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THE GENERATION OF FERRYL OR HYDROXYL RADICALS DURING INTERACTION OF HAEMPROTEINS WITH HYDROGEN PEROXIDE[1]

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The oxidation of 2-keto-4-thiomethyl butyric acid (KTBA) and methionine to ethylene has been used to evaluate generation of ferryl species or hydroxyl radicals by H_2O_2-activated haemproteins or free ferric ions. Hydrogen peroxide was generated by a glucose oxidase-glucose system at a rate of 1 μM/min. Free ferric in the presence of H_2O_2 oxidizes KTBA, and this was highly inhibited by hydroxyl radical scavengers, caeruloplasmin, superoxide dismutase (SOD) and EDTA. However, when metmyoglobin, methaemoglobin (MtHb) or horseradish peroxidase (HRP) were tested in the same model system, hydroxyl radical scavengers suppressed partially KTBA oxidation and caeruloplasmin, SOD and EDTA failed to inhibit the reaction. Cytochrome-c was found to be a weak promoter of KTBA oxidation in the presence of H_2O_2. Methionine was oxidized to ethylene by an active system which generates hydroxyl radicals, but not by H_2O_2-activated metmyoglobin. Ferric ions chelated to membranes or ADP in the presence of H_2O_2 generated enzymatically, initiated membranal lipid peroxidation only in the presence of ascorbic acid, and this was inhibited by EDTA. In contrast, metmyoglobin and methaemoglobin activated by H_2O_2 generated by the same system, initiated membranal lipid peroxidation and this was not inhibited by EDTA. It is concluded that ferryl and not HO· is the main oxidant in systems containing myoglobin and haemoglobin activated by low concentrations of H_2O_2.

ABBREVIATIONS: MetMb, metmyoglobin; MetHb, methaemoglobin; Cyt-c, cytochrome c; TBA, thiobarbituric acid; MDA, malondialdehyde; DMSO, dimethyl sulphoxide; SOD, superoxide dismutase; DETA; diethylenetriaminepenta acetic acid.

INTRODUCTION

Methaemoglobn and metmyoglobin, the ferric states of these proteins, are activated by H_2O_2 producing a short-lived intermediate with one oxidizing equivalent on the haem, and one on the globin, giving an oxene-ferryl haemglobin radical,[1,2] Studies of H_2O_2-activated metmyoglobin show that although it is not identical with Compound I or II of horseradish peroxidase, it has some structural features in common with both.[3]

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The iron in cytochrome c is covalently bound to the polypeptide chain by all six coordinates.[4] Iron cytochrome c could interact with H_2O_2[5] and with hydroperoxides[6] without forming a ferryl compound.

Hydrogen peroxide-activated metmyoglobin and methaemoglobin were found to oxidize a series of phenols,[7] uric acid,[8] and to cause protein cross-linking.[9] Recently, we reported that H_2O_2-activated metmyoglobin and methaemoglobin could initiate membrane lipid peroxidation[10] and also oxidize several molecules, such as methional, ascorbic acid, phenol-red and β-carotene.[11] In addition to metmyoglobin and methaemoglobin, in enzymes such as peroxidases, catalase[12] and cyclooxygenase,[13] hydrogen peroxide is known to activate haem iron to higher oxidation states.

Sadrzadeh *et al.*[14] postulated that haemoglobin in the presence of O_2^- or H_2O_2 promotes hydroxyl-radical formation, acting as a biological Fenton reaction. This work was recently criticized by Whitburn,[15] Gutteridge[16] and Puppo and Halliwell,[17] who suggested that iron released from haemoglobin during the interaction with H_2O_2 or hydroperoxides was the true generator of hydroxyl radicals in a model system containing this reagent. More recently, it was found that oxyhaemoglobin reacts with low concentration of H_2O_2 to form a "reactive species" that degrades deoxyribose but does not hydroxylate phenylalanine.[17] Previous evidence for hydroxyl radical generation by ferric ion-derived Fenton reactions was presented by Walling *et al.*[17] and Gutteridge.[19] This reaction was inhibited partially by superoxide dismutase (SOD).[19] Currently, iron-dependent Fenton reaction and other oxidative processes are highly implicated in the pathogenesis of several human diseases,[20] and the identification and evaluation of potential catalysts are very important in our efforts to understand and prevent these diseases.

In this study we have adopted KTBA, methionine and membrane lipids as probes for hydroxyl radical and ferryl ion activities. KTBA has been used in numerous *in vitro* studies to detect hydroxyl radicals[21–23] and more recently also in *in vivo* studies.[24] Earlier studies showed also that ethylene was produced from KTBA via peroxidase-H_2O_2[25] and cyclooxygenase-H_2O_2.[26] KTBA and related methionine analogs, which interact with HO· or ferryl ions to produce ethylene, are useful in studies of oxidation-mediated processes, however, only if we are aware of the possibility that several other radicals, such as alkoxyl and peroxyl, could also generate ethylene from these compounds.[27]

The present study was conducted to evaluate the possible action of H_2O_2 on haemproteins as activator of iron to ferryl or generator of HO· radicals.

MATERIALS AND METHODS

Myoglobin type I from equine skeletal muscle, haemoglobin type I (from bovine blood), cytochrome c (horse heart), glucose oxidase (from *Aspergillus niger*), superoxide dismutase (bovine erythrocytes), caeruloplasmin, catalase-free thymol, thiobarbituric (TBA), formic acid sodium salt, dimethyl sulphoxide (DMSO), diethylenetriaminepentaacetic acid (DETA), β-carotene, all-*trans*-linoleic acid and bovine serum albumin were obtained from Sigma Chemical Compnay (St. Louis, MO, U.S.A.). EDTA and glucose were purchased from BDH Chemicals Ltd. (Poole, England); mannitol from May & Baker Ltd. (Dagenham, England); ferric chloride from Mallinckrodt Chemical Works (St. Louis, MO, U.S.A.); and hydrogen peroxide (30% for synthesis) and trichloroacetic acid from Merck (Darmstadt, W. Germany).

Formate-Na (^{14}C) with a specific activity of 5 mCi/mmole was obtained from New England Nuclear, Boston. Haemproteins treated with Chelex 100 and neutralised to pH 7.0 were separated from low molecular weight compounds by a column of Sephadex 15.

Degradation of KTBA 1 mM (in 50 mM acetate buffer, pH 7.0) to ethylene was conducted in a glass tube closed with a serum cap lined with Teflon and incubated in a shaking bath at 25°C. The total volume of the gas phase and the liquid phase in each ampule was measured. The ethylene production in the reaction was identified with the use of a 6 ft. Porapak Q column and a flame ionization detector in a Packard gas chromatograph. The amount of ethylene was calculated from a standard sample (28). Degradation of methionine (10 mM) to ethylene was conducted under the same conditions which oxidize KTBA. The oxidation of formate-Na (^{14}C) to $^{14}CO^2$ was determined using a method developed by May and Haen[29] as used by us previously.[11]

Isolation of the microsome fraction from muscle tissues was by a procedure described previously.[10,30] Protein determinations were conducted by the modified Lowry procedure[31] using bovine serum albumin as standard. Microsomes for lipid peroxidation assays were incubated under a shaking water bath at 37°C. The reaction mixture contained 1 mg of microsomal proteins/ml and 4 ml of 50 mM acetate buffer, pH 7.0. Thiobarbituric acid-reactive substances (TBA-RS) were determined by the procedure of Bidlack *et al.*[32] The results are reported as nanomoles of malondialdehyde (MDA) per milligram of protein, using a molar extinction coefficient of $E_{532} = 1.56 \times 10^5\,M^{-1}\,cm^{-1}$. β-Carotene co-oxidation in the presence of linoleate at pH 7.0 and 37°C, was determined by a method described previously.[33] The results are means of triplicates of two separate experiments. In the figures, each error bar denotes the standard deviation.

RESULTS

The rate of KTBA oxidation by the glucose–glucose oxidase system containing horseradish peroxidase or metmyoglobin was, respectively, almost 4- and 3-fold higher than that of ferric chloride. Cytochrome c in the same system oxidized KTBA at half the rate of ferric chloride; however, its oxidative effect increased during incubation. The oxidation of KTBA by ferric chloride and cytochrome c was inhibited by EDTA, but that of horseradish peroxidase or metmyoglobin was not affected by the chelator (Figure 1). The differences between metmyoglobin and ferric chloride or cytochrome c as catalysts of membrane lipid peroxidation were evaluated in a model system containing microsomes and glucose–glucose oxidase (Figure 2). Metmyoglobin, which was activated by H_2O_2 generated enzymatically, initiates membranal lipid peroxidation and was not affected by EDTA. Ferric chloride with or without ADP and cytochrome c in the same model system did not initiate membranal lipid peroxidation. An effective iron redox cycle from ferric chloride, in the same model system, was produced by ascorbic acid, which peroxidized membrane lipids, but this reaction was inhibited by EDTA.

The oxidation of KTBA was also determined in the presence of an added 100 μM of H_2O_2. This oxidation was found to be highly dependent on the concentration of the catalysts in the respective order: horseradish peroxidase > metmyoglobin > - $FeCl_3$ > cytochrome c (Figure 3). The oxidation of KTBA to ethylene by metmyoglobin, $FeCl_3$ and cytochrome c was affected by H_2O_2 concentration (Figure 4). The

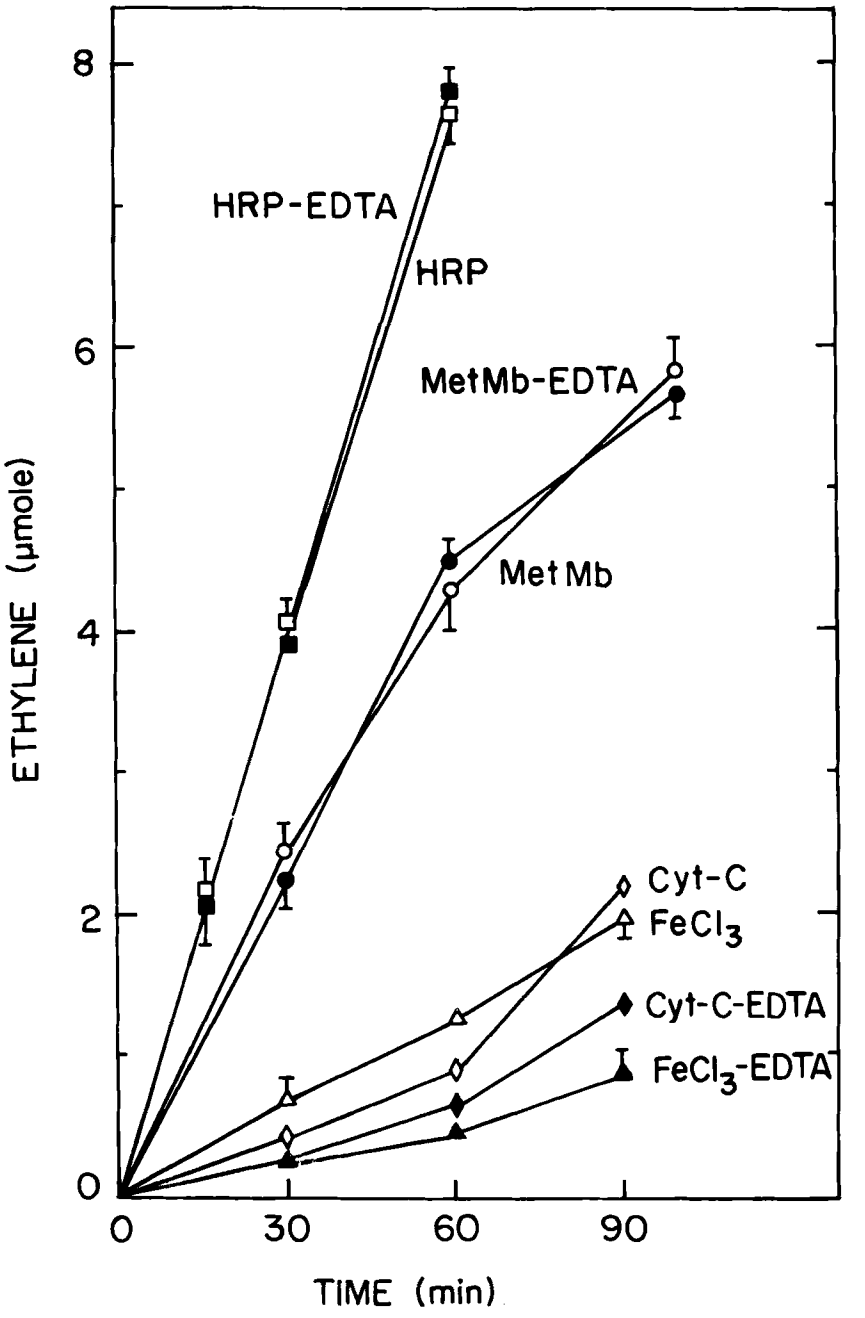

FIGURE 1 Ethylene generation during KTBA oxidation by several haemproteins activated by enzymatically-produced H_2O_2 at a rate of 1 nmole/ml/min at pH 7.0, 25°C. metmyoglobin or other reagents, 30 μM; EDTA, 33 μM.

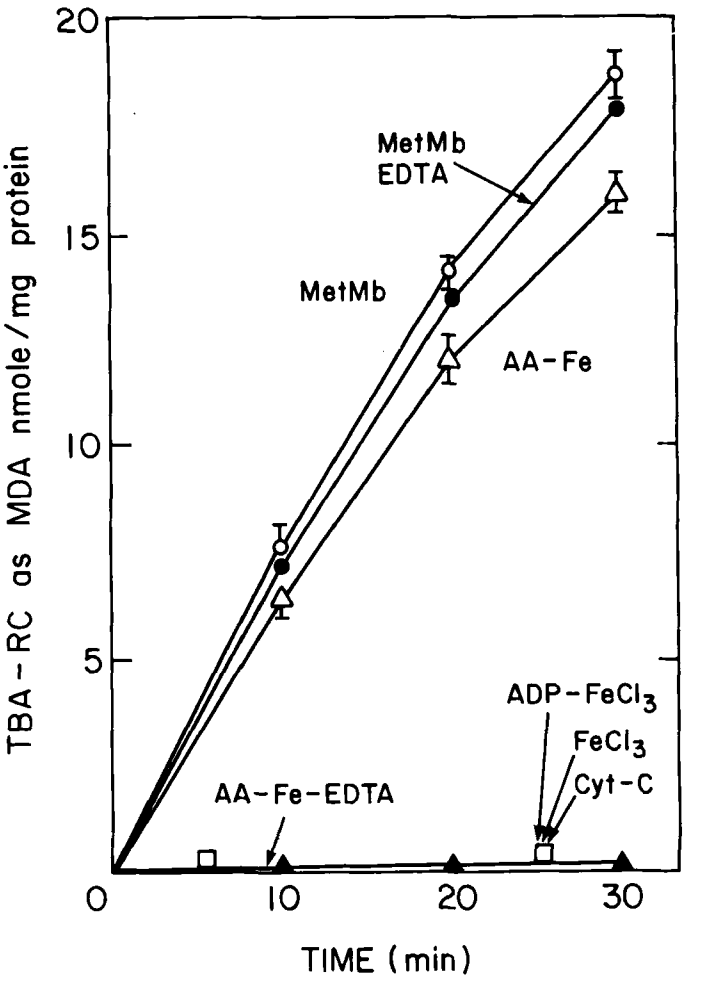

FIGURE 2 Membrane lipid peroxidation by several catalysts activated by enzymatically-generated H_2O_2, as in Figure 1. metmyoglobin, 30 μM; AA-Fe (ascorbic acid, 200 μM; $FeCl_3$, 10 μM); ADP-$FeCl_3$, 33 μM-30 μM; cytochrome c, 30 μM; $FeCl_3$, 30 μM; EDTA, 33 μM.

rate of KTBA oxidation by metmyoglobin and $FeCl_3$ decreased at a high concentration of H_2O_2, but that of cytochrome c increased. EDTA inhibited KTBA oxidation by ferric chloride or cytochrome c in a system which contained added H_2O_2, very similar to the system by which H_2O_2 was generated enzymatically. Cytochrome c was found to be a weak catalyzer of KTBA oxidation in the presence of H_2O_2. As cytochrome c is known as a good catalyzer of linoleate peroxidation,[6] this reaction was compared with the catalysts of metmyoglobin. Figure 5 demonstrates that cytochrome c's ability to oxidize β-carotene in a β-carotene-linoleate model system was almost 50% of that of metmyoglobin. However, cytochrome c efficiency to oxidize KTBA was only about 10% of that of metmyoglobin.

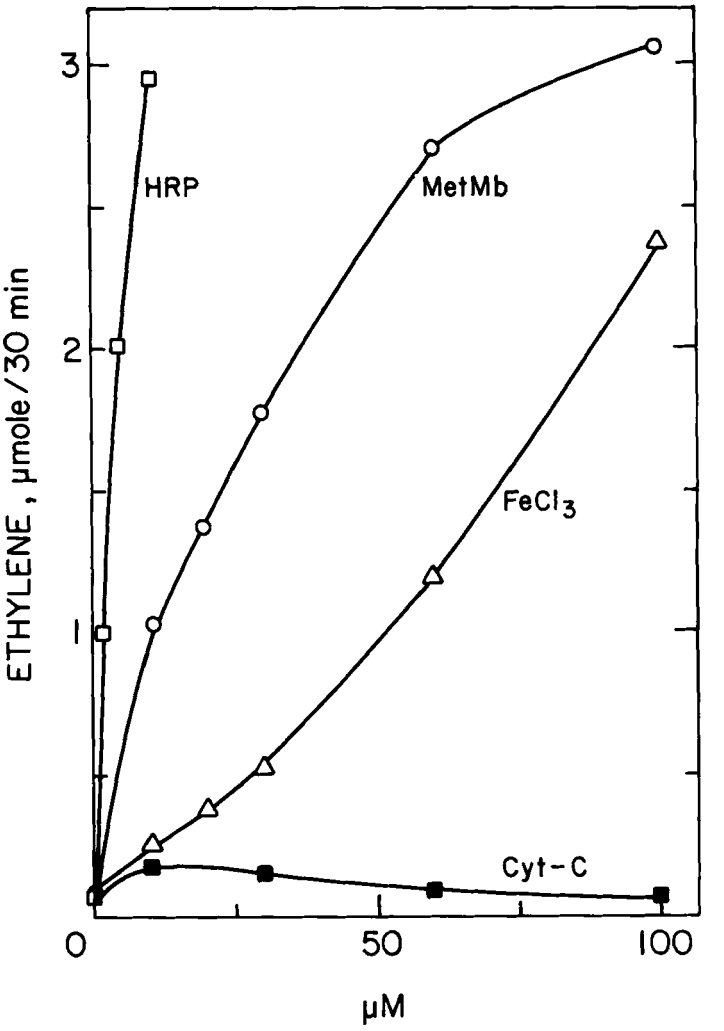

FIGURE 3 The effect of haemprotein concentration on KTBA oxidation during incubation witn 100 μM H_2O_2 at pH 7.0 and 25°C.

The incubation of KTBA in the presence of the glucose–glucose oxidase system generated ethylene at a very low rate (0.08 μmole/30 min). Addition of a fresh solution of ferric chloride at a concentration of 30 μM increased ethylene generation to almost 1.0 μmole/30 min. The breakdown of KTBA by ferric chloride was inhibited by hydroxyl radical scavengers (mannitol, formate and DMSO), superoxide dismutase, caeruloplasmin and catalase. EDTA and DETA were also found to inhibit ferric chloride catalysis of KTBA oxidation (Table I). Ethylene generated by metmyoglobin and methaemoglobin activated by the same system was almost 3-fold higher than that produced by ferric chloride. The oxidation of KTBA by H_2O_2-activated metmyoglobin and methaemoglobin was inhibited partially by mannitol and formate, and very

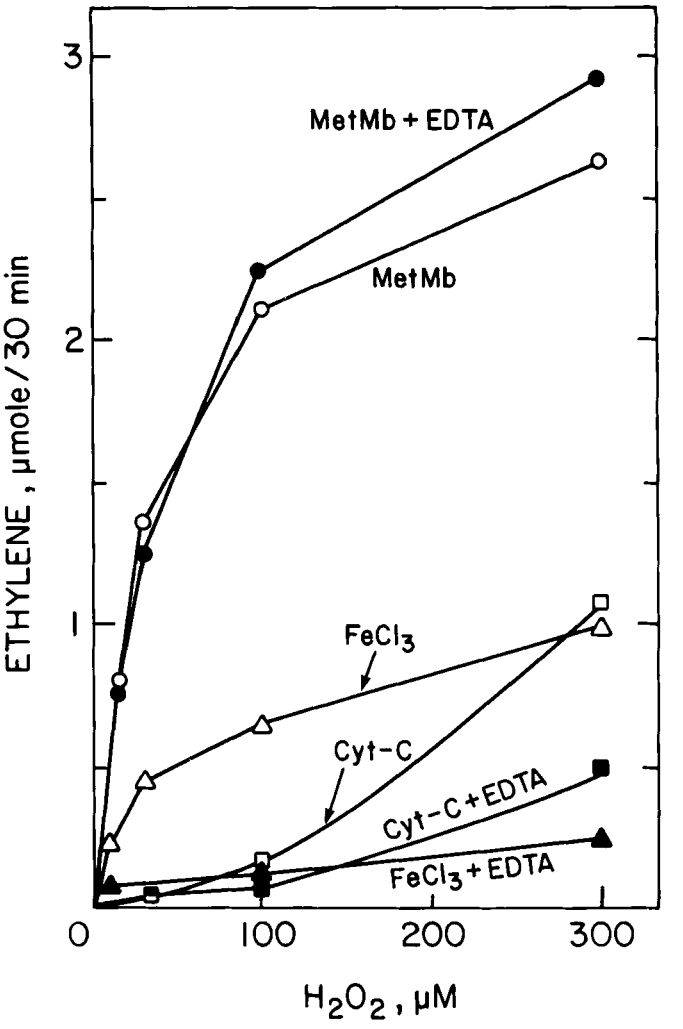

FIGURE 4 The effect of H_2O_2 concentration on KTBA oxidation by metmyoglobin, cytochrome c and $FeCl_3$ (30 μM), with or without EDTA (33 μM).

slightly by DMSO. Superoxide dismutase inhibits only partially KTBA oxidation by activated methaemoglobin. Caeruloplasmin, DETA and EDTA, which inhibit 98%, 85% and 60%, respectively, of KTBA oxidation by ferric chloride, failed to inhibit the catalysis of this reaction by activated metmyoglobin and methaemoglobin. Horseradish peroxidase activated by the glucose–glucose oxidase system oxidized KTBA, forming ethylene at a rate higher than other haemproteins; however, its inhibition by all the reagents treated in this research resembles that of metmyoglobin and methaemoglobin. The inhibitory effect of SOD, caeruloplasmin and catalase toward KTBA oxidation by free iron/H_2O_2 system was eliminated by autoclaving the enzymes at 120°C for 15 min.

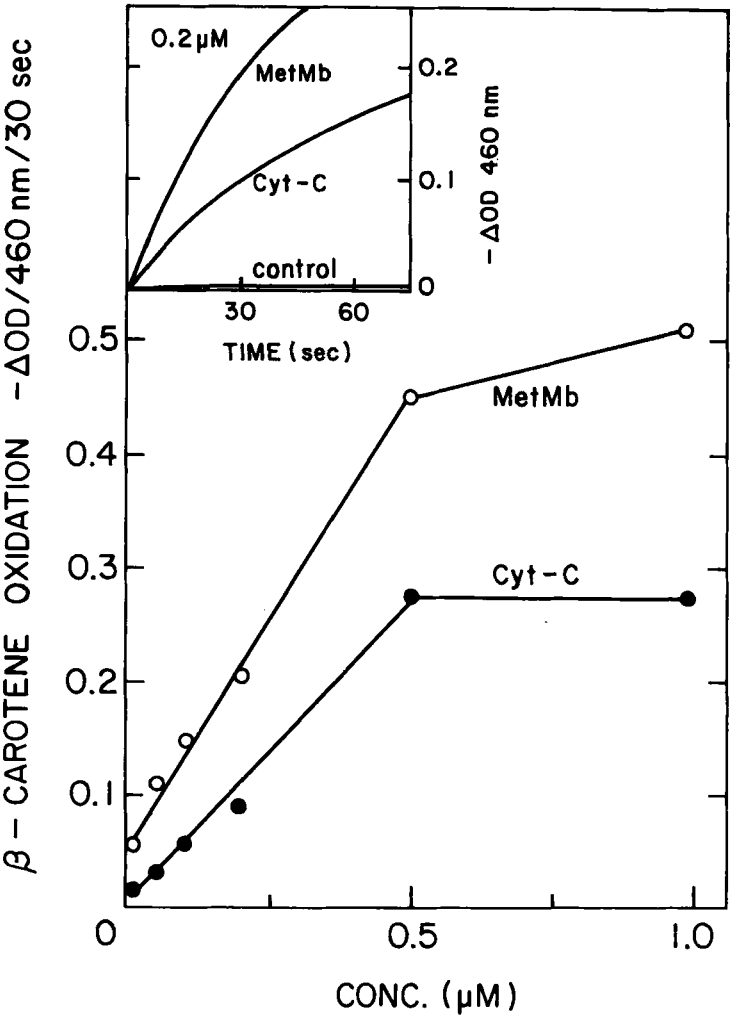

FIGURE 5 β-carotene linoleate coupled peroxidation as affected by metmyoglobin or cytochrome c concentration.
The model system contained β-carotene (14 μM); linoleate (2 mM); linoleate hydroperoxides (25 μM); Tween-20, 0.05%; Upper figure, the rate of β-carotene oxidation by haemproteins (0.2 μM).

The oxidation of KTBA by H_2O_2-destroyed metmyoglobin was inhibited by almost all the reagents which inhibit the catalysis by ferric chloride (Table I).

Formate was found to inhibit the generation of ethylene by H_2O_2-activated haemproteins by almost 50% (Table I). However, H_2O_2 activated metmyoglobin and methaemoglobin did not decompose formate to CO_2 (results not shown). Methionine was decomposed by free iron in a model system containing H_2O_2 produced enzymatically in the presence of ascorbic acid and especially when EDTA was added. However, in the same model system, metmyoglobin even in the presence of ascorbic acid and EDTA did not decompose methionine significantly (Table II).

TABLE I
KTBA oxidation by several catalysts activated by H_2O_2 generated enzymatically

Treatments		$FeCl_3$	Ethylene μmole/30 min			
			MetMb[1] destroyed	MetMb	MetHb	HRP
Control, glucose/glucose oxidase		0.99 (−)	1.11 (−)	2.76 (−)	3.12 (−)	4.11 (−)
+ Mannitol	(100 mM)	0.22 (78)[2]	0.40 (64)	1.72 (48)	2.38 (24)	2.92 (65)
+ Formate	(50 mM)	0.04 (96)	0.30 (73)	1.41 (49)	0.91 (71)	2.50 (40)
+ DMSO	(200 mM)	0.35 (65)	0.27 (75)	2.60 (6)	2.37 (24)	3.71 (10)
+ SOD	(200 U)	0.47 (56)	0.46 (58)	2.82 (−)	3.09 (−)	4.32 (−)
+ Caeruloplasmin	(10 U)	0.02 (98)	0.80 (28)	2.68 (3)	3.23 (−)	4.21 (−)
+ Catalase	(200 U)	0.20 (75)	0.20 (82)	0.34 (88)	0.54 (83)	1.60 (61)
+ EDTA	(33 μM)	0.40 (60)	0.62 (45)	2.70 (−)	3.20 (−)	4.09 (−)
+ DETA	(33 μM)	0.14 (85)	0.78 (30)	2.65 (4)	3.21 (−)	4.10 (−)

Reaction mixture contained, in a final volume of 10 ml, the following reagents added in the order to give the final concentration in brackets; acetate buffer (50 mM); glucose (4 mM); KTBA (1 mM); scavengers, enzymes and chelates; glucose oxidase injected to produce H_2O_2 at a rate of 1 nmole/ml/min at 25°C. Autoclaved enzymes for 15 min gave results very similar to control.

[1] MetMb (30 μM) was destroyed by 600 μM of H_2O_2 and incubated for 18 h, reaction was done with more 200 μM H_2O_2 without glucose oxidase.
[2] % inhibition.

TABLE II
Methionine oxidation by several catalysts activated by H_2O_2 generated enzymatically

Treatment		Ethylene/μmole/30 min	
		EDTA present	EDTA absent
$FeCl_3$	(30 μM)	0	0
AA	(200 μM)	0.01	0
$FeCl_3$ + AA		18.58	0.38
MetMb	(30 μM)	0	0
MetMb + AA		0.01	0

Reaction mixture contained, in a final volume of 10 ml, the following reagents added in the order stated to give the final concentration in brackets: acetate buffer (50 mM), pH 7.0; glucose (4 mM); methionine (10 mM); EDTA (33 μM); ascorbic acid (200 μM); $FeCl_3$ (30 μM); MetMb (30 μM); glucose oxidase injected for generation of H_2O_2 at a rate of 1 nmole/ml/min, at 25°C.

Incubation of metmyoglobin with glucose–glucose oxidase system for 30 min releases 10–15% of the haemprotein iron, producing a concentration of 3–4 μM iron ions (results not shown).

DISCUSSION

Iron in several haemproteins is activated by H_2O_2 to higher oxidation states.[3,34]

Haemproteins are also destroyed when they are incubated with peroxides and free iron release. Iron release from haemproteins is rapid when they are incubated with a high concentration of H_2O_2 in the absence of reducing agents.[16,34,36] In order to decrease iron release from haemproteins in our model system, we hept consecutively a low concentration of H_2O_2 by generating it enzymatically.

The results demonstrated that free ferric ions derived Fenton reaction oxidize KTBA, and this was strongly inhibited by hydroxyl radical scavengers, such as mannitol, formate or DMSO and by antioxienzymes like caeruloplasmin, superoxide dismutase and catalase. The results resemble those obtained by Gutteridge *et al.*[19] However, when metmyoglobin, methaemoglobin or horseradish peroxidase was tested in the same model system, hydroxyl radical scavengers suppressed only partially KTBA oxidation and caeruloplasmin, or SOD failed to inhibit the reaction.

The partial suppression of KTBA oxidation by H_2O_2-activated haemproteins with mannitol and formate could be explained by the interaction of the hydroxyl radical scavengers with free radical intermediates of degradated KTBA. Ethylene production from KTBA is a complex process, involving several products and at least three short-lived intermediates.[37] It is proposed that scavengers may inhibit oxidation by reacting with the initiating species, but their effects can also be due to reaction with other intermediates.[38] H_2O_2-activated metmyoglobin and methaemoglobin did not decompose formate to CO_2[11] and for this reason it seems that the inhibitory effect of formate is by interaction with KTBA intermediates and not directly with oxene-ferryl metmyoglobin. Very similar results were obtained by Winterbourn[38] determining KTBA oxidation by a xanthine oxidase/iron system in the absence of EDTA. Formate was found to inhibit ethylene production from KTBA; however a xanthine oxidase/iron system failed to break down directly formate to CO_2.[38]

Dimethylsulphoxide seems to be resistant to oxidation by oxene ferryl-haemproteins or by KTBA breakdown intermediates. For this reason, DMSO inhibits KTBA oxidation especially by ferric ions-dependent reaction.

Methionine, another known hydroxyl radical scavenger,[39] was found to be oxidized to ethylene only in the presence of an active system which generates hydroxyl radicals (Table II), and not by H_2O_2-activated haemproteins. Metmyoglobin activated by H_2O_2 generated enzymatically, even in a system containing ascorbic acid and EDTA, did not oxidize methionine at a significant amount. Very similar effects were obtained by the Elstner group[40] using oxoferrin to activate haemoglobin, myoglobin or peroxidase. Methionine was not oxidized by oxoferrin-activated haemproteins, but only by hydroxyl radicals.

Superoxide-dismutase and caeruloplasmin, which totally failed to inhibit H_2O_2-activated haemproteins catalysis, were found to be very efficient in inhibiting the free ferric ions-dependent Fenton reaction. These results could be explained only if ferric ions during interaction with H_2O_2 produce superoxide and ferrous ions. Our data support those obtained by Gutteridge *et al.*[19] concerning oxidation of deoxyribose caused by hydrogen peroxide and ferric-EDTA.

EDTA is a known enhancer of the superoxide iron-dependent Fenton reaction.[28,41,42] However, in our model system EDTA was found to inhibit the degradation of KTBA by ferric ions and not to affect those of H_2O_2-activated haemproteins. Similar inhibitory effects of EDTA were found in systems in which the superoxide iron-derived Fenton reaction decomposes DMSO,[43] and deoxygalactose or deoxyglucose is known to chelate iron.[41] KTBA also chelates iron,[44] and hydroxyl radicals seem to be generated by a site-specific mechanism, attached to a target molecule. EDTA seems to remove iron from KTBA, which causes hydroxyl radicals to be generated in free solution, thus reducing radical attack; this could explain our data. It is also known that the redox potential of the Fe^{2+}/Fe^{3+} pair can vary by complexing ligands.[45–47] EDTA decreases the redox potential of the electron from H_2O_2 to Fe^{3+} and thereby affects the rate of KTBA oxidation. In the system containing methionine

and ascorbic acid, the reduction of Fe^{+3} is not the limiting factor in this system; EDTA alters the potential of Fe^{+2} to reduce H_2O_2 to hydroxyl radicals. As EDTA could not remove iron from haemproteins, it is not surprising that the chelator did not affect KTBA oxidation by activated haemproteins.

Our data demonstrated that in systems containing myoglobin and haemglobin, where H_2O_2 was kept at a low concentration, ferryl ions seem to be the main oxidant affecting target molecules. Similar conclusions were assumed analyzing the data collected using membrane lipid peroxidation as a model for determining the differences between the activity of haemproteins and ferric ions.

Membrane lipid peroxidation, initiated with metmyoglobin or methaemoglobin (results not shown) activated with enzymatically-generated H_2O_2 was not affected by EDTA. Ferric ions chelated to membranes or ADP failed to initiate lipid peroxidation in the same model system which generates hydrogen peroxide enzymatically. Membrane lipid peroxidation was initiated by ferric ion only in the presence of ascorbic acid and enzymatically-generated H_2O_2; however, this reaction was inhibited by a low concentration of EDTA. EDTA, which could transfer iron from a low molecule target of biological complexes, seems to remove chelatable iron also from membranes into the exogenous environment and by this to decrease membranal lipid peroxidation.[28] This assumption, made previously, was more recently proved by Vile and Winterbourn.[48] The results provide further evidence on the differences between the behavior of ferric ions and ferric-haemproteins as oxidants activated by H_2O_2.

The iron in cytochrome c is covalently attached to the polypeptide chain by thioether bridges from cysteines 14 and 17, so that the 5th and 6th iron coordination is occupied. The reduction-oxidation of cytochrome c is often used as a "marker" protein for detection of superoxide anions and hydroxyl radicals.[4] Iron cytochrome c could interact also with H_2O_2[5] and hydroperoxides[6] without forming a complex. This interaction seems to form hydroxyl and lipid radicals. Hydroxyl radicals generated in such a system will most probably interact with the haem pocket, leading to its oxidative degradation and release of iron, as was actually found in our study[49] and by others.[36]

The low efficiency of ferricytochrome c to oxidize KTBA, in the presence of low concentrations of enzymatically-generated H_2O_2, seems to be due to the fact that very small amounts of HO· escape the haem edge. The "site specific" generation of hydroxyl radical by cytochrome c seems to be in the vicinity of the iron ion. The hydroxyl radical is so reactive[18] that it will attack the haem edge with a rate-constant usually of the order of $10^9 M^{-1} s^{-1}$. It may also be assumed that, in part, KTBA oxidation by ferricytochrome c is affected by free iron ion release from the molecule and thus was affected by the addition of EDTA.

The interaction of ferricytochrome c with hydroperoxides leads to the formation of lipid free radicals, which seem to diffuse more efficiency out of the haem edge, oxidizing β-carotene. The exact mechanism by which ferricytochrome c produces active oxygen species from H_2O_2 or hydroperoxides is unknown. We assume that it could derive from the following reactions:

$$\text{Cyt-Fe}^{3+} + \text{ROOH} \longrightarrow \text{CytFe}^{2+} + \text{ROO}\cdot$$

$$\text{Cyt-Fe}^{2+} + \text{ROOH} \longrightarrow \text{CytFe}^{3+} + \text{RO}\cdot + \text{HO}-$$

R = lipid or hydrogen.

Graf *et al.*[43] assumed that occupation of all iron coordination sites by a chelator precludes the binding of H_2O_2 to Fe^{2+} and the subsequent Fenton reaction. The observation reported by us and others[5,6] was that occupation of all iron coordination sites does not prevent the interaction of iron valence electrons from eg and t2g orbitals with H_2O_2 or ROOH valence orbitals. However, the availability of a free coordination site is a stringent requirement for H_2O_2 activation of ferric-haemproteins to ferryl species.

In summary, our results demonstrated that most of the KTBA oxidation and membrane lipid peroxidation, in a system containing low concentrations of H_2O_2 and metmyoglobin or methaemoglobin, is catalyzed mostly by ferryl species. Only high concentrations of H_2O_2, 10–30-fold higher than that of the haemproteins, destroyed haem molecules and released iron significantly forming a system by which oxidation is dependent on free iron and HO·.

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