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Comparative Studies of Protein Modification Mediated by Fenton-like Reactions of Iron, Hematin, and Hemoglobin: Generation of Different Reactive Oxidizing Species

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The reactive oxygen species oxidatively modify the biological macromolecules, including proteins, lipids, and nucleic acids. Iron- and heme-mediated Fenton-like reactions produce different pro-oxidants. However, these reactive products have not been clearly characterized. We examined the nature of the oxidizing species from the different iron sources by measuring oxidative protein modification and spectroscopic study. Hemoglobin (Hb) and methemoglobin (metHb) were oxidatively modified in O_2^- and H_2O_2 generating systems. Globin and bovine serum albumin (BSA) were also modified by iron, iron-EDTA, hematin, and Hb in an O_2^- generating system. In a H_2O_2 generating system, the iron- and iron–EDTA-mediated protein modifications were markedly reduced while the Hb- and hematin-mediated modifications were slightly increased. In the O_2^- generating system, the iron- and iron-EDTA-mediated protein modifications were strongly inhibited by superoxide dismutase (SOD) or catalase, but heme- and Hb-mediated protein modifications were inhibited only by catalase and slightly increased by SOD. Mannitol, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), deoxyribose, and thiourea inhibited the iron-EDTA-mediated protein modification. Mannitol and DMPO, however, did not exhibit significant inhibition in the hematin-mediated modification. Desferrioxamine (DFO) inhibited protein modification mediated by iron, but cyanide and azide did not, while the hematin-mediated protein modification was inhibited by cyanide and azide, but not significantly by DFO. The protein-modified products by iron and heme were different. ESR and UV-visible spectroscopy detected the DMPO spin adduct of the hydroxyl radical and ferryl ion generated from iron-EDTA and metHb, respectively. These results led us to conclude that the main oxidizing species are hydroxyl radical in the iron-EDTA type and the ferryl ion in the hematin type, the latter being more effective for protein modification.

Keywords: Fenton reaction, Hemoglobin, Hydroxyl radical, Iron, Reactive oxygen species.

Introduction

Biological generation of activated oxygen species such as O_2^-, H_2O_2 and hydroxyl radical (·OH) has been implicated as a causative factor in many biological disorders (Halliwell and Gutteridge, 1986). Although O_2^- and H_2O_2 are generated by activated phagocytes (Makino *et al.*, 1986; Forehand *et al.*,1989; Jackson *et al.*,1989), mitochondria (Chance *et al.*, 1979; Schulze-Osthoff *et al.*, 1992) and enzymes (Nakamura and Yamazaki, 1969) in biological systems, they are not very toxic on their own. Hydroxyl radical, however, generated by the iron-catalyzed Haber-Weiss reaction (reactions 1 and 2) are believed to be a major species in oxygen toxicity in biological systems (Halliwell and Gutteridge, 1986), because they indiscriminately react with numerous biomolecules such as membrane lipid, proteins, and DNA.

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2 \quad [1]$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH \quad [2]$$

The Fenton reaction (reaction 2) produces not only ·OH but can also produce the ferryl ion ($Fe^{4+} = O$) and the ratio of these two species formed is dependent on the nature of any iron chelator present (Yamazaki and Piette,

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1990; 1991). Although the ferryl ion is different from ·OH in terms of reactivity with detector molecules, they both can cause deleterious biological effects through lipid peroxidation (Kim *et al.*, 1994).

Free hemoglobin (Hb) can cause serious harmful effects, such as increased mortality in bacteria peritonitis (Pruett *et al.*, 1984), inactivation of neutrophils (Kim *et al.*, 1994), and damage to the central nervous system (Sadrzadeh and Eaton, 1988) through lipid peroxidation. These deleterious effects have been reported to be caused by strong oxidants produced in the reaction of Hb with H_2O_2, suggesting that Hb acts as a type of Fenton reagent (Sadrzadeh *et al.*, 1984; Puppo and Halliwell, 1988). Although there is no evidence that the Hb molecule directly produces ·OH (Gutteridge, 1986; Puppo and Halliwell, 1988), it is known that Hb is oxidized to the highly reactive form upon reaction with H_2O_2 (Kanner and Harel, 1985). At high concentrations of H_2O_2, iron can be released from Hb and Mb and act as Fenton reagents, producing ·OH (Gutteridge, 1986; Prasad *et al.*, 1989). Studying the mechanism of Hb-mediated inactivation of stimulated neutrophils, we have concluded that the major oxidizing species produced from the reaction of Hb or hematin with H_2O_2 is not ·OH (Kim *et al.*, 1994).

Hydroxyl radicals generated by irradiation or the iron-catalyzed Haber-Weiss reaction cause oxidative protein modifications in various ways, such as aggregation, fragmentation, and conformational change (Levine, 1983; Davies, 1987; Meucci, 1991). It has also been reported that hematin mediates oxidative degradation of proteins (Aft and Mueller, 1984), amino acid oxidation (Stadtman and Berlett, 1991), and inactivation of phagocytes. We thought it important therefore to compare the mechanisms of protein modification in these systems and have thus examined the roles of iron, iron-EDTA, hematin, and Hb in the presence of O_2^- and H_2O_2. In this paper, we classify these reactions into the iron-EDTA- and hematin-type on the basis of their mechanisms of oxidative protein degradation.

Materials and Methods

Materials Bovine serum albumin (BSA), bovine globin, heme, xanthine oxidase (XO), glucose oxidase (GO), catalase, superoxide dismutase (SOD), 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), and phosphate-buffered saline (PBS) (pH 7.4) were purchased from Sigma. Desferrioxamine (DFO) was obtained from CIBA-Geigy. Acetaldehyde obtained from Aldrich and DMPO were used after redistilling. All reagents for electrophoresis were obtained from Bio-Rad. Hb, MetHb, and hematin solutions were prepared according to Kim *et al.* (1994). BSA and globin were used after purification by Sephadex column chromatography.

Protein modification (breakdown and aggregation) Typical protein modification in the XO system was carried out in a reaction mixture containing 0.13 mg of protein, 14 μM of an iron species, 7.5 milliunits of XO, 10 mM acetaldehyde, and PBS in a final volume of 300 μl. The mixture was incubated at room temperature and shaken for 10 s every 15 min. The incubation time was 2 h for the experiments listed in Tables 1–5. The oxidation state of the iron and iron-EDTA used in this study was ferric ion, but no essential difference was observed when ferric ion was replaced by ferrous ion. Protein modification was measured from the decrease in the intensity of the protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Gel Electrophoresis SDS-PAGE was carried out according to Laemmli (1970) using 10% and 15% acrylamide gels. At indicated times, the incubated solution (5 μl) was mixed with an equal volume of treatment buffer containing 4% SDS and 10% mercaptoethanol and then heated at 100°C for 150 s. After chilling on ice, the mixture was deposited on the gel and run at constant voltage of 100 V. The gels were stained with Comassie

Table 1. Effect of antioxidant enzymes on iron-mediated protein modification in the XO system.

Incubation mixtures	Protein modification in the XO system (%)			
	No addition	SOD	CAT	SOD/CAT
Gb/Fe^{3+}	20.0 ± 4.9	9.2 ± 2.4	2.9 ± 0.4	0.7 ± 0.2
Gb/Fe^{3+}EDTA	25.2 ± 5.7	7.3 ± 3.1	2.6 ± 0.3	0.8 ± 0.2
Gb/Hm	53.8 ± 8.1	58.2 ± 4.1	3.2 ± 0.4	0.8 ± 0.4
Gb/Hb	44.6 ± 5.9	49.1 ± 3.8	3.1 ± 0.9	0.9 ± 0.3
BSA/Fe^{3+}	19.3 ± 3.4	11.4 ± 2.9	2.2 ± 0.7	0.9 ± 0.6
BSA/Fe^{3+}EDTA	28.7 ± 4.5	9.3 ± 1.6	2.5 ± 0.4	1.3 ± 0.7
BSA/Hm	44.8 ± 4.9	50.5 ± 3.9	2.9 ± 0.6	0.8 ± 0.4
BSA/Hb	35.5 ± 3.8	42.6 ± 4.5	3.4 ± 0.9	0.8 ± 0.3

The concentration of SOD and catalase was 30 units in an incubation solution of 300 μl.
Abbreviations: CAT, catalase; Gb, globin; Hm, hematin.

Brilliant Blue R-250. The intensities of the protein bands were measured with a Bio-Rad densitometer. Protein concentration was calculated from the integration of the peak area in comparison with standards.

Binding of iron complexes to proteins A protein solution (0.5 mg/ml) was placed in one compartment (1.2 ml) of an equilibrium dialysis chamber separated by a membrane disc (MW cutoff: 6000–8000) and an iron compound (360 μM) was placed in the other. After equilibration for 12 h, the iron concentration of both compartments was measured according to Kim *et al.* (1994). The amount of iron bound to proteins was calculated from the difference in the iron contents in the two compartments.

Table 2. Effect of hydrogen peroxide sources on protein modification.

Incubation mixtures	Protein modification (%)		
	XO system	GO system	H_2O_2
Gb	10.1 ± 2.5	4.9 ± 1.9	5.9 ± 1.8
Gb/Fe^{3+}	21.9 ± 8.4	9.1 ± 1.6	10.4 ± 1.6
Gb/Fe^{3+}EDTA	26.4 ± 6.4	5.3 ± 2.8	6.6 ± 2.4
Gb/Hm	53.9 ± 5.2	57.8 ± 5.8	59.2 ± 7.6
Gb/Hb	42.1 ± 9.2	46.2 ± 3.9	48.8 ± 8.4
Hb	48.5 ± 8.7	55.2 ± 6.1	57.2 ± 5.1
BSA	7.2 ± 3.1	2.4 ± 1.9	4.3 ± 1.0
BSA/Fe^{3+}	20.8 ± 3.8	9.5 ± 3.6	12.4 ± 3.8
BSA/Fe^{3+}EDTA	32.6 ± 2.1	5.8 ± 3.1	7.2 ± 0.7
BSA/Hm	41.8 ± 4.9	46.2 ± 1.5	48.9 ± 2.1
BSA/Hb	34.1 ± 2.2	38.2 ± 1.9	39.5 ± 4.7

The GO system consisted of 7.3 milliunits of GO and 0.1 M glucose in 300 μl. H_2O_2 concentration was 250 μM. Abbreviations: see Table 1. The concentration of H_2O_2 in the XO and GO systems was ~250 μM after 1h incubation.

Electron spin resonance (ESR) and UV–Visible spectroscopy The reaction mixture consisted of 14 μM iron source, 100 μM H_2O_2, and 100 μM DMPO (only for ESR). After incubation for 4 min, the mixture was transferred to a standard quartz flat ESR cell and then the magnetic field was scanned. All spectra were recorded at room temperature. Unless otherwise noted, the following were applied: modulation frequency, 100 kHz; modulation amplitude, 1 G; scan speed, 100 gauss/min; and receiver gain, 3.2×10^3 for iron and 8×10^4 for heme compounds. UV–visible spectra were recorded with a Shimazu UV260 spectrophotometer at room temperature using a 1-cm pathlength cell.

Results

Incubation of Hb or MetHb in the XO system resulted in rapid protein modification, as indicated by a decrease in the intensity of the Hb band on SDS-PAGE (Figs. 1A and B). Hb was slightly less modified than MetHb under the same conditions. It has been reported that Hb and hematin induce lipid peroxidation through the same mechanism, but different from that of iron alone (Kim *et al.*, 1994). To further characterize this difference, we examined the oxidative modification of bovine globin and BSA by various iron sources in the XO system (Figs. 1C and 1D). Exposure of globin or BSA to the $O_2^{\cdot-}$ generating system caused modification of only a small amount of protein, but the addition of iron or its EDTA complex resulted in a rapid increase in protein modification. Hb and hematin were more effective than the iron ion or its EDTA complex. To compare the roles of iron ions and heme compounds in protein modification, we investigated the effects of SOD and catalase on Hb modification (Figs. 1A and 1B) and on the modification of globin and BSA (Table 1) in the XO system. Table 1 shows that iron- and iron–EDTA-mediated protein modifications were strongly

Table 3. Effect of radical scavengers on protein modification in the XO system.

Incubation mixtures	Fe^{3+}–EDTA		Hm	
	Protein modification (%)	Inhibition (%)	Protein modification (%)	Inhibition (%)
Globin/XO	28.3 ± 3.5	0	58.5 ± 4.8	0
+DR	13.0 ± 2.6	54	45.3 ± 3.6	23
+DMPO	13.3 ± 6.2	53	52.0 ± 5.4	11
+Man	13.8 ± 0.5	51	53.0 ± 5.0	9
+TU	17.0 ± 7.4	40	34.5 ± 9.0	42
BSA/XO	32.8 ± 0.5	0	45.0 ± 2.8	0
+DR	12.8 ± 6.2	61	34.5 ± 2.6	23
+DMPO	15.8 ± 3.5	52	41.0 ± 4.2	9
+Man	16.8 ± 1.5	49	45.0 ± 1.6	0
+TU	19.5 ± 0.6	41	32.5 ± 1.3	28

Abbreviations: Gb, globin, DR, deoxyribose; Man, mannitol; TU, thiourea. The concentration of scavengers was 30 mM.

Table 4. Effect of ligands and DF on protein modification in the XO system.

Incubation mixtures	Protein modification (%)		
	None	Fe^{3+}-EDTA	Hm
Globin/XO System	11.5 ± 1.8 (0)[a]		
+DF	10.9 ± 1.3 (5)		
+Cyanide	2.4 ± 1.4 (79)		
Globin/XO System		29.5 ± 2.4 (0)	60.3 ± 4.5 (0)
+DF		6.5 ± 3.5 (78)	57.7 ± 3.3 (4)
+Cyanide		22.6 ± 2.4 (23)	8.3 ± 2.9 (86)
+Azide		24.8 ± 2.7 (16)	10.9 ± 2.5 (82)
BSA/XO System	8.3 ± 0.8 (0)		
+DF	1.8 ± 1.3 (78)		
+Cyanide	7.6 ± 1.2 (8)		
BSA/XO System		32.9 ± 3.6 (0)	49.3 ± 2.9 (0)
+DF		7.2 ± 3.7 (78)	44.8 ± 4.9 (9)
+Cyanide		28.6 ± 2.5 (13)	6.9 ± 4.3 (86)
+Azide		29.2 ± 2.9 (11)	9.8 ± 3.6 (80)

[a] Numerical values in parentheses are percent inhibition of protein degradation. The concentration of ligands and DF was 26 μM.

Table 5. Binding of iron to globin and BSA.

Iron species	Iron bound (mol/mol protein)	
	Globin	BSA
None	0.40 ± 0.03	0.02 ± 0.01
Hm	4.12 ± 0.32	3.38 ± 0.12
Fe^{3+}	3.04 ± 0.38	1.44 ± 0.07
Fe^{3+}-EDTA	0.92 ± 0.14	0.63 ± 0.03

inhibitory by SOD, catalase, or the two together. SOD was slightly more inhibitory in the reaction of iron-EDTA than iron alone. However, the modification of Hb itself and hematin-mediated modification of globin and BSA were markedly decreased by the addition of catalase and slightly enhanced with the addition of SOD (Fig. 1 and Table 1).

The role of $O_2^{\cdot-}$ in protein modification became clear when investigation was carried out in a GO system or in the presence of H_2O_2 (Table 2). Hb and hematin were both effective in protein modification in these H_2O_2 systems as well as in the XO system. However, protein modification by iron alone and iron-EDTA in these H_2O_2 systems was nearly the same as the control system without added iron. The results show clearly that an $O_2^{\cdot-}$ generating system was needed for protein modification mediated by iron and iron-EDTA, but not for that mediated by hematin and Hb.

To characterize the nature of the oxidizing species responsible for protein modification, the effects of several scavengers were investigated (Table 3). When iron-EDTA was incubated with globin or BSA in the XO system, ·OH scavengers such as deoxyribose, mannitol, thiourea, and DMPO inhibited protein modification. On the other hand, protein modification by hematin was inhibited by deoxyribose or thiourea, but not by the more specific ·OH scavengers DMPO or mannitol. Table 4 shows that the modification of globin and BSA by iron-EDTA was strongly inhibited by DFO, but not by cyanide or azide. Protein modification by hematin was inhibited in the presence of cyanide or azide, but not DFO. Globin modification without added iron sources was inhibited by cyanide, but not DFO, while the reverse was true for the case with BSA modification (Table 4). These results suggest that globin and BSA were contaminated by small amounts of heme and an iron ion, respectively. It appears that the oxidizing species from iron-EDTA and heme compounds are different in biological reactivity.

Since the spin-trap technique is useful for identification of free radicals, we examined the nature of oxidizing species produced from iron- and heme-compounds in the presence of H_2O_2 and the spin-trap reagent DMPO. When DMPO was mixed with H_2O_2 in the presence of Fe^{2+}-EDTA, a typical DMPO-spin adduct of ·OH was observed (Fig. 2B). Although not shown, Fe^{2+} showed the same ESR spectrum as that of its EDTA complex. Hematin did not show any DMPO-spin adduct (Fig. 2C), while metHb generated the ESR spectra of the slowly-tumbling nitroxide radical adduct with 6 lines of different intensities (Fig. 2D). It suggests that the DMPO-spin adducts produced by metHb are protein radicals, but not ·OH. The nature of the oxidizing species produced by metHb was further analyzed by UV–visible spectroscopy. Figure 2E

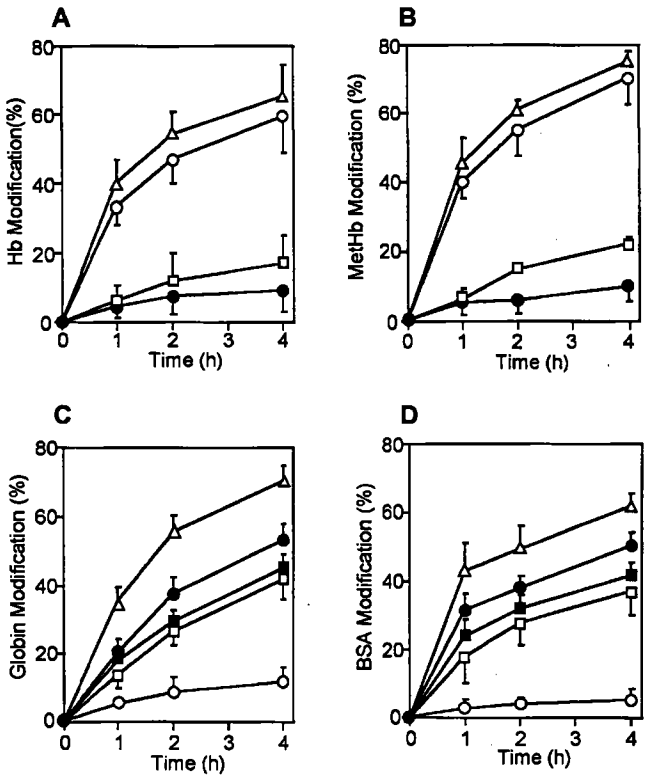

Fig. 1. Effect of iron species on the protein modification in the XO system. A and B: Effect of antioxidant enzymes on the oxidative modification of Hb and MetHb (14 μM on the heme basis) in the XO system. At indicated times, an aliquot (12 μl) of the mixture was subjected to the SDS-PAGE analysis. Modification of Hb (A) and MetHb (B) in the absence (○) and presence of SOD (△), catalase (□), or both (●).The concentration of SOD and catalase in the incubation mixture was 100 units/ml. C and D: Effect of iron species on the modification of globin and BSA. Proteins (0.13 mg/300 μl) was incubated with an iron species (14 μM) in the XO system. Aliquots (6 μl) were subjected to the SDS-PAGE analysis for modification of globin (C) and BSA (D) in the absence (o) and presence of iron ions (□), iron-EDTA (■), hematin (△), or Hb (●).

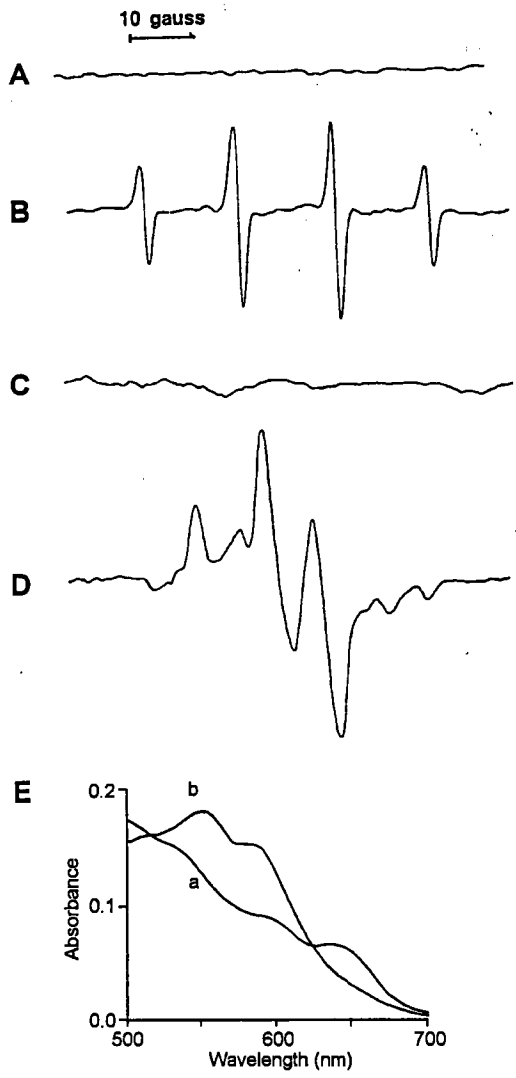

Fig. 2. ESR and UV–visible spectra of iron(II)–EDTA, hematin, and metHb. The reaction mixture consisted of 250 μM H_2O_2, 100 μM DMPO (A, B, C, and D) with PBS (A), iron(II)–EDTA (B), hematin (C), and metHb (D). All iron concentrations were 14 μM. ESR spectra (A, B, C, and D) and UV–visible spectra (E) were recorded in the reaction mixture of metHb alone (a) and metHb and H_2O_2 (b) at room temperature.

showed that the spectra were of the same ferryl ions as described previously in the reaction of metmyoglobin with H_2O_2 (Davies, 1990).

Examples of SDS-PAGE chromatograms of globin and BSA exposed to H_2O_2 and the XO system are shown in Fig. 3. Globin incubated with iron, iron-EDTA, heme, or Hb in the XO system produced several fragments of between 10 and 12 kDa (Fig. 3A), but in an H_2O_2 solution globin did not degrade to specific peptide fragments. When globin was incubated with hematin or Hb, the XO system produced a major dimeric product and a highly aggregated product appeared in the presence of H_2O_2. When BSA was incubated with iron and iron-EDTA in the XO system, seven peptide fragments were observed, viz., four major bands at 59, 53, 47, and 28 kDa, and three minor bands at 20.2, 16.5, and 12 kDa (Figs. 3B and 3C). Treatment of BSA with hematin or Hb in the XO system produced two additional major bands at 62 and 49 kDa and one minor band at 14 kDa. In the H_2O_2 system, however, BSA produced a smeared broad band around the BSA band when incubated with hematin and Hb, and produced no specific fragment when incubated with iron or iron-EDTA. The incubation of BSA with Hb gave two additional bands (>200 and ~28 kDa) originated from Hb (Fig. 3B).

Since the binding of iron to a target protein appeared to be a prerequisite to protein modification, the binding of hematin, iron, and iron-EDTA were measured. Table 5 shows that the affinity of hematin to proteins was the highest and also that EDTA inhibited the binding of iron to proteins.

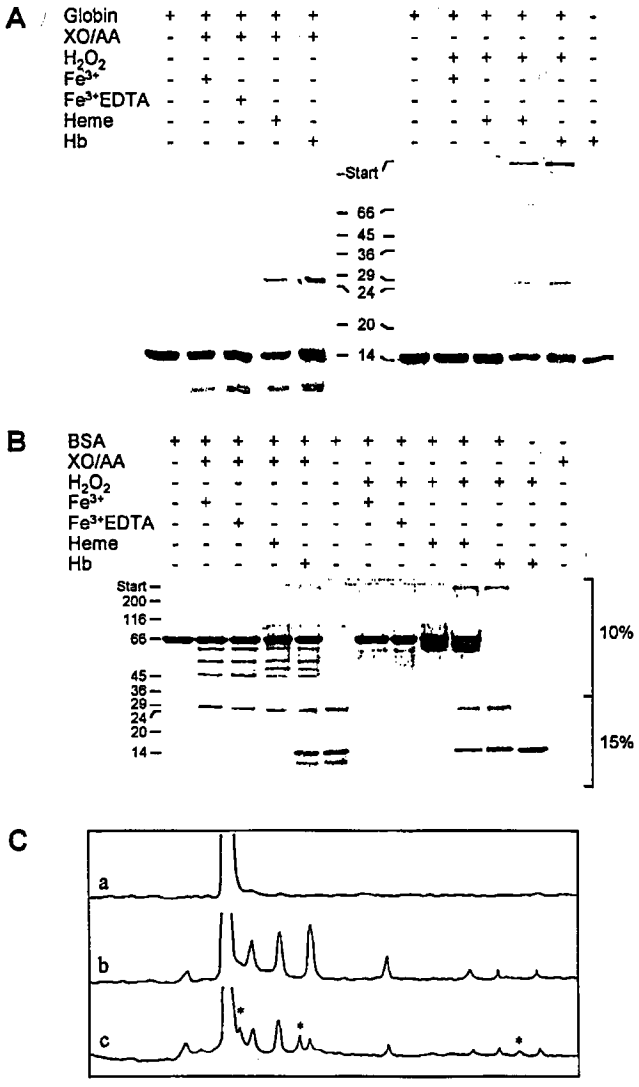

Fig. 3. SDS-PAGE of globin (A) and BSA (B) modified by iron species in the XO system or in the presence of 250 μM H_2O_2. The incubation time was 80 min. Concentration of the iron species was 14 μM. Densitometer traces (C) of SDS-PAGE were BSA alone (a), with iron–EDTA (b), or with hematin (c) in the XO system. Asterisks indicate the additional peptide fragments in hematin-treated BSA.

Discussion

Oxygen radicals generated by irradiation or from metal ion-mediated reactions are capable of modifying proteins through aggregation, fragmentation, and conformational change, to destroy biological functions of the proteins and increase their proteolytic susceptibility (Levitzki and Anbar,1967; Fucci *et al.*, 1983; Levine, 1983; Schnessler and Schilling, 1984; Wolff and Dean, 1986; Davies, 1987; Davies and Goldberg, 1987; Meucci, 1991; Stadtman and Oliver, 1991). Besides numerous reports on hematin-mediated lipid peroxidation (Ursini *et al.*, 1981; Kalyanaraman *et al.*, 1983; Kim and Sevanian, 1991; Koga *et al.*, 1991; Kim *et al.*, 1994), it has been also reported that hematin mediates oxidative degradation of proteins in the presence of mercaptoethanol and oxygen (Aft and Mueller, 1984). Our results clearly show that Hb and hematin increase protein modification more effectively than iron ions and iron–EDTA in an $O_2^{\cdot-}$ generating system, and that hematin is also the most effective among them (Fig. 1).

SOD prevents oxidative damage in tissues and cells, providing therapeutic potential for many diseases associated with oxygen free radicals (Fridovich, 1986). The protective effect of SOD was observed in iron- and iron–EDTA-mediated protein modification in an $O_2^{\cdot-}$ generating system, but not in hematin- and Hb-mediated modification (Table 1). Catalase was effective as an inhibitor in both cases. The results are also reflected on the data in Table 2, which shows that significant protein modification in the presence of H_2O_2 is caused by hematin and Hb, but not by iron and iron–EDTA. These results support the assumption that deleterious oxidizing species are formed as a result of metal-catalyzed Haber-Weiss reactions (reactions 1 and 2) in the cases of iron and iron-EDTA, and from reaction 3 in the cases of hematin and Hb.

$$\text{Porphyrin-Fe}^{3+} + H_2O_2 \rightarrow \text{Porphyrin-Fe}^{4+} = O + \cdot\text{OH-derived species} \quad [3]$$

The ·OH-derived species is still unknown, but is assumed to be either the porphyrin π-cation radical or free radicals of amino acid residues. In this study, the radical generated by metHb was a protein radical. ESR spectra of this radical was identical to that of the tyrosyl radical (Miki *et al.*, 1989).

It has been shown (Gutteridge, 1986; Puppo and Halliwell, 1988; Prasad *et al.*, 1989) that excessive H_2O_2 degrades the heme moiety, releasing iron that promotes ·OH formation in $O_2^{\cdot-}$ generating systems. This may not be the case in our system. Hematin- and Hb-mediated protein degradation was inhibited by azide and cyanide, but not by DFO which abrogates biological toxicity of iron ions through inhibiting the recycling of the Fenton reaction (Graf *et al.*, 1984; Gutteridge, 1984; Gutteridge, 1986;). Since hematin and Hb were more active than iron ions as a mediator in protein modification and their mechanism of action appeared to be different from that of iron alone, we ruled out the possibility that they release free iron and then act as a Fenton reagent.

The addition of SOD slightly increased protein modification by hematin and Hb in the XO system. This could explain the slight increases in protein modification in the H_2O_2 and GO systems, because the H_2O_2 concentration was adjusted to that accumulated in the XO system (Table 2). $O_2^{\cdot-}$ appears to inhibit protein modification by reducing oxidants formed from reactions of Hb and hematin with H_2O_2. Davies *et al.* (Davies *et al.*, 1987) have suggested that $O_2^{\cdot-}$ has a dual function in protein damage by oxygen free radicals. The first function is acceleration of

fragmentation by reacting with α-carbon radicals rather than with peptide bonds (Davies *et al.*, 1987). The second is repair of the amino acid radical, probably reducing tyrosyl radicals which contribute to protein aggregation through the formation of intermolecular bityrosine bonds (Davies and Delsignore, 1987). It was, therefore, notable that polymerized products appeared during incubation of globin and BSA with Hb or hematin in the presence of H_2O_2, but not in the O_2^- generating system (Fig. 3).

The ·OH scavengers such as mannitol, DMPO, deoxyribose, and thiourea, all effectively inhibited protein modification mediated by iron–EDTA (Table 3), but only thiourea and deoxyribose significantly inhibited the hematin-mediated protein modification. DMPO specifically scavenges ·OH (Finkelstein *et al.*, 1980; Buettner, 1987; Yamazaki and Piette, 1991), but deoxyribose may react with both ·OH and ferryl species (Flitter and Mason, 1990; Winterbourn, 1991). The action of thiourea is complex because it reacts with both ·OH and H_2O_2 (Cederbaum *et al.*, 1979) and inhibits the XO reaction as assayed by O_2 uptake (Halliwell, 1978). We have reported that ·OH was not detected with the DMPO-spin trap technique in the reaction mixture of Hb and H_2O_2 (Kim *et al.*, 1995). Our UV–visible spectroscopic data showed that the ferryl heme was the major oxidizing species produced by the reaction of metHb with H_2O_2. With the ESR technique, Yamazaki and Piette, (1990) showed that the major oxidizing product was ·OH in the reaction of iron and iron–EDTA with H_2O_2. Our result indicated that ·OH and ferryl ion are the main oxidizing species involved in iron–EDTA- and heme-mediated protein modification, respectively.

Binding of Hb and hematin to proteins and membrane lipids is an essential step in biological deleterious reactions. As reviewed by Stadtman and Olivers (1991), metal-catalyzed oxidation of proteins is a site-specific process involving the interaction of H_2O_2 and Fe^{2+} at metal binding sites on the protein. Aft and Mueller (1984) have also reported that protein degradation becomes sensitive when the heme is coordinated to a susceptible target site of the protein. BSA has a high affinity binding site for cupuric ion (Bradshaw *et al.*, 1968). This protein has also one high affinity binding site for hemin and additional sites of much lower affinity (Beaven *et al.*, 1974; Adams and Berman, 1980), indicating that it can be degraded at specific sites by hematin in the presence of H_2O_2. The affinity of proteins for heme, however, is not always consistent with their susceptibility to heme-mediated degradation (Aft and Mueller, 1984). Figure 3 demonstrates that hematin mediates site specific modification (mostly degradation) of globin and BSA in the XO system, but contrary to hematin, Hb does not seem to bind with these proteins at specific sites. The pro-oxidants generated from hemoproteins, however, can oxidize lipid (Kim *et al.*, 1994) and styrene (Ortiz de Montellano and Catalano, 1985). Our data also showed that iron–EDTA can effectively cause protein modification without direct interaction, indicating that binding of iron or its complexes to target proteins may not be the critical factor.

We conclude, therefore, that in spite of the presence of antioxidant enzymes, SOD, catalase, peroxidases, there is a steady state concentration (~1 μM) of reactive oxygen species in biological systems, which may lead to interaction with iron ions and heme-compounds with concomitant production of hydroxyl radicals and ferryl ions, respectively. The pro-oxidant activity of these oxidizing species could be responsible for many deleterious biological processes. The mechanism of the protein modification by the oxidizing species is under investigation.

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