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ESR-Spin Trapping Detection of Radical Center Formed on the Reaction of Metmyoglobin with Hydrogen Peroxide

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Abstract: The radical centers detected in the reaction of metmyoglobin (MetMb) with hydrogen peroxide (H_2O_2) have been studied by using a spin trapping technique. A broad 5-line asymmetric electron spin resonance (ESR) spectrum, with $2A_{max}=4.07$ mT and $2A_{min}=2.97$ mT, obtained after incubation of MetMb with H_2O_2 in the presence of a spin trap, 5,5-dimethyl pyrroline-N-oxide (DMPO) was gradually weakened with time and disappeared completely by 6 min after addition of guanidine-HCl (1.4 M). When a higher concentration (6 M) of the agent was added, the signal disappeared within 40 sec and the DMPO/OH signal appeared immediately. Then, a new 8-line signal with similar intensities grew gradually and was fixed by 45 min, coexisting with the DMPO/OH signal. This new signal was found to be composite, consisting of two different radical species. One of the 6-line signals, with a_N 1.49 mT and a_H 0.988 mT, was assigned to the DMPO/phenoxyl radical adduct. The second 6-line signal with a_N 1.55 mT and a_H 2.22 mT was assigned to carbon-centered radical adduct. When 3,3,5,5-tetramethylpyrrolin-N-oxide (TMPO), was employed in the place of DMPO, another broad asymmetric 5-line signal was detected with $2A_{max}=3.99$ mT and $2A_{min}=3.04$ mT, which is virtually identical to that obtained from the DMPO system. The shape of the spectrum of the TMPO adduct changed drastically, with lapse of time resulting in a broad singlet after 40 min. The broad singlet was assigned to the porphyrin radical adduct. Incubation of globin with Fenton reagent in the presence of DMPO initially gave a DMPO/OH signal. Then, a new 12-line signal began to grow after one minute and fixed after 15 min, coexisting with the DMPO/OH signal. This 12-line signal was assigned to DMPO/phenoxyl with a_N 1.47 mT, $a_{\beta H}$ 0.99 mT and $a_{\gamma H}$ 0.13 mT. A minor concentration of carbon-centered radical adduct was also detected. This radical composition is identical to that of guanidine-HCl treated MetMb/DMPO/H_2O_2 system, indicating that the radical producing conditions are somehow common in both systems. Heme iron can be released by excess H_2O_2 in the MetMb/H_2O_2 system, providing for Fenton reagent. When MetMb was pretreated with tyrosine blocking agent, KI_3, the broad 5-line MetMb-derived signal was not detected in the MetMb/DMPO/H_2O_2 system, whereas no such effect was detected on such system of Hb in which the radical center was assigned to cysteine residue not tyrosine, indicating that tyrosine residue is a main radical center produced in the MetMb/H_2O_2 system. Thus, the present data strongly support the previous indication that the apomyoglobin-derived radical center formed in the reaction of MetMb with H_2O_2 is a tyrosine residue.

Key words: hydrogen peroxide, metmyoglobin, radical center, spin trapping.

Reactive oxygen species such as O^{2-}, H_2O_2, are implicated in several biological processes including cellular damage, carcinogenesis, and phathogenesis (Halliwell *et al.*, 1986). These species are produced by phagocytes, enzymatic reaction, redox reaction of xenobiotics, and mitochondrial respiration in biological systems.

The oxygen species produce highly reactive toxic hydroxyl radical (·OH) through metal-catalyzed Haber-Weiss reaction.

$$O^{2-} + 2H^{+} \longrightarrow H_2O_2 \quad (1)$$

$$F^{3+} + O^{2-} \longrightarrow Fe^{2+} \quad (2)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{-} + \cdot OH \quad (3)$$

Of these reaction systems, the reaction of ·OH generation has been known to be a Fenton reaction.

Although ·OH has been considered a major source of deleterious reactions with biological molecules, most of the iron is sequestered from the reaction with reactive oxygen species to produce ·OH in biological systems. Ferritin, a typical iron storage protein, therefore,

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prevents cellular components from ·OH-mediated oxidative damage. In addition, several cellular components such as ascorbic acid, vitamin E, and glutathione can attenuate oxidative damage of biological molecules by scavenging oxygen centered free radicals.

There are many papers (Sadrzadeh *et al.*, 1984; Poppo and Halliwell, 1988; Galaris *et al.*, 1990) showing that hemoproteins such as myoglobin (Mb) as well as hemoglobin (Hb) can act as a Fenton reagent in the presence of H_2O_2 in biological systems, resulting in peroxidative damage through two different pathways in a reactive oxygen species-generating system. The first hypothetical pathway is that hemoproteins produce a highly oxidizing iron species, ferryl (Fe(IV)=0) (Harel and Kanner, 1985; Galaris *et al.*, 1990), and/or an amino acid radical (Catalano *et al.*, 1989). The other process is ·OH generation through the reaction of free iron released from hemoproteins with H_2O_2 in the presence of excessive H_2O_2 (Puppo and Halliwell, 1988). Although the ferryl myoglobin is a stable and oxidizing species, amino acid radicals can also initiate the oxidative damage of biological molecules (Kim *et al.*, 1994).

In a previous paper (Hong and Piette, 1989), we first reported the distinctive, five broad line ESR spectrum, detected after incubation of MetMb with H_2O_2 in the presence of a spin trap DMPO. The spectrum then was ascribed to the MetMb-derived radical DMPO adduct which is strongly immobilized (Hong and Piette, 1976). But the assignment of the radical species formed was difficult due to the anisotropic nature of the signal. Thereafter, Davies (1990 and 1991), using the same technique and stopped-flow ESR, proposed the radical center formed and trapped by DMPO to be assigned to a sterically constrained tyrosine phenoxyl radical, arising from oxidative damage to the globin moiety, which can react with oxygen to give tyrosine peroxyl radical. But the direct, unambiguous assignment of the immobilized ESR signal is yet to be determined.

In the present paper, therefore, a number of alternative approaches to detect the radical center have been introduced; 1) the spectral changes of the immobilized spectrum, ideally to a free spin spectrum, was monitored after appropriate alteration to the protein structure by denaturation; 2) Another type of spin trap, TMPO, was employed in the same system in place of DMPO in an attempt to see any difference; 3) Reaction of globin with Fenton reagent in the presence of DMPO was employed, in the hopes of finding some clue as to the identification of the radical center produced in the Mb/H_2O_2 system; 4) Iodinated MetMb was employed to see whether or not the tyrosine blocking agent blocks the free radical production in the system.

The results obtained in the present experiments strongly support the previous data (Miki *et al.*, 1989; Davies, 1990; 1991) that the radical center produced by Mb/H_2O_2 system is tyrosine phenoxyl.

Materials and Methods

Horse heart MetMb, Hb (from bovine blood, containing up to 75% methemoglobin), globin (from bovine hemoglobin), guanidine hydrochloride and spin trapping agent, DMPO and TMPO were purchased from Sigma Chemical Co. (St. Louis MO), and Sephadex G-15 gel was purchased from Pharmacia. Iodine and potassium iodide were purchased from Kanto and Showa Chemical Inc., respectively.

DMPO and TMPO were purified before use as described previously (Buettner, 1978) and the others were used without further purification.

For protein denaturation effect, MetMb was mixed with H_2O_2 in the presence of DMPO or TMPO and stirred for 30 sec. Solid guanidine-HCl was added to that solution and the resultant solution was examined by ESR.

Iodinated MetMb was prepared by the method described by Kretsinger (1968).

60 mM KI_3 solution was prepared by dissolving equimolar amounts of KI and of sublimed I_2 in 5% ethanol in 0.1 M sodium phosphate buffer (pH 7.0). To this solution, MetMb was added to a protein/KI_3 molar ratio of 1 : 6 and then incubated for 1 h at room temperature. Following the incubation, excess iodinating agent was removed by gel chromatography using a column of Sephadex G-15 gel. The concentration of iodinated MetMb was determined following sodium dithionite ($Na_2S_2O_4$) reduction using an extinction coefficient of 13,800 $M^{-1}\cdot cm^{-1}$ at 560 nm for deoxyMb. A similar procedure was applied for iodination of hemoglobin.

ESR measurements were carried out on a Bruker ER 200D-SRC ESR spectrometer equipped with a 100 KHz modulation frequency and a quartz flat liquid cell at room temperature. Samples were prepared in 0.1 M phosphate buffer (pH 7.0).

ESR spectra were recorded immediately after mixing (approx. 1 min) with the following instrumental settings : microwave power, 5 dB; microwave frequency, 9.76~9.77 GHz; receiver gain, 1×10^3~5×10^5; modulation amplitude, 1~2G; time constant, 50~200 ms; scan range, 20 mT; scan time, 10~200 s.

Results and Discussion

The ESR spectrum in Fig. 1A, obseved in the reac-

tion of MetMb (5 mM) and H_2O_2 (8 mM) in the presence of DMPO (0.1 M), was assigned (Hong and Piette, 1989) to a sterically constrained DMPO-adduct of radical centered on apomyoglobin.

Reaction of MetMb with H_2O_2 generates not only a ferryl species (Fe(IV)=O) (George and Irvine, 1952; Yonetani and Schleyer, 1967) but also protein radical (Gibson *et al.*, 1958; Harada and Yamazaki, 1987; King and Winfield, 1963; King *et al.*, 1967; Yonetani and Schlerer, 1967). Although the former species is a strong enough oxidizing species to react with many kinds of biomolecules (Harel and Kenner, 1985), it is not easily trapped by spin trap reagent, DMPO. Therefore, the DMPO adduct might be ascribed to amino acid radicals of apomyoglobin. The protein radical is generated by transferring one of two oxidizing equivalents trapped in heme center from H_2O_2 to the surrounding globin. The fact indicates that the adduct can be generated either in the surface or the vicinity of heme pocket of MetMb. It is difficult, however, to find the localization of radical center since DMPO, hydrophilic and small, is able to access to the radicals on surface and hydrophobic environment.

Davies (1990) obseved the same ESR spectrum as shown in Fig. 1A (Hong and Piette, 1989) for the same MetMb/DMPO/H_2O_2 mixing system. This signal, then, was assigned to a slowly-tumbling MetMb-derived spin adduct in which spin is centered on an aromatic residue, probably a tyrosine peroxyl radical. By using stopped-flow ESR mixing equimolar amount of equine MetMb and H_2O_2, he also obtained (1991) a 6-line signal (Harada and Yamazaki, 1987) with coupling constants of approx. a_H 2.26 mT and a_{2H} 0.75 mT, which was assigned to a sterically-constrained tyrosine phenoxyl radical generated from the readily accessible Tyr-103 residue. Futhermore, he suggested that the phenoxyl radical reacts with oxygen to give the tyrosine peroxyl radical which has been previously trapped with the spin trap DMPO. However, these interpretations have not allowed unambiguous assignment because the assignment by means of ESR of amino acid radicals formed in proteins is in general very difficult due to the isotropic hyperfine splitting being smeared out by anisotropic terms (Ehrenberg, 1972).

The best way to identify free radicals by ESR is to measure the spectral parameters from a well resolved spectrum which may be obtained by the alteration of the spin center from immobilized state to free spin state, if possible.

In an attempt to investigate the well-resolved spectrum, the effect of guanidine-HCl, a protein denaturing agent, on the ESR spectrum was recorded in the mixture of MetMb and H_2O_2. When solid guanidine-HCl

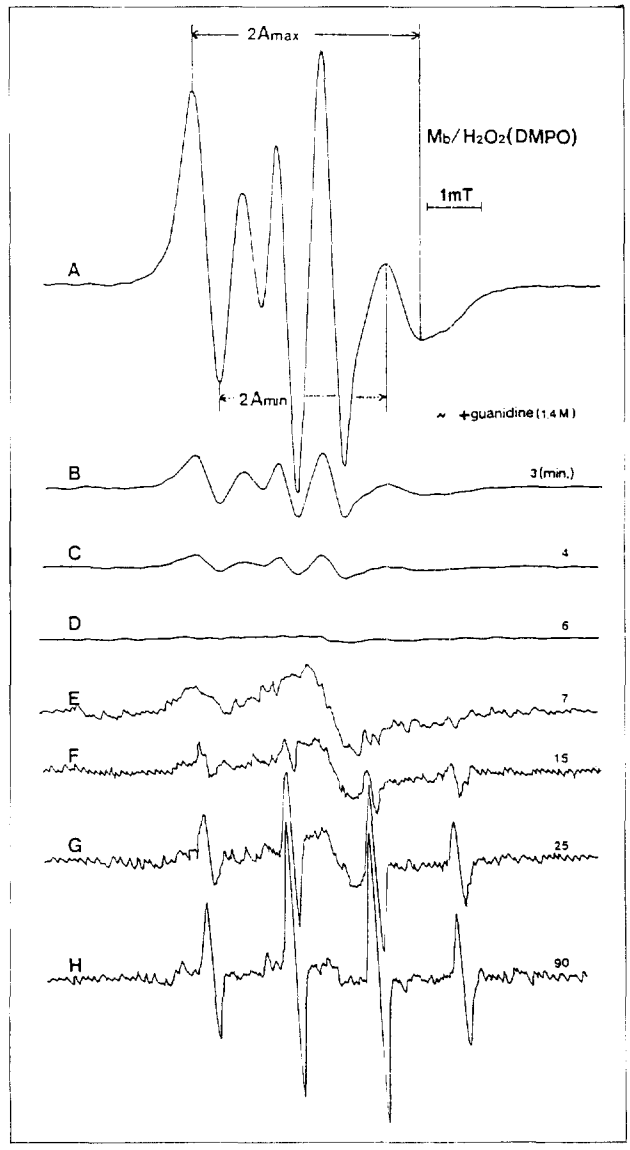

Fig. 1. The effect of guanidine-HCl on the system containing MetMb, H_2O_2 and DMPO. A. The ESR spectrum observed after mixing MetMb with H_2O_2 in the presence of DMPO. The final concentrations of each solution were 5 mM MetMb, 8 mM H_2O_2 and 0.1 M DMPO. B-H, ESR spectra recorded 3 min (B), 4 min (C), 6 min (D), 7 min (E), 15 min (F), 25 min (G) and 90 min (H) after addition of guanidine (1.4 M) to the mixture A.

(1.4 M) was added to the system in Fig. 1A, the intensity of the 5-line spectrum was weakened gradually with time and disappeared after 6 min (Fig. 1D). After 25 min, it was replaced by a DMPO/OH signal (Fig. 1G), well defined 1 : 2 : 2 : 1 line intensity of ·OH, in which the intensity increased about 10 fold in 45 min and then finally diappeared after 5 h (data not shown). During this experiment, no other new signal was observed.

At higher levels of guanidine-HCl (6 M), the 5-line spectrum disappeared within 40 sec and the 1 : 2 : 2 : 1 DMPO/OH signal appeared immediately. However, when the residual broad-immobilized spectrum disap-

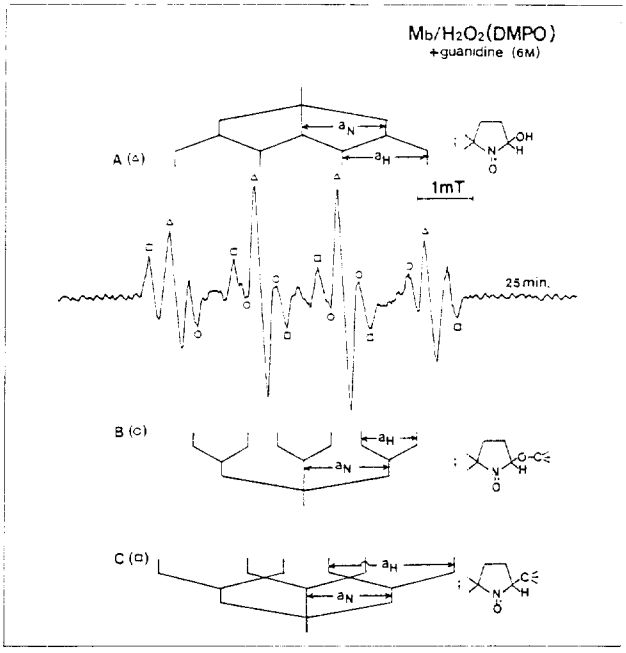

Fig. 2. The ESR spectrum observed 45 min after addition of guanidine-HCl (6 M) to the mixture of Mb/DMPO/H_2O_2. Mark △(A), O(B), and □(C) indicate the signals ascribing to DMPO-OH, DMPO-O-C≡ and DMPO-C≡ adduct, respectively. It lasts longer than 4 h.

peared completely, an 8-line spectrum with similar intensities appeared and coexisted with the DMPO/OH signal (Fig. 2) for over 1 h. Obviously this signal is not due to the free spin signal of an immobilized DMPO/MetMb-derived radical adduct since this signal did not show up during the decay of the immobilized signal but slowly emerged long after the appearance of DMPO/OH signal.

Although the exact molecular events that eventually yield these results can not be clear upon these grounds alone, the signal in Fig. 2 was interpreted to be a mixture of two different new signals coexisting with DMPO/OH signal (Fig. 2A), the intensities of which are about equal. The first of these, 6-line signal (Fig. 2B) was assigned, on the basis of its hyperfine coupling constants, aN 1.49 mT, aH 0.988 mT and their similarity to previously reported values for DMPO/OCH_3, a_N 1.42 mT, a_H 0.94 mT (Makino *et al.*, 1990), to phenoxyl radical adducts. The second 6-line signal (Fig. 2C), with a_N 1.55 mT, a_H 2.22 mT, was assigned to well characterized carbon-centered radical adduct (Garry, 1987).

The result that ·OH did not simultaneously appear with the disappearance of the 5-line spectrum supports the idea that the radical is generated by iron released from the heme component by excess H_2O_2 (Gutteridge, 1986; Harel *et al.*, 1988; Puppo and Halliwell, 1988), but not by heme (Sadrzadeh *et al.*, 1984). This type of iron provided for a Fenton reagent to produce ·OH. ·OH liberated by a redox cycle of iron in the presence of H_2O_2 can attack amino acid residues (Cooper *et al.*, 1985) to produce free radicals, particularly centered on tyrosine residues in apomyoglobin (Davies, 1991), providing the possibility that ·OH generated by released iron in the heme pocket as well as ferryl may be a source of protein radical formation.

The rapid decrease of the ESR signal intensity by adding guaninine-HCl to the mixture of MetMb and H_2O_2 (Fig. 1A-D) may be ascribed to pH change of reaction mixture and/or conformational change. Addition of 6 M guanidine-HCl actually decreased pH from 7.4 to 5.9, giving rise to instability of the spin adducts. Protein denaturation exposes hemes and amino acid residues to solution and so increases the intermolecular interaction between them and the spin adducts. The interaction between spin adducts and ferryl, produced by reacting heme with H_2O_2, as well as amino acids such as tryptophan, tyrosine, and cystine, dramatically decreases the ESR signal intensity of the spin adducts (unpublished data). ESR signal intensity is dependent on both the rate of radical formation and the stability of spin adducts. Tyrosine radical in proteins is generated by electron migration in pulse radiolysis (Farver and Pecht, 1991) and in the system of MetMb and H_2O_2 (Miki *et al.*, 1989). Since electron transfer reaction in proteins is largely dependent on distances between electron acceptor and donor, change of protein configuration is a critical factor for the electronic interaction. The rate of intramolecular electron migration in globular portion is faster than in extended structures such as fibrous or denatured proteins. It is also possible that guanidine-mediated denaturation of MetMb is responsible for the rapid decrease of spin adduct signal.

Another kind of spin trap, TMPO, was also employed to see whether or not it was possible to obtain comparable results. TMPO is more hydrophobic and bulkier than DMPO because of two additional methyl substituents, resulting in steric hinderance as well as less diffusion to small cavities in aqueous solution. Since these physical properties are assumed to be a major factor for trapping radicals formed in the surface of protein molecules (Ortiz and Catalano, 1985), the change of spectrum by TMPO, in the place of DMPO, was measured in the mixture of MetMb and H_2O_2 (Fig. 1A) to see radical species and its localization in protein.

MetMb (4 mM) when incubated with H_2O_2 (7 mM) in the presence of TMPO (0.1 M) produced an asymmetric 5-line ESR spectrum as shown in Fig. 3A, which appears to be practically identical to that obtained from the MetMb/DMPO/H_2O_2 mixing system (Fig. 1A), suggesting that the same protein radicals was adducted

by both spin-trapping reagents and the main radical detected by this technique was formed on the surface of the protein rather than inside of globin molecule. This result supports the previous data that the ESR spectra of DMPO-adduct closely resemble to those of the TMPO-adducts (Janzen, 1984). The splitting constants of DMPO-adduct signal, $2A_{max}=4.07$ mT and $2A_{min}=2.97$ mT and TMPO adduct signal, $2A_{max}=3.99$ mT and $2A_{min}=3.04$ mT were very similar to previous parameters of tyrosine radical, observed in the mixture of MetMb and H_2O_2 (Davies, 1990; Miki *et al.*, 1989), indicating that the main radical center trapped by both of the spin traps DMPO and TMPO in the MetMb system is tyrosine.

Since TMPO adducts are more persistent than for DMPO, stability of the adducts by TMPO was compared to that of DMPO under the same reaction conditions as those above. As shown in Fig. 3A-E, half-life of TMPO adduct was about 11 min, while the spin adduct of DMPO was shorter, 3 min. In the case of TMPO, spin adduct was stable for 5 min, followed by rapid destruction. The shape of the spectrum of the DMPO adduct was not changed with lapse of time (data not shown), while the spectrum of the TMPO adduct was drastically changed after 10 min, resulting in singlet spectrum (see Fig. 3E) by about 40 min. The decay curve suggests that the TMPO adduct signal probably consisted of two different components, labile and stable, but the same species, produced from two different tyrosine residues in different environments. The Tyr-103 is partially exposed to solution even though closed to the heme center, while the Tyr-146 is buried in a hydrophobic environment (Uyeda and Peisach, 1981). Although no definitive evidence has yet been obtained for such a species, the radical centered on Tyr-146 may be more stable because the radical adduct on Tyr-103 was found to be destroyed by ferryl ion (data not shown). A new singlet signal appeared in the TMPO system after 40 min is probably assigned to porphyrin radical (Van Stevenick, 1988).

The spectrum in Fig, 3F was obtained immediately after guanidine-HCl was added to the system Fig. 3A. This signal appears to be virtually identical to that shown in Fig. 1E and Fig. 3E as well as to that obtained in the MetMb/H_2O_2 flow-mixing after stopping the flow (Miki *et al.*, 1989). These results strongly indicate that the stability of the radical center giving rise to this signal is less dependent on the protein denaturation. This signal, therefore, may be ascribed to porphyrine radical adduct.

In an alternative approach to validate that the radical center produced in the MetMb/H_2O_2 system is a tyrosine residue (Davies, 1991) within an apoprotein, we

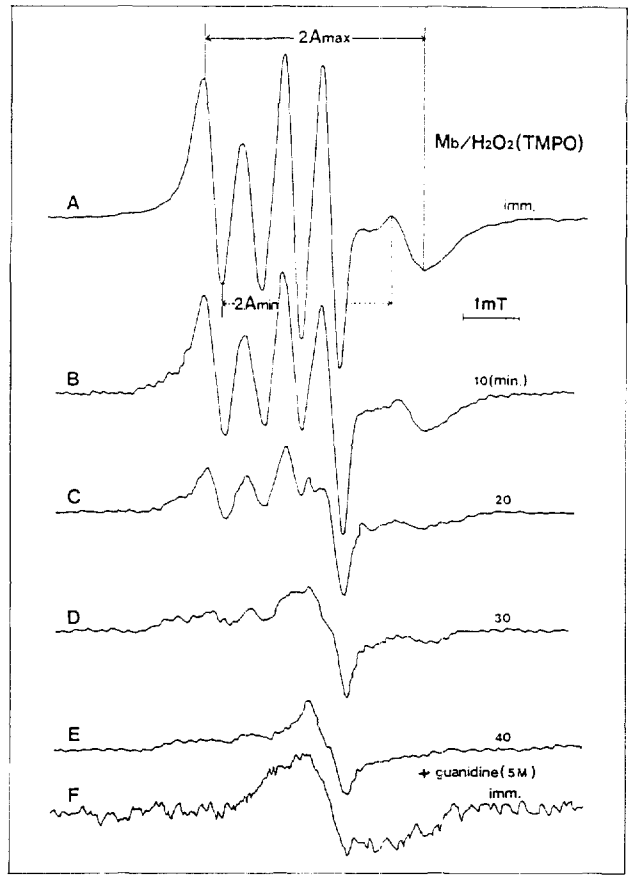

Fig. 3. Time course and the effect of guanidine on the ESR spectrum observed in MetMb/TMPO/H_2O_2 system. A. The ESR spectrum observed immediately after mixing for the system containing 4 mM MetMb, 7 mM H_2O_2 and 0.1 M TMPO. Time course was checked 10 min (B), 20 min (C), 30 min (D) and 40 min (E) after mixing of system A, respectively. Spectrum F was recorded immediately after addition guanidine (5 M) to the mixture A, 30 sec after mixing. Gains were 8×10^4 for A, 3×10^5 for B and 5×10^5 for F.

investigated ·OH-mediated protein radical generation in the system of globin and Fenton reagents. Globin contains three tyrosine residues in α-chain and two in β-chain. Incubation of globin (240 μM) with Fenton reagent (60 μM Fe(II) and 5 mM H_2O_2) in the presence of DMPO (0.1 M), in 0.1 M phosphate buffer, gave new ESR signals as shown in Fig. 4. The time course of the signal shows that only the DMPO/OH appears initially (Fig. 4A) and then 12-line signal with equal intensity slowly grows for 5 min (Fig. 4E). Then, after 15 min, two lines at low and high magnetic fields disappeared and the 12-line signal was fixed (Fig. 4F), coexisting with the DMPO/OH signal in about equal concentration.

This spectrum appears to be the same signal as that shown in Fig. 2, obtained by incubation for 45 min after addition of guanidine-HCl (6 M) to the mixture of MetMb/DMPO/H_2O_2. The hyperfine splitting con-

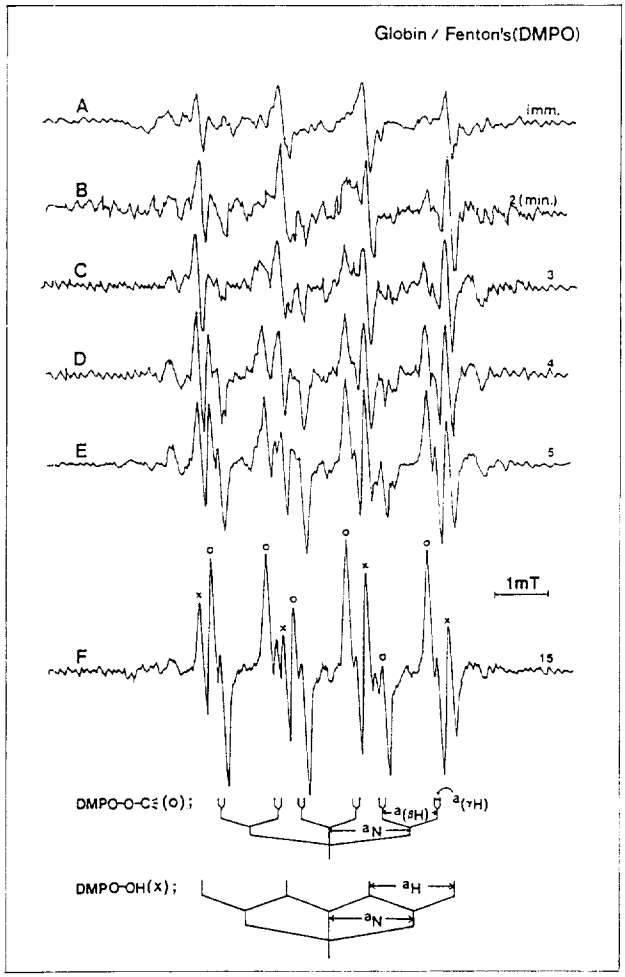

Fig. 4. ESR spectra observed after mixing of globin with Fenton's reagent in the presence of DMPO. The mixture contains 240 μM globin, 60 μM Fe(II), 6 mM H_2O_2 and 0.1 M DMPO. Each spectrum was recorded immediately (A), 2 min (B), 3 min (C), 4 min (D), 5 min (E) and 15 min (F) after mixing. Mark O and X indicate the signals ascribing to DMPO/Tyr adduct and DMPO/OH adduct, respectively.

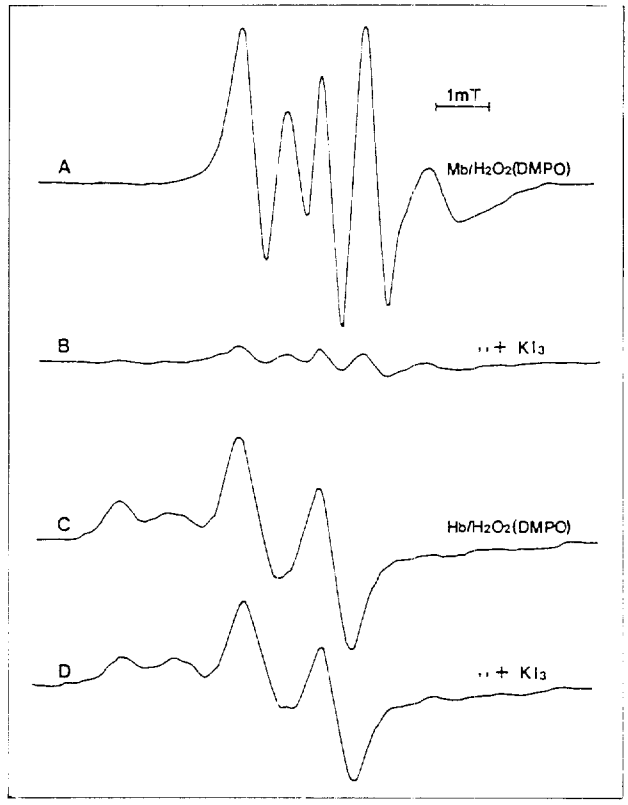

Fig. 5. Effects of pretreatment of MetMb and Hb with iodinating agent, KI_3, on the MetMb and Hb/H_2O_2 mixing system in the presence of DMPO. (A) ESR spectrum observed immediately after mixing of MetMb (1 mM) with H_2O_2 (8 mM) in the presence of DMPO (0.1 M). (B) ESR spectrum observed immediately after mixing system A, except the same concentration of iodinated MetMb in the place of MetMb. ESR settings unchanged. (C) ESR spectrum observed immediately after mixing of Hb (300 μM) with H_2O_2 (7 mM) in the presence of DMPO (0.2 M). (D) ESR spectrum observed immediately after mixing system A, exept the same concentration of iodine-treated Hb in the place of Hb. ESR settings unchanged.

stants of 12-line signal were a_N 1.47 mT, $a_{\beta H}$ 0.99 mT and $a_{\gamma H}$ 0.13 mT, virtually identical to that shown in the Fig. 2 signal, even though the relative intensities of each radical species are somewhat different and $a_{\gamma H}$ splitting was smeared out in the case of Fig. 2. The signal, shown as two lines at low and high magnetic field, with lower intensity, was assigned to carbon-centered radical adducts (Yamazaki and Piette, 1990). The inner four-lines of the typical 6-line signal (see Fig. 2C) for carbon-centered radical adducts must have been burried in the composite spectrum.

This result together with the data shown in Fig. 2 strongly indicates that the radical species formed in both of the ·OH-generating systems could be phenoxyl and carbon-centered radical, coexisting with the hydroxyl radical, indicating that the free iron released from the heme component by excess H_2O_2, in Fig. 2 mixing system, provided for a Fenton reagent to produce the same results as the globin/Fenton system. It is thought that when electron vacancy, arising from α-H abstraction in globin by OH radical, migrates along the protein backbone to a tyrosine residue, one of the radical "sinks" (Adams *et al.*, 1972), phenoxyl radicals are generated through electron-relay mechanism as a secondary species by H-abstraction from hydroxyl group of the tyrosine residues (Henriksen and Pihl, 1961; Makino *et al.*, 1990). Thus, both of the 6-line signal in Fig. 2 and 12-line signal in Fig. 4F were assigned to phenoxyl radical DMPO adducts.

This interpretation is supported further by the relative time course of the observed DMPO/OH signal and the 12-line signal (data not shown). The intensity of the DMPO/OH signal increased rapidly and saturated at around 10 min and lasted for over 90 min, whereas that of the 12-line signal appeared later and then in-

creased abruptly, reaching its maximum at around 20 min and slowly decreased. These results may be interpreted in such a way that the DMPO/OH adducts are maintained in relatively stable saturation concentration whereas the radical center for the 12-line signal appears later than the DMPO/OH adducts since it may be trapped by DMPO after H-abstraction and migration to tyrosine residue through electron-relay mechanism, and then decayed.

Based on these results, the radical species for the 12-line signal was assigned to the DMPO/phenoxyl radical adduct formed at a tyrosine residue in globin. Alkoxy radical can be generated on serine, but serine residue is not good radical sink (Adams, 1972), indicating that the possibility of a DMPO/alkoxyl radical adduct formation is excluded.

Definitive evidence as to the residue which gives rise to MetMb-derived radical was obtained by employing iodinated MetMb. It is now well known that the reaction of KI_3 with MetMb occurs only at tyrosine residue (Kretsinger, 1968). Fig. 5A shows the ESR spectrum obtained immediately after mixing MetMb (1 mM) with H_2O_2 (8 mM) in the presence of DMPO (0.1 M). When MetMb was displaced by iodinated MetMb, the 5-line signal shown in Fig. 5A almost disappeared (Fig. 5B), indicating that blocking tyrosine residue resulted in loss of the MetMb-derived radical in the system. This result strongly supports the previous data that the multiplet ESR signal was not observed when iodinated equine MetMb was employed in place of untreated MetMb in the stopped-flow ESR of the MetMb/H_2O_2 mixing system (Davies, 1991). The residual intensity of the signal in Fig. 5B may be due to the uniodinated MetMb in excess (Kretsinger, 1968).

In the case of Hb/H_2O_2 system, however, no such effect of the iodination treatment was detected as shown in Fig. 5C and D, supporting the previous indication that the radical center formed in Hb is cysteine residue, not tyrosine (Maples *et al.*, 1988).

Thus, though the direct detection of free-spin ESR signal was not successful, the data presented here strongly supports the previous indication that the apomyoglobin-derived radical center formed in the reaction of MetMb with H_2O_2 is a tyrosine residue (Miki *et al.*, 1989; Davies, 1988, 1990, 1991).

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