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DECAY STUDIES OF DMPO-SPIN ADDUCTS OF FREE RADICALS PRODUCED BY REACTIONS OF METMYOGLOBIN AND METHEMOGLOBIN WITH HYDROGEN PEROXIDE

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The 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) spin adduct of myoglobin (Mb) or hemoglobin (Hb) was formed when metmyoglobin (MetMb) or methemoglobin (MetHb) reacted with H_2O_2 in the presence of DMPO, and both decayed with half-life of a few minutes. The DMPO spin adduct of Mb decayed with biphasic kinetics with $k_1 = 0.645\ min^{-1}$ and $k_2 = 0.012\ min^{-1}$, indicating that the spin adduct consisted of two kinetically heterogeneous species, stable and unstable ones. The DPMO spin adduct of Hb, however, was homogeneous. Decay of both spin adducts was accelerated in the presence of tyrosine, tryptophan or cysteine, but not phenylalanine, methionine or histidine. The decay obeyed the first order kinetics at varying concentrations of the spin adducts. The decay was accelerated by denaturation and proteolysis of protein moiety. The decay rate was not affected by the extra addition of MetMb or MetHb to each spin adduct. The decay rate of the spin adduct of Mb was increased by hematin in the presence of H_2O_2 and decreased by catalase. Decay of stable spin adduct of Mb, however, was not significantly changed under any experimental conditions used. These results led us to conclude that instability of the DMPO-spin adducts of Mb and Hb is due to intramolecular redox reactions between the spin adducts and amino acid residues and/or products of the reaction between heme and H_2O_2.

KEY WORDS: ESR, Spin adduct, Protein radical, MetMb, MetHb, Spin trapping.

Abbreviations: DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; ESR, electron spin resonance; Hb, hemoglobin; Mb, myoglobin; MetMb, metmyoglobin; MetHb, methemoglobin; PBS, phosphate-buffered saline (pH 7.4); BSA, bovine serum albumin; CAT, catalase; Hm, hematin.

INTRODUCTION

Oxidative damage of biological molecules is often mediated by myoglobin (Mb) or hemoglobin (Hb),[1-5] and the oxidative damage inflicted by reactive oxygen species is associated with the induction of carcinogenesis, aging, inflammation, and

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radiation damage.[6] The reaction of metmyoglobin (MetMb) with H_2O_2 generates a ferryl species, Fe(IV) = O with one oxidizing equivalent.[7,8] The second oxidizing equivalent from the H_2O_2 molecule is rapidly transferred from the heme center to surrounding amino acid residues, resulting in the formation of an apoprotein radical.[9–12] These products are regarded as oxidizing agents capable of peroxidation of fatty acids and oxidation of β-carotene, ascorbic acid, methional, uric acid and phenol as well as epoxidation of styrene.[13]

Protein free radicals of Mb and Hb have been detected by ESR at liquid nitrogen temperature[9,10] and also at room temperature with a flow apparatus.[12] Recently, ESR-spin trapping techniques have been used to characterize the protein radicals.[14–16] Although these protein radicals are stabilized when reacting with spin-trapping reagents, the spin adducts also decay with half-life of a few minutes[14,16] and the nature of their decay has not been characterized. We have examined the decay mechanism and the reactivity of these spin adducts. In this paper we demonstrate that the decay of these spin adducts with a short half-life is due to intramolecular interactions between the spin adducts and amino acid residues of hemoproteins.

MATERIALS AND METHODS

Horse heart myoglobin, bovine hemoglobin, bovine serum albumin (BSA), bovine globin, hemin, bovine liver catalase, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), amino acids, and phosphate-buffered saline (PBS: pH 7.4) were purchased from Sigma. DMPO was used after redistilling. Hematin solutions were obtained by dissolving hemin in a weak alkaline solution. MetMb and MetHb were prepared by treating with potassium ferricyanide and desalting with Sephadex G-25 column chromatography. The concentration of hemoproteins was determined on the basis of heme content measured by pyridine-hemochromogen method.[17]

The ESR measurement was started 1 min after H_2O_2 was added to a mixture of DMPO (final concentration = 0.1 M) and MetMb (or MetHb) in 30 mM PBS. Concentrations of Mb and Hb used in this study denote the heme concentration. To remove excessive H_2O_2, catalase (final concentration = 300 unit/ml) was added 12 sec after the addition of H_2O_2. The reaction mixture was then transferred to a flat cell and magnetic field was scanned. All spectra were recorded at room temperature. Unless otherwise noted, modulation frequency, 100 kHz; modulation amplidude, 1G; Scan speed, 100 gauss/min; and receiver gain, 3.2×10^3 for Mb and 3.2×10^4 for Hb.

RESULTS

As already described,[14–16] upon addition of equimolar amount of H_2O_2 at room temperature in the presence of DMPO, MetMb produced a distinctive and asymmetric ESR spectrum, consisting of 5 lines of different intensities (Figure 1A). The observed signal was highly anisotropic with considerable broadening of the high field lines. This is characteristic of an immobilized spectrum with hyperfine splitting constants of $2A_{max} = 40.7$ G and $2A_{min} = 29.7$ G, indicating that the radical adduct formed in the reaction was confined to restricted surroundings. When MetMb was replaced by MetHb, the ESR spectrum observed was different from that of MetMb

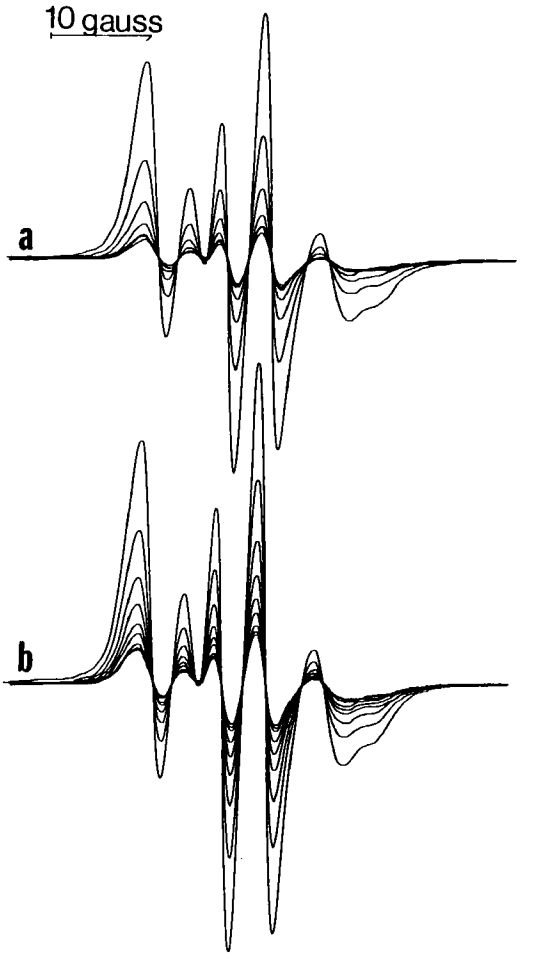

FIGURE 1 ESR spectra of DMPO spin adduct of Mb. **a**. Spectra were scanned every 1 min after H_2O_2 (0.8 mM) was added to a mixture of MetMb (0.4 nM) and DMPO. **b**. Same as **a** except that catalase (300 unit/ml) was added 12 sec after the addition of H_2O_2. Gain was 3.2×10^3.

and consisted of 6 lines of very different intensities, resulting in formation of a slowly-tumbling nitroxide radical adduct (Figure 2A).

Decay of the Mb-spin adduct obeyed biphasic process (Figure 3A), with first order rate constants of $k_1 = 0.648\,min^{-1}$ and $k_2 = 0.012\,min^{-1}$ (Table I), indicating that the ESR spectrum of Mb-spin adduct consisted of at least two kinetically different species, stable and unstable ones. The addition of catalase 12 sec after the reaction of MetMb and H_2O_2 decreased the decay rate of the unstable spin adduct to $k = 0.450\,min^{-1}$ (Table I), but did not significantly change that of the stable adduct (Figure 1B and 3A). The spin adduct of Hb decayed according to first order kinetics with $k = 0.425\,min^{-1}$ (Figure 3B and Table I). The effect of catalase on the MetHb system differed from that on the MetMb system. The addition of catalase decreased the formation of the Hb spin adduct by about 25% (Figure 3B) and did not significantly change the decay rate (Table I). This could be explained

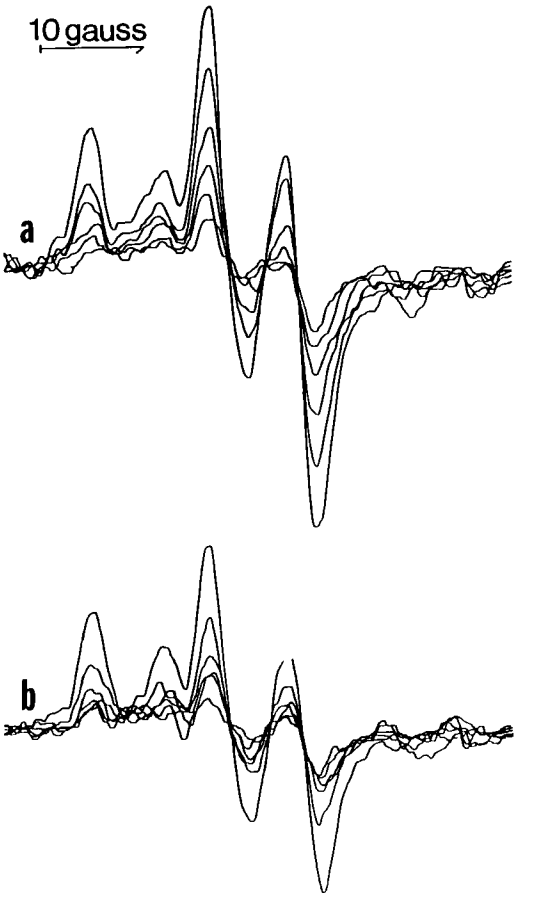

FIGURE 2 ESR spectra of DMPO spin adduct of Hb. The experimental procedure was as described in Figure 1 except that MetMb was replaced by MetHb. Gain was 3.2×10^4.

by the fact that the reaction of H_2O_2 with MetHb was slower than with MetMb. The further increase in the H_2O_2 concentration, however, did not increase the steady state level of the spin adduct because the decay was also accelerated by H_2O_2. Figure 4 shows difference in the optimum concentration of H_2O_2 required to produce the spin adduct of Mb and Hb. The signal intensity of spin adduct reached a maximum at a concentration of H_2O_2 equimolar to that of MetMb, but a double ratio was needed to obtain a maximum in the case of MetHb.

In order to check whether or not the spin adducts decay via intermolecular reactions, we examined the effect of hemoprotein concentrations on the decay kinetics. Figure 5 shows that the amount of the spin adducts accumulated was nearly proportional to the concentration of MetMb (Figure 5A) and MetHb (Figure 5B) and also that the first order rate constants were not changed by the MetMb or MetHb

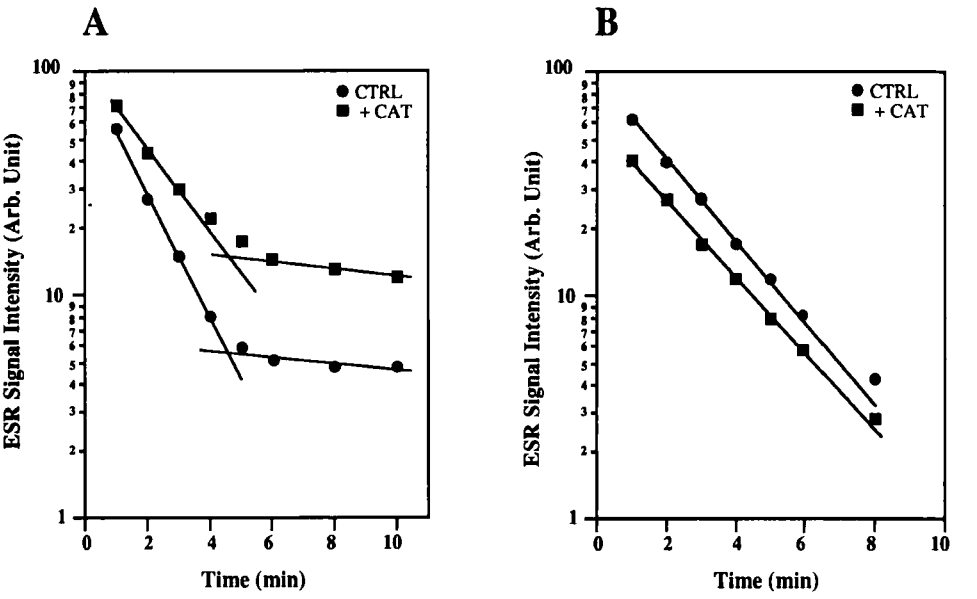

FIGURE 3 Effect of catalase on the decay of DMPO spin adducts of Mb (A) and Hb (B). The experimental procedure was as described in Figure 1 and 2. The ESR signal intensity was measured in the reaction mixture of 0.4 mM MetMb or MetHb, 0.8 mM H_2O_2, and 100 mM DMPO in the presence (■) or absence (●) of catalase (300 unit/ml).

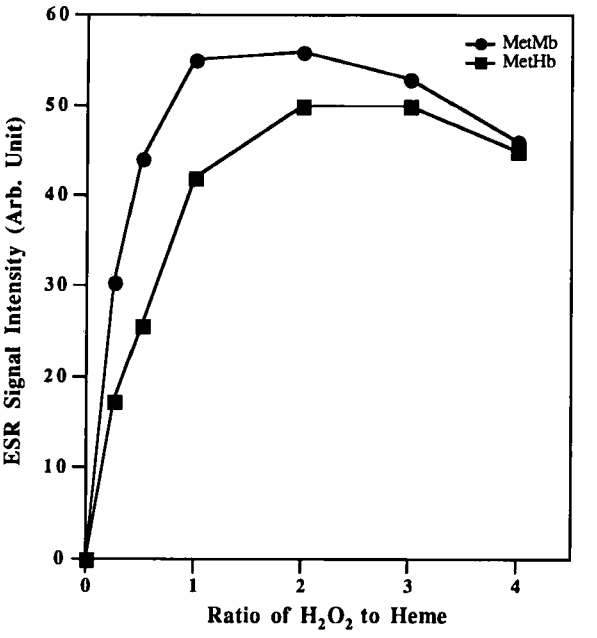

FIGURE 4 Effect of the H_2O_2 concentration on the formation of DMPO spin adducts of Mb and Hb. The ESR signal intensity was measured 1 min after the addition of H_2O_2. The concentration of MetMb (●) and MetHb (■) was 0.4 mM.

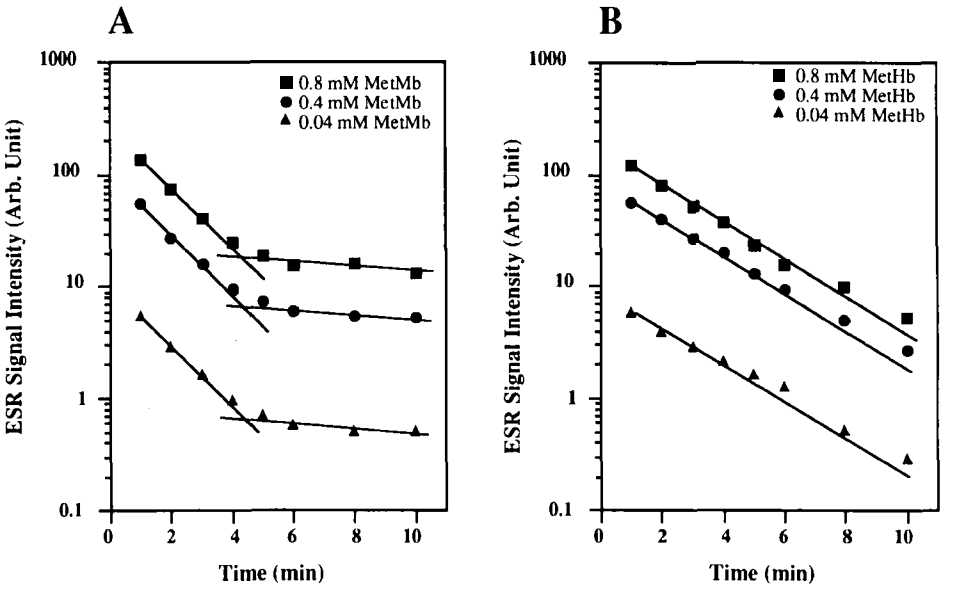

FIGURE 5 Effect of the hemoprotein concentration on the decay of DMPO spin adducts of Mb (A) and Hb (B). The experimental condition was the same as Figure 3 except for the concentrations of hemoprotein and H_2O_2. ■, 0.8 mM MetMb or MetHb plus 1.6 mM H_2O_2; ●, 0.4 mM MetMb or MetHb plus 0.8 mM H_2O_2; ▲, 0.04 mM MetMb or MetHb plus 0.08 mM H_2O_2.

TABLE I

Effect of catalase, amino acids, proteins, and hematin on rate constants for decay of DMPO spin adducts of Mb and Hb. Control was a mixture of 0.4 mM MetMb or MetHb, 0.8 mM H_2O_2, and 100 mM DMPO. Catalase (CAT: 300 units/ml) was added 12 sec after the addition of H_2O_2. Amino acids (0.15 mM), proteins (0.4 mM), and hematin (Hm: 0.4 mM) were added 24 sec after the addition of H_2O_2

	MetMb/H_2O_2/DMPO		MetHb/H_2O_2/DMPO
Treatment	k_1	k_2	k
CTRL	0.648	0.012	0.425
+CAT	0.450	0.009	0.382
+CAT/Trp	1.421	0.015	0.719
+CAT/Tyr	0.851	0.013	0.839
+CAT/Cys	0.690	0.012	1.073
+CAT/BSA	0.472	0.012	0.402
+CAT/Globin	0.488	0.016	0.381
+CAT/MetMb	0.471	0.014	
+CAT/MetHb			0.395
+Hm	0.975	0.008	
+CAT/Hm	0.463	0.009	
MetMb[1]	0.632	0.010	
MetHb[1]			0.434

[1]Reaction mixture contained 0.8 mM MetMb or MetHb, 1.6 mM H_2O_2, and 100 mM DMPO and ESR spectra was measured 1 min after the addition of H_2O_2.

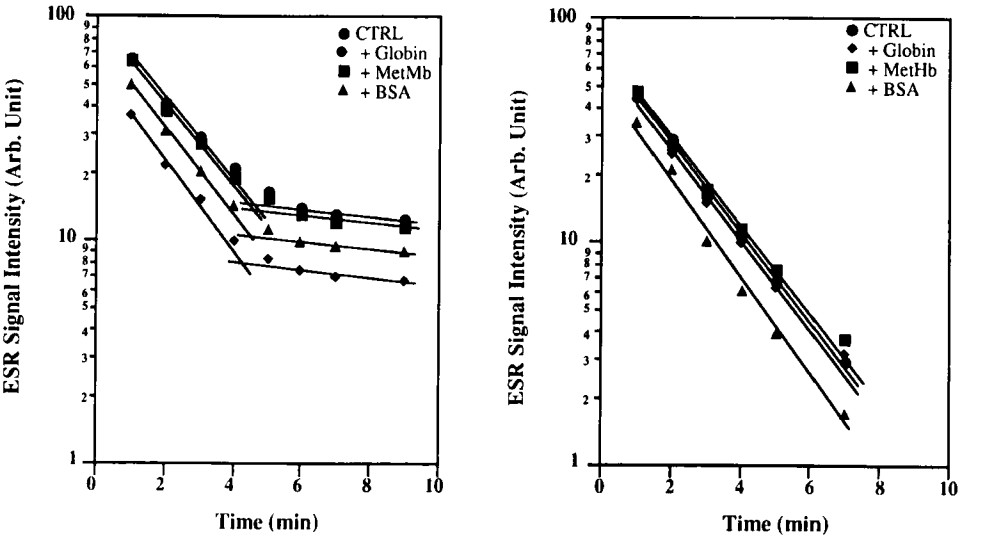

FIGURE 6 Effect of proteins on the decay of DMPO spin adducts of Mb (A) and Hb (B). Catalase and proteins were added 12 and 24 sec after the addition of H_2O_2, respectively. The ESR signal intensity was measured every 1 min after the addition of H_2O_2. The ESR signal intensity was recoreded in the mixture (●) of 0.4 mM MetMb or MetHb, 0.8 mM H_2O_2, DMPO, and catalase in the presence of 0.4 mM globin (◆), metMb or MetHb (■), or BSA (▲).

concentration (Table I), indicating that the decay rate-determining factor is intramolecular reactions. The effect of addition of some proteins is shown in Figure 6. In this case, proteins were mixed with the spin adducts after removal of H_2O_2 by catalase. Similarly as seen in Figure 5, the additions of globin, BSA, and hemoproteins to the spin adducts of Mb and Hb did not change the decay rates (Table I). However, in both cases the addition of BSA slightly decreased initial level of the spin adducts. Globin decreased initial level of the spin adduct of only Mb, but not Hb.

Contrary to the weak effect of proteins, reducing amino acids such as cysteine, tyrosine, and tryptophan markedly accelerated decay of the Mb and Hb spin adducts (Figure 7). There was an interesting difference in the effect of these amino acids on the decay of the spin adducts of Mb and Hb. The most effective amino acid was tryptophan for the decay of the spin adduct of Mb (Figure 7A) and cysteine for that of the spin adduct of Hb (Figure 7B). The rate constants are summarized in Table I. The level of stable component of the Mb-spin adduct was decreased proportionally with the decay rate of the unstable spin adduct. Effect of the amino acid concentration on the decay rate was different in these spin adducts (Figure 8). The Mb-spin adduct was markedly decayed in the presence of 150 μM of the amino acids and the maximum decay was observed at about 500 μM (Figure 8A). The decay of Hb-spin adduct was less sensitive to the same concentration of the amino acids compared with that of Mb-spin adduct (Figure 8B). No significant effect was observed with methionine, phenylalanine, and histidine in both cases.

It is clear from Figure 3A that the removal of excessive H_2O_2 slightly stabilized the unstable spin adduct of Mb. Decay of the spin adduct of Mb by H_2O_2 was accelerated by the presence of hematin. Figure 9 shows that hematin-mediated decay

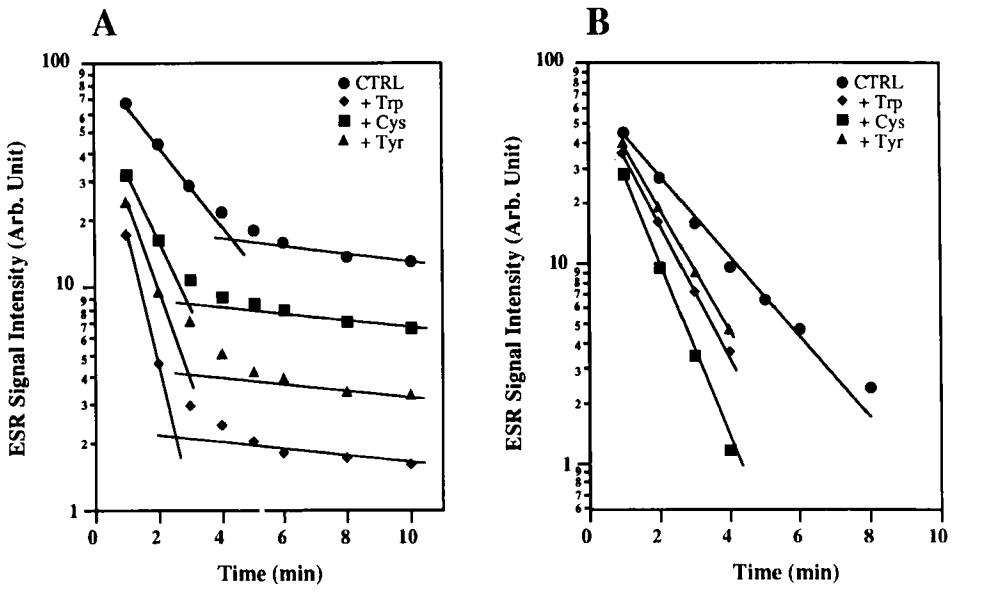

FIGURE 7 Effect of amino acids on the decay of DMPO spin adducts of Mb (A) and Hb (B). The experimental procedure was as described in Figure 6 except that proteins were replaced by amino acids. The ESR signal intensity was recorded in the mixture (●) of 0.4 mM MetMb or MetHb, 0.8 mM H_2O_2, DMPO, and catalase in the presence of 0.15 mM tryptophan (♦), cysteine (■), or tyrosine (▲).

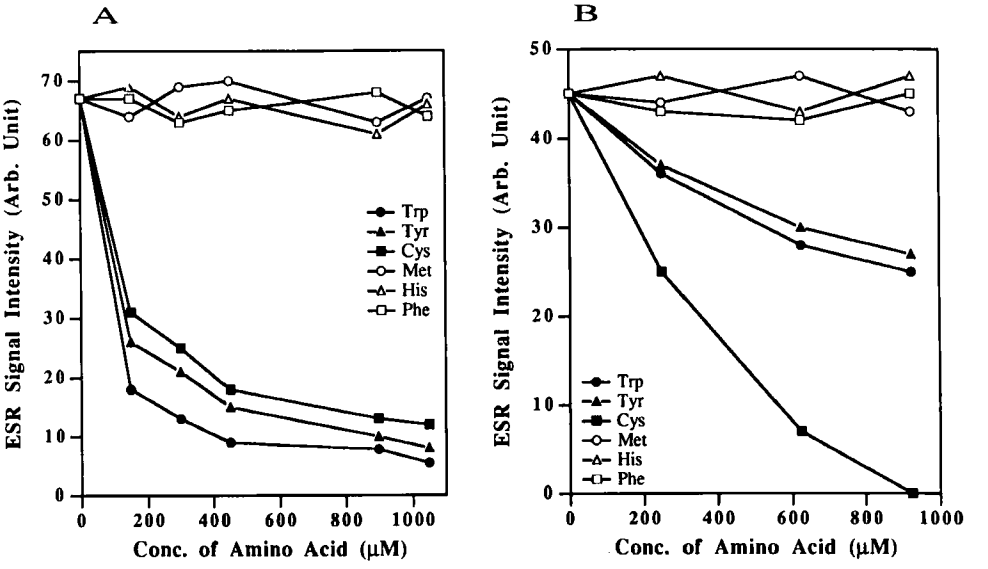

FIGURE 8 Effect of the amino acid concentration on the decay of DMPO spin adducts of Mb (A) and Hb (B). The experimental procedure was as described in Figure 7. The ESR signal intensity was recoreded in the mixture of 0.4 mM MetMb or MetHb, 0.8 mM H_2O_2, DMPO, and catalase in the presence of tryptophan (●), cysteine (■), tyrosine (▲), methionine (○), histidine (△), or phenylalanine (□).

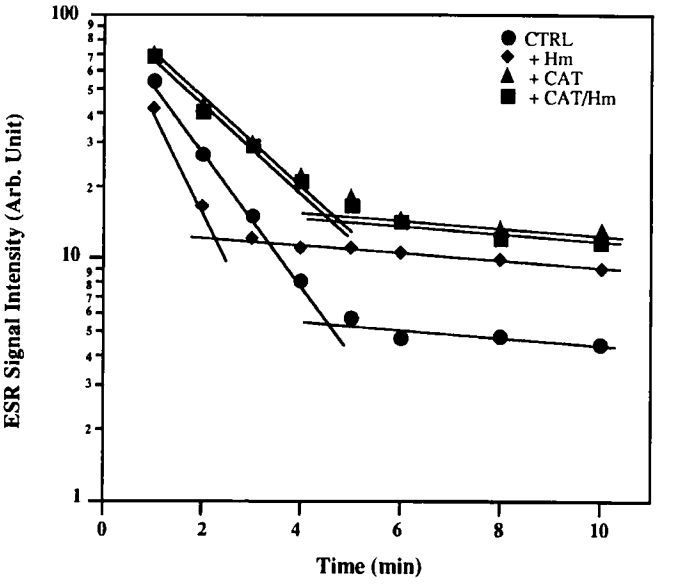

FIGURE 9 Effect of hematin on the decay of DMPO spin adduct of Mb. The ESR signal intensity was measured in the reaction mixture (●) of 0.4 mM MetMb, 0.8 mM H_2O_2, and DMPO in the presence of 0.4 mM hematin (♦), 300 unit/ml catalase (▲), or combination of both (■). Catalase and hematin were added 12 and 24 sec after the addition of H_2O_2.

of the spin adduct of Mb took place only in the presence of H_2O_2. The remaining level of the stable spin adduct depended on the experimental conditions. Although hematin accelerated the decay of the spin adduct, the remaining level of the stable spin adduct was higher than that of the control. When the spin adducts were treated with denaturing reagents (urea and guanidine-HCl) or protease, the ESR signal intensity rapidly decayed (data not shown).

DISCUSSION

The ESR spectrum of DMPO-spin adducts of Mb radical formed in the reaction of MetMb with H_2O_2 have already been reported by Hong and Piette[14] and Davies.[15,16] Although this spin adduct is more stable than protein radicals, they are still unstable compared with other spin adducts such as DMPO-OH and DMPO-OOH. The instability of the Mb-spin adduct has not been thoroughly investigated. From our present study the decay of the Mb-spin adduct is kinetically further characterized as follows, (1) the decay is slightly retarded by removing excessive H_2O_2 by catalase, (2) the rate-determining factor of the decay is an intramolecular reaction of the spin adduct, probably with amino acid residues or ferryl, an oxidized hematin, (3) the decay is accelerated by denaturation and proteolysis of the protein moiety, (4) the decay was accelerated by exogenous electron donors such as tyrosine, trypthophan, and cysteine, and (5) there are at least two kinetically different species, stable and unstable ones, although these are not spectroscopically distinguishable.

The presence of the relatively stable spin adduct becomes clearer when catalase and hematin are added. Hematin accelerates the decay of the spin adduct of Mb only in the presence of H_2O_2, but increases the level of stable spin adduct. This curious effect of hematin may be explained in terms of hematin-accelerated decay of the unstable spin adduct and concomitant removal of H_2O_2. We assume that the H_2O_2-mediated decay of the unstable spin adduct occurs via an intramolecular oxidation catalyzed by the endogenous heme.

The ESR spectrum of the DMPO spin adduct of Hb is similar to that of DMPO spin adduct formed *in vivo* by the administration of phenylhydrazine, which has been concluded by Maples *et al.*[18] to be the spin adduct of Hb thiyl free radical. This spin adduct also decays through a similar mechanism as does the spin adduct of Mb although there are some differences in the specificity for electron donors and effect of catalase. The Mb spin adduct is most sensitive to tryptophan while the Hb spin adduct is to cysteine. These spin adducts both decay with half-life of a few min. As compared with MetMb, the accumulation of the spin adducts is lower in the reaction of MetHb, mostly because of the slow reaction of MetHb with H_2O_2. This fact explains the results that the ESR signal of the Hb-spin adduct was decreased by catalase and that a double amount of H_2O_2, compared with Mb, was needed to get maximum signal intensity. The observation that the decay rate of the Hb-spin adduct was not affected by catalase suggests that the decay of the Hb-spin adduct is not mediated by the endogenous heme.

In the reaction of MetMb (or MetHb) with H_2O_2 two different oxidizing species are formed.

$$\text{MbFe(III)} + H_2O_2 \rightarrow \text{Mb}\cdot\text{Fe(IV)} = O + H_2O$$

Fe(IV) = O species of Mb and Hb are relatively slow oxidants. On the other hand, the free radicals of amino acid residues (Mb· or Hb·) are very reactive and decay very fast. Hb has long been known to catalyze the formation of a potent oxidant, which is assumed to be the hydroxyl radical.[19,20] Since the spin-trapping detection of the hydroxyl radical has not been successful, there is a possibility that this potent oxidant is not the hydroxyl radical, but a free radical of an amino acid residue of Hb. Since half-life of the Mb free radical increases from about 10 sec[12] to a few min when the free radical is trapped by DMPO, it would be reasonable to assume that the free radical is more reactive than its spin adduct. Since the DMPO-spin adduct is also a free radical, it can react with electron-donating amino acid residues or ferryl to yield non-paramagnetic products.

In conclusion, we demonstrated that the instability of DMPO-spin adducts of Mb and Hb is due to intramolecular redox reaction between the nitroxide radical of the DMPO-spin adducts and the ferryl or an amino acid residue (probably tyrosine, tryptophan, or cysteine), which is located near the spin adducts in the protein molecules. It is also suggested that electron-donating amino acids may be responsible for short half-life of protein radicals produced in the reaction of MetMb with peroxide.[12]

Acknowledgements

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