Free Rad. Res. Comms., Vol. 5, No. 1, pp. 11-19
Reprints available directly from the publisher
Photocopying permitted by license only

IRON RELEASE FROM METMYOGLOBIN, METHAEMOGLOBIN AND CYTOCHROME c BY A SYSTEM GENERATING HYDROGEN PEROXIDE†

STELLA HAREL, MENAHEM AVIOR SALAN and JOSEPH KANNER‡

Agricultural Research Organization, The Volcani Center, Department of Food Science, Bet-Dagan, Israel

(Received October 22, 1987; in final form December 18, 1987)

The reaction of H_2O_2 with resting metmyoglobin (MetMb), methaemoglobin (MetHb) and cytochrome-c (Cyt-c) was studied in the Soret and visible regions. The differences between the original and the final peak heights of the native haemproteins at 408 nm was found to be directly proportional to the loss of iron from the molecule. The release of iron from haemproteins was studied in a system generating H_2O_2 continuously at a low rate by an enzymic system, or by addition of large amounts of H_2O_2. Cytochrome-c, methaemoglobin and metmyoglobin during interaction with H_2O_2 at a concentration of 200 μM release 40%, 20% and 3%, respectively, of molecular iron after 10 min. The inhibition of haem degradation and iron release by enzymatically-generated H_2O_2 was determined using several hydroxyl radical scavengers, reducing agents and antioxienzymes, such as superoxide dismutase, catalase and caeruloplasmin.

ABBREVIATIONS: MetMb, metymoglobin; MetHb, methaemoglobin; Cyt-c, cytochrome-c; DMSO, dimethyl sulphoxide; SOD, superoxide dismutase; DETA, diethylenetriaminepenta acetic acid.

INTRODUCTION

The oxidative degradation of the haem molecule *in vivo* requires the participation of an enzyme, haem-oxygenase.[1,2] In addition to haem-oxygenase, molecular oxygen, NADPH (NADH), as well as the microsomal NADPH-Cytochrome c (P-450) reductase, are required for oxidation of the molecule at the α-methene bridge to form biliverdin IXα.[2] Purified NADPH-Cytochrome c (P-450) reductase alone will also degrade haem, but biliverdin is a minor product. The activated oxygen species involved in NADPH-Cytochrome c (P-450) reductase-mediated haem degradation is unknown, but the evidence is strongly suggestive of the involvement of hydrogen peroxide.[2]

It was shown earlier that under certain chemical conditions, such as during ascorbic acid oxidation or in the presence of H_2O_2, haem molecules degrade, forming several by-products.[3,4] It was also found that during this reaction free iron is formed.[5] Haemproteins are destroyed when they are directly incubated with hydrogen peroxide,[4] and the method was adopted by several authors to release iron from myoglobin.[6,7]

†No. 2101-E, 1987 series. This work was supported by the U.S.-Israel Binational Agricultural Research and Development Fund (BARD), grant no. I-801-84.

‡To whom correspondence should be addressed.

Metmyoglobin and methaemoglobin were found to be activated by H_2O_2 into a species, most probably an oxene ferryl, which could initiate membrane lipid peroxidation[8] and other peroxidizing reactions.[9] Recently, it was suggested that the haemglobin molecule is a catalyst for the biological Fenton reaction.[10] This suggestion was rejected by Gutteridge,[11] who believes that iron released from the haem molecule during interaction of those molecules with hydrogen peroxide is the true generator of hydroxyl radicals in this system.

"Free" iron is implicated now as a major factor in the etiology of several degenerative diseases,[12] and the source of such iron is very important for understanding these processes in *in vitro* model systems and *in vivo*.

The purpose of this study was to broaden our knowledge on the mechanism by which H_2O_2 releases iron from haemproteins, in order to understand better the involvement of iron-haem or free iron in several oxidative processes.

MATERIALS AND METHODS

Myoglobin type I from equine skeletal muscle, haemoglobin type I (from bovine), Cytochrome C (horse heart), glucose oxidase (from *Aspergillus niger*), superoxide dismutase (bovine erythrocytes), caeruloplasmin, catalase-free thymol, formic acid sodium salt, dimethylsulphoxide (DMSO), diethylenetriaminepenta-acetic acid (DETA), bovine serum albumin, histidine and imidazole were all obtained from Sigma Chemical Company (St. Louis, MO, USA); EDTA, and glucose were purchased from BDH Chemicals Ltd. (Poole, England); mannitol was obtained from May & Baker Ltd. (Dagenham, England); ferric chloride was purchased from Mallinckrodt Chemical Works (St. Louis, MO, USA); hydrogen peroxide (30% for synthesis), ascorbic acid and trichloroacetic acid were purchased from Merck (Darmstadt, FRG).

Spectral properties of MetMb and Cyt-c upon addition of H_2O_2 or a glucose-glucose oxidase system generating H_2O_2 at a rate of 1 nmole/ml/min, in 50 mM acetate buffer at pH 7.0 were monitored by recording UV-visible absorption in the Soret region (350–450 nm) and visible (450–600 nm) on an Avikon model 810 spectrophotometer combined with an Avikon 21 recorder using serial overlog at 1-min intervals. The reaction was stopped by 400 U of catalase. Ferryl compounds were reduced by the addition of 200 μM potassium ferrocyanide.

Haemproteins treated with Chelax 100 and neutralised to pH 7.0 were separated from low molecular mass compounds by a column of Sephadex 15. The purified haemproteins were incubated with H_2O_2 or a glucose-glucose oxidase system. Iron released from haemproteins was determined by the ferrozine method developed by Carter.[13] The reaction was stopped by ascorbic acid (5 mM) previously treated with Chelex 100.

The results are means of triplicates of two separate experiments; in the figure each error bar (I) denotes the standard deviation.

RESULTS

The reaction of H_2O_2 with resting MetMb and Cyt-c was studied using a scanning double-beam spectrophotometer in the Soret and the visible 450–600 nm regions. The

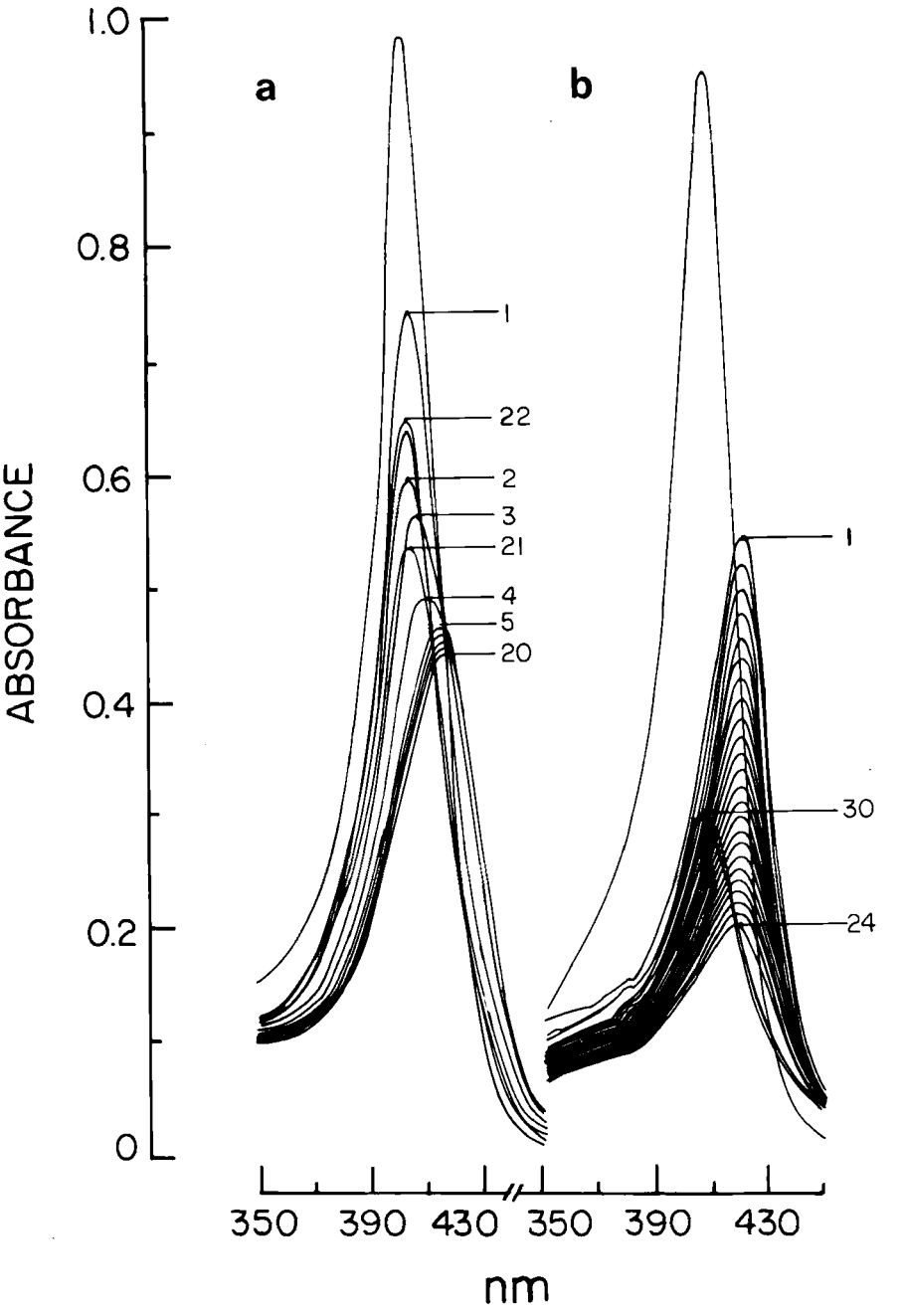

FIGURE 1 Spectral changes in the Soret region of metmyoblobin (7 μM) incubated with H_2O_2. a, 7 μM H_2O_2; b, 500 μM H_2O_2. Ferryl compounds were reduced by the addition of 200 μM potassium ferrocyanide.

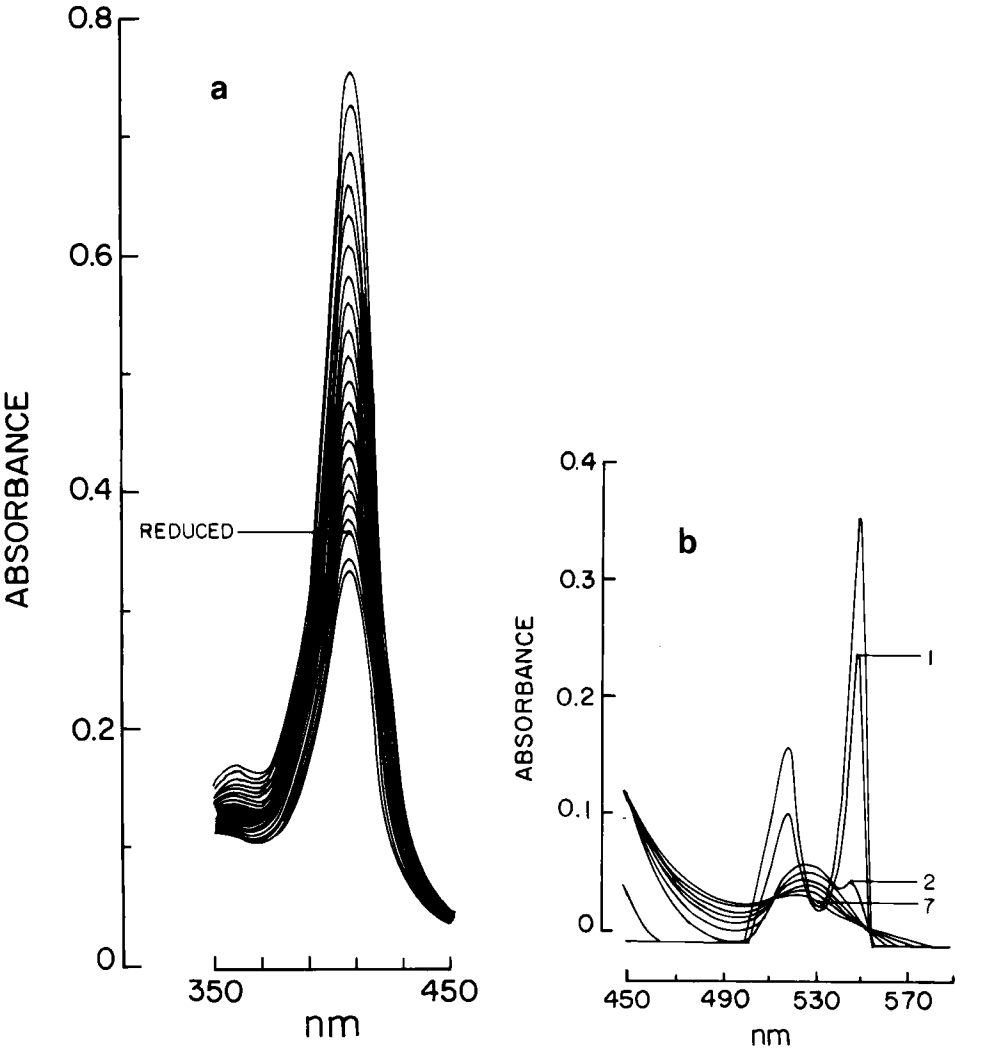

FIGURE 2 Spectral changes in the Soret region of ferric cytochromne c (7 μM) (a); and ferrous cytochrome c (30 μM) (b) incubated with 500 μM H_2O_2.

scan performed was taken at 1-min intervals (Figures 1 and 2). Metmyoglobin in the presence of a low concentration of H_2O_2, slowly formed an intermediate which shows a bathochromic shift from 408 to 420 nm (Figure 1a). Hydrogen peroxide at a high concentration produced very rapidly the metmyoglobin intermediate which lost its absorption with time (Figure 1b). The addition of catalase and potassium ferrocyanide reduced this oxene ferryl intermediate to the native form (Figure 1a min 22), but its absorption remained very low (Figure 1b min 30). Ferric Cyt-c was affected especially by high concentrations of H_2O_2, which did not shift the spectrum of the molecule, but decreased significantly its absorption (Figure 2a). Ferrous cytochrome c interacted rapidly with H_2O_2, producing ferric cytochrome c (Figure 2b). A high

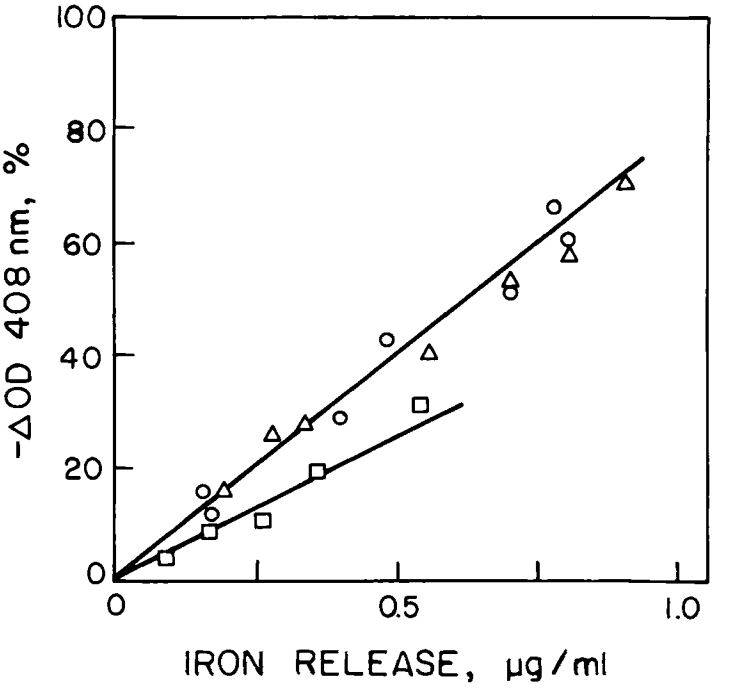

FIGURE 3 Interrelationship between the decrease in haemproteins absorption at 408 nm and iron release by H_2O_2 generated enzymatically. The system contained glucose-glucose oxidase, which generates H_2O_2 at a rate of 1 nmole/ml/min in 50 mM acetate buffer, pH 7.0, and 25°C. The concentration of each haemprotein was 30 μM.
○, MetMb; △, MetHb; □, Cyt-c.

concentration of H_2O_2 decreased also the absorption of Cyt-c at 528 nm (Figure 2b). The intermediate formed during the incubation of metmyoglobin and H_2O_2 shows absorption in the visible region at 549 and 586 nm. In the presence of a high concentration of H_2O_2, the absorption of those peaks decreases (results not shown). As the extinction coefficient of metmyoglobin and Cyt-c at the Soret region is almost 5–10-fold higher than that of the visible region, the Soret region was chosen for studying the correlation between spectrophotometric changes and the release of iron from the molecule.

The difference between the original and the final peak heights of the native haemproteins at 408 nm was found to be directly proportional to the loss of iron from the molecule (Figure 3). Methaemoglobin incubated in an enzymatic system of glucose-glucose oxidase, which generated H_2O_2 at a low rate of 1 nmole/ml/min, degraded and released iron at a rate which was higher than that of MetMb or Cyt-c (Figure 4). However, when a solution of H_2O_2 was added directly by one step to the haemproteins, the release of iron was more rapid from Cyt-c and MetHb than from MetMb. Cyt-c and MetHb during interaction with H_2O_2 at a concentration of 200 μM after 10 min released iron in very significant amounts (Figure 5).

Metmyoglobin and Cyt-c degradation, during incubation with H_2O_2 generated enzymatically, was inhibited by several reducing agents, such as uric acid, thiourea

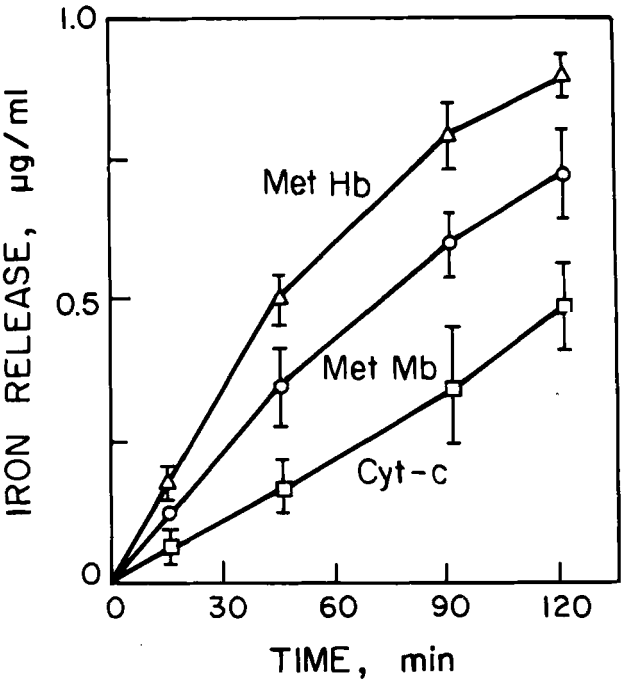

FIGURE 4 Iron release from haemproteins incubated in a system with H_2O_2 generated enzymatically (for conditions, see Fig. 3).
○, MetMb; △, MetHb; □, Cyt-c.

and ascorbic acid (Table 1). The degradation of these haemproteins was also inhibited by histidine, imidazole and caeruloplasmin. Caeruloplasmin was found to inhibit haemproteins degradation and iron release (results not shown), also if the enzyme was autoclaved for 10 min at 120°C. Several hydroxyl radical scavengers, such as formate, DMSO, mannitol, and chelators such as DETA or EDTA (results not shown) failed to inhibit haemproteins degradation by H_2O_2. Glucose oxidase was not affected by any of the reagents tested in our study.

DISCUSSION

Haemproteins are destroyed when they are directly incubated with hydrogen peroxide.[3,4,11] The incubation of MetMb (and MetHb, results not shown) with low or high concentrations of H_2O_2 produced only one intermediate, most probably the oxene-ferryl MetMb, which absorbed light at 420 nm. In contrast with our results, Jenzer *et al.*[14] found recently that lactoperoxidase was activated by a low concentration of H_2O_2 to compound I and compound II; however, a high concentration of H_2O_2 produced also compound III, which is the oxy-ferrous peroxidase. Incubation of MetMb and H_2O_2 destroyed the haem molecule which was recorded by a decrease in the absorbance of 420 nm and by a decrease in the ability of the molecule to be reduced with potassium ferrocyanide to the native ferric myoglobin. As the absorption at 408 nm is affected by the bonding of iron to haem structure, it is not surprising

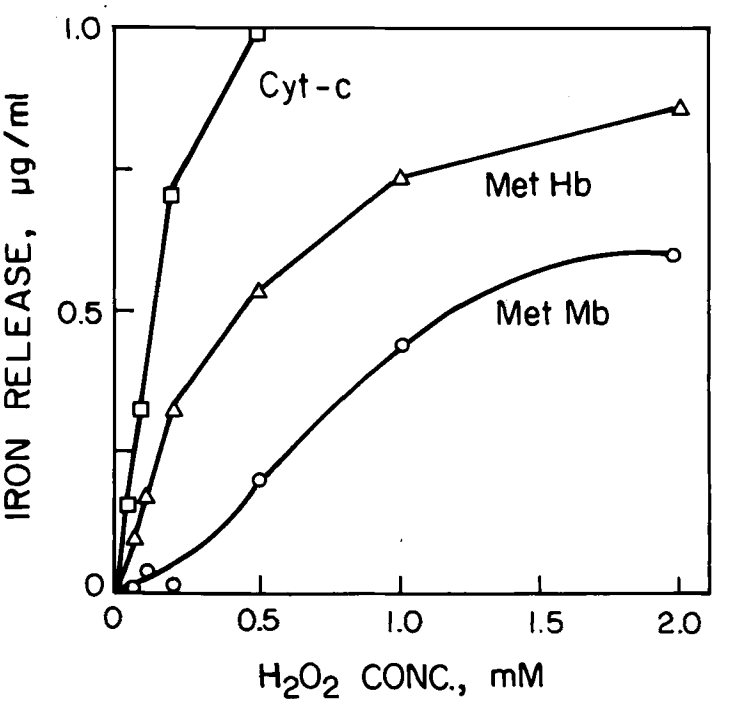

FIGURE 5 Iron release from haemproteins incubated with added H_2O_2. The system contained haemproteins (30 μM) in 50 mM acetate buffer pH 7.0 and 25°C, incubation time – 10 min.
○, MetMb; △, MetHb; □, Cyt-c.

that the decrease in Soret band absorption of MetMb correlates well with iron release from the molecule. A relatively similar correlation was found between the decrease in Soret region absorption of Cyt-c and iron release.

The mechanism by which H_2O_2 released iron from both molecules seems to be

TABLE I
The effects of hydroxyl radical scavengers, reducing agents and antioxienzymes on metmyoglobin and cytochrome c deterioration by H_2O_2 generated enzymatically

Treatment	*MetMb*		*Cyt-c*	
	– A 408 nm/30 min	Inhibition (%)	– A 408 nm/30 min	Inhibition (%)
Control	0.365 ± 0.05	0	0.135 ± 0.05	0
Formate (50 mM)	0.365	0	0.135	0
DMSO (200 mM)	0.365	0	0.135	0
Mannitol (100 mM)	0.365	0	0.135	0
Histidine (25 mM)	0.170	53	0.040	70
Imidazole (25 mM)	0.050	87	0	100
Uric acid (10 mM)	0.260	22	0.095	30
Thiourea (10 mM)	0.080	76	0.050	62
Ascorbic Acid (5 mM)	0.080	76	0	100
SOD (100 U)	0.335	8	0.132	0
Ceruloplasmin (30 U)	0.190	43	0.007	46
Catalase (400 U)	0	100	0	100

different. We assume that the degradation of haem structure and iron release from MetMb and MetHb is effected mostly by oxene-ferryl species and those of Cyt-c by HO·. Hydrogen peroxide, which activated MetMb to oxene-ferryl species,[15] seems to attack and oxidize the edge haem of a second molecule, producing a porphyrin radical. The possible interaction between two haem molecules, such as Cyt-c peroxidase and Cyt-c, is well known.[16]

The iron in Cyt-c is bound covalently to the polypeptide chain by all six coordinations. Iron Cyt-c interacts with H_2O_2[17–19] without producing an intermediate compound I. This was also demonstrated in our study, in which H_2O_2 did not shift but rapidly decreased the spectrum of ferric Cyt-c at 408 or 528 nm. Ferrous cytochrome-c was also found to be oxidized very rapidly by H_2O_2. We suggest that ferric Cyt-c interacts with H_2O_2, producing HO· by the following reactions:

$$\text{Cyt-c } Fe^{+3} + H_2O_2 \rightarrow \text{Cyt-c } Fe^{+2} + O_2^{\cdot-} + 2H^+ \quad (1)$$

$$\text{Cyt-c } Fe^{+3} + O_2^{\cdot-} \rightarrow \text{Cyt-c } Fe^{+2} + O_2 \quad (2)$$

$$\text{Cyt-c } Fe^{+2} + H_2O_2 \rightarrow \text{Cyt-c } Fe^{+3} + HO\cdot + HO^- \quad (3)$$

$$\text{Cyt-c haem} + HO\cdot \rightarrow \text{Cyt-c porphyrin radical} + HO^- \quad (4)$$

In our model systems, a small amount of H_2O_2 was generated continuously by an enzymic system, or by the addition of large amounts of H_2O_2 (50–500 μM). In both systems, Cyt-c and other haemproteins were destroyed and free iron was released. However, in the enzymic system which generates H_2O_2 at a low level MetHb and MetMb were destroyed more rapidly than Cyt-c, whereas in the system containing large amounts of H_2O_2, Cyt-c was destroyed and iron released more rapidly than this from MetMb or MetHb.

It was found than the reaction of small amounts of hydroxyl radicals (produced by γ-radiation) with ferric Cyt-c was fast and led spectroscopically to the reduction of the iron, most probably by an electronic tunnelling process; however, excess of HO· led to the degradation of Cyt-c.[20] These results could explain the differences obtained in our model system between the degradation of Cyt-c by small or large amounts of H_2O_2. At low H_2O_2 concentration, HO· is generated at a low level by Cyt-c; these species, in part, reduce but also oxidise the molecule. However, at a high H_2O_2 level, oxidation is the dominant process.

It seems that Cyt-c oxidation and degradation by HO· are site-specific, formed in the endogenous region of haem molecule, since the hydroxyl radical scavengers used did not efficiently prevent iron release. The inability of HO· scavengers to inhibit iron release could be explained also if it is assumed that H_2O_2 itself interacts with the haem edge molecule.[11] As the haem edge molecule is not known to work as a good electron donor, we believe that if such a reaction occurs its rate will be very slow.

Histidine, imidazole and several reducing agents, such as uric acid, thiourea and ascorbic acid, were found to inhibit haem degradation and iron release (Table 1). We assume that these results were obtained by the interaction of the reducing agents with H_2O_2-activated haemproteins[9,21] and/or with the oxidised haem edge, preventing the breakdown of the porphyrin ring and the release of iron.

We do not have a reasonable explanation for the differences in iron release between MetMb and MetHb. Caeruloplasmin was found to inhibit the degradation of MetMb and Cyt-c by H_2O_2. As an autoclaved enzyme inhibits haem degradation, very similarly to those unheated enzymes, the activity of caeruloplasmin is not attributed

to an enzymic, but to a possible masking or antioxidant effect of the protein. As EDTA and DETA do not affect haemprotein degradation in the presence of H_2O_2, it is concluded that in our systems free iron is not involved in the breakdown of the haem molecule.

Summing up, MetMb, MetHb and other haemproteins bonded to iron by five coordinations interact with H_2O_2, forming a complex which activates iron to a ferryl species. This active species seems to attack other haem edge molecules, producing a porphyrin radical which destabilises the molecule.

In contrast, Cyt-c protein is bound to iron by all six coordinations, which prevents the complexing of H_2O_2 or O_2 by the molecule. The valence Cyt-c iron electrons interact with H_2O_2 producing HO· which attacks the haem pocket from the endogenous region of the molecule. This oxidation seems to be sterically protected from the interference of HO· scavenger molecules. The interaction of low concentrations of H_2O_2 generated continuously in injured cells or tissues, could release iron from haemprotein molecules, but only if the pool of reducing agents is very low or the reaction is affected by a compartmentalisation environment which prevents the reaction of H_2O_2-activated haemproteins with reducing agents.

References

1. Tenhunen, R., Marver, H.S. and Schmid, R. *J. Biol. Chem.*, **244,** 6288–6394, (1969).
2. Dochert, J.C., Firneisz, G.D. and Schacter, B.A. *Arch. Biochem. Biophys.*, **235,** 657–664, (1984).
3. Lemberg, R., Legge, J.W. and Lockwood, W.H. *Biochem. J.*, **35,** 339–352, (1941).
4. Cantoni, L., Gibbs, A.H. and De Matteis, F. *Int. J. Biochem.*, **13,** 823–830, (1981).
5. Legge, J.W. and Lemberg, R. *Biochem. J.*, **35,** 353–362, (1941).
6. Lin, H.P. and Watts, B.M. *J. Food Sci.*, **35,** 596–598, (1970).
7. Igene, J.D., King, J.A., Pearson, A.M. and Gray, J.I. *J. Agric. Food Chem.*, **27,** 838–842, (1979).
8. Kanner, J. and Harel, S. *Arch. Biochem. Biophys.*, **237,** 314–324, (1985).
9. Kanner, J. and Harel, S. *Lipids*, **20,** 625–628, (1985).
10. Sadrzadeh, S.M., Graf, E., Panter, S.S., Hallaway, P.E. and Eaton, J.W. *J. Biol. Chem.*, **259,** 15354–15356, (1984).
11. Gutteridge, J.M.C. *FEBS Letters*, **201,** 291–295, (1986).
12. Halliwell, B. and Gutteridge, J.M.C. *Molec. Aspects Med.*, **8,** 89–193, (1985).
13. Carter, P. *Anal. Biochem.*, **40,** 450–458, (1971).
14. Jenzer, H., Jones, W. and Kohler, H. *J. Biol. Chem.*, **261,** 15550–15556, (1986).
15. Chance, M., Powers, L., Kumor, C. and Chance, B. *Biochemistry*, **25,** 1259–1265, (1986).
16. Yonetani, T. and Schleyer, H. *J. Biol. Chem.*, **242,** 1974–1979, (1967).
17. Vandewalle, P.L. and Petersen, N.O. *FEBS Letters*, **210,** 195–198, (1987).
18. Koppenol, W.H. *J. Free Rad. Biol. Med.*, **1,** 281–285, (1985).
19. Florence, T.M. *J. Inorg. Biochem.*, **23,** 131–141, (1985).
20. Koppenol, W.H. and Butler, J. *Israel J. Chem.*, **24,** 11–16, (1984).
21. Shiga, T. and Imaizumi, K. *Arch. Biochem. Biophys.*, **167,** 469–479, (1975).

Accepted by Dr B. Halliwell