Biol. Chem. 259, 14354–14356.
[8] Taniguchi, H., Hasumi, H. and Hatano, H. (1972) Bull. Chem. Soc. Jap. 45, 3380–3383.
[9] Rice, R.H., Lee, Y.M. and Brown, W.D. (1983) Arch. Biochem. Biophys. 221, 417–427.
[10] King, N.K., Looney, F.D. and Winfield, M.E. (1964) Biochim. Biophys. Acta 88, 235–236.
[11] Kanofsky, J.R. (1983) J. Biol. Chem. 258, 5591–5593.
[12] Repine, J.E., Fox, R.B. and Berger, E.M. (1981) J. Biol. Chem. 256, 7094–7096.
[13] Aft, R.L. and Mueller, G.C. (1984) J. Biol. Chem. 259, 301–305.