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HAEMOGLOBIN AND MYOGLOBIN AS INHIBITORS OF HYDROXYL RADICAL GENERATION IN A MODEL SYSTEM OF "IRON REDOX" CYCLE

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Methionine was oxidized to ethylene by an "Iron Redox" system containing H_2O_2, Fe-EDTA and ascorbate, generating hydroxyl radicals or another species of similar reactivity. Oxy or met forms of haemoglobin and myoglobin were found to inhibit methionine oxidation. Methionine oxidation was elevated in the "Iron Redox" system by increasing ascorbic acid concentration. However, in the presence of metmyoglobin or methaemoglobin, the increases in ascorbic acid did not lower the haemproteins' inhibitory effects but rather increased them.

The pro-oxidative or anti-oxidative activities of haemproteins in biological oxidative reactions seem to be dependent on compartmentalization and on the presence and concentrations of reducing compounds and H_2O_2.

KEY WORDS: Haemoglobin, myoglobin, hydroxyl radical, ascorbic acid, ferric chloride, hydrogen peroxide.

INTRODUCTION

Oxygen species such as superoxide, hydrogen peroxide and hydroxyl radical, or species of similar reactivity are implicated by many authors as important causative agents of aging and of several human diseases.[1,2]

Hydroxyl radical species are frequently considered one of the most important cytotoxic species generated in biological tissues.[1-5] The generation of this radical seems to occur by a reaction dependent on transition metal ion complexes and H_2O_2 via the following mechanism (5-7):

$$Fe^{+2}(\text{complex}) + H_2O_2 \longrightarrow Fe^{+3}(\text{complex}) + HO^- + HO\cdot \qquad [1]$$

This reaction is cycled by O_2^- (Haber-Weiss) or by raducing agents, e.g. ascorbic acid (Redox-Cycle). Haemoglobin and myoglobin are molecules of great importance in the metabolism of oxygen in our organism. The involvement of haemoglobin as a catalyzer of oxidative reactions was first described by Robinson[8] in 1924, who found that the haemprotein accelerates the peroxidation of unsaturated fatty acids. Typical lipid peroxidation by haem compounds exhibits an induction period which was postulated by Banks[9] to be dependent on preformed hydroperoxides. Haemproteins such as haemoglobin and myoglobin accelerate the decomposition of hydroperoxides[10] to free radicals which, in the presence of oxygen, propagate lipid peroxidation. Haemoglobin and myoglobin are also known as hydrogen peroxide decomposers.[11] The interaction

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of oxymoglobin[12] or metmyoglobin with H_2O_2 generates a ferryl-myoglobin.[12,13] A similar reaction occurs between oxyhaemoglobin or methaemoglobin with H_2O_2.[12] Ferrylmyoglobin or haemoglobin which contain tetravalent iron[13] could oxidize many molecules such as membrane fatty acids,[14] or free fatty acids,[15] β-carotene and methional,[16] KTBA,[17] desferrioxamine,[18] proteins[19] or reducing compounds ,[16] but not methionine.[17] The reaction of haemoglobin or myoglobin with H_2O_2 in the presence of reducing compounds resembles peroxidase activity, a reaction which was described in the literature.[11,18,20,21]

Sadrzadeh *et al.*[22] postulated that haemoglobin in the presence of $O_2^{\cdot-}$ or H_2O_2 promotes hydroxyl-radical formation, acting as an effective biological Fenton raction. This work was recently criticized by several authors who suggested that iron released from haemoglobin during the interaction with a high concentration of H_2O_2 was the true generator of hydroxyl radicals in a model system containing these reagents.[23,24,25] Most recently, it was found that oxy or met forms of haemogobin or myoglobin in the presence of a low concentration of H_2O_2 form the ferryl species and not free hydroxyl radicals.[17]

In this study we examined how haemoglobin and myoglobin in both oxy and met forms affect the production of hydroxyl radicals in a model system of iron-dependent "Redox Cycle" reaction.

In contrast with the Eaton group,[22] who postulated that oxyhaemoglobin in the presence of H_2O_2 and ascorbic acid could work as an efficient biological Fenton reagent, we will show that oxy and met forms of haemoglobin or myoglobin in the presence of ascorbic acid are inhibitors of hydroxyl radical generation.

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), catalase-free thymol, and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). EDTA and glucose were purchased from BDH Chemicals Ltd. (Poole, England); ferric chloride form Mallinckrodt Chemical Works (St. Louis, MO, USA); and hydrogen peroxide (30% for synthesis) from Merck (Darmstadt, W. Germany).

Haemproteins treated with Chelex 100 and neutralized to pH 7.0 were separated from low molecular weight compounds on a column of Sephadex G-15. Oxymyoglobin and oxyhaemoglobin were produced by reducing the methaemproteins by dithionite under aerobic conditions and purified by gel filtration on a column of Sephadex G-15.

Degradation of methionine 10 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 reaction mixture contained, in a final volume of 10 ml, the following reagents added in that order to give the final concentration: acetate buffer (50 mM), pH 6.5; glucose (4 mM); methionine (10 mM); EDTA (33 μM); ascorbic acid (200 μM); $FeCl_3$ (30 μM); glucose oxidase injected for generation of H_2O_2 at a rate of 2 nmole/ml/min, at 25°C.

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 form a standard sample

Spectral properties of myoglobin and haemoglobin were monitored by recording UV-visible absorption in the Soret region (350–450 nm) and visible region (450–600 nm) on a Uvikon model 810 spectrophotometer combined with a Uvikon 21 recorder using serial overlap at 1-min intervals.

The reaction was stopped by adding 400 U catalase and the ferryl compounds were reduced by potassium ferrocyanide or by ascorbic acid.

Peroxidase activity was determined spectrophotometrically by a method based on the peroxidation of pyrogallol to purpurogallin. The absorbance changes at 300 nm were recorded by a DB Varian spectrophotometer. The reaction was determined in acetate buffer 50 mM, at pH 6.5 and 25°C.

Ascorbic acid oxidation by ferric ions with and without EDTA was monitored by its absorbance at 265 nm. The 50 mM acetate buffer at pH 6.5 was treated with Chelex 100 to remove transition metal impurities. Reaction of ferryl myoglobin (7 μM – 7 μM H_2O_2) with ascorbate in acetate buffer at pH 6.5, was determined by recording spectral changes at 426 nm. The results are means of triplicates of two separate experiments. In the figure, each error bar (I) denotes the standard deviation.

RESULTS

The "Iron Redox" system containing H_2O_2 generated enzymatically, produced hydroxyl radicals[17,27] which oxidize methionine to ethylene. Increasing the amount of H_2O_2 in this system, by elevating the amount of glucose oxidase, increased methionine oxidation (Figure 1). Glucose was used at a concentration of 4 mM which is far from these concentrations (200–400 mM) which could efficiently scavenge HO· in competition with methionine. The oxidation of methionine by the active "Iron Redox" system was inhibited effectively by metmyoglobin (Figure 1). The generation of active species by the "Iron Redox" system was also inhibited by methaemoglobin and only slightly by ferric cytochrome c (Figure 2). Metmyoglobin, methaemoglobin and ferric cytochrome c at a concentration of 5 μM haem inhibit ethylene generation in the "Iron Redox" system by 84%, 45% and 0%, respectively.

One could assume that the inhibitory effect of metmyoglobin or methaemogoblin is connected with a rapid depletion of ascorbic acid by the H_2O_2-activated metmyglobin. In order to evaluate this assumption, we carried out an experiment to determine methionine oxidation by increasing the concentration of ascorbic acid, with and without metmyoglobin.

The results showed (Figure 3) that elevating the levels of ascrobic acid in our reaction increased methionine oxidation. However, these increases in ascorbic acid did not lower the inhibitory effects of metmyoglobin but rather increased them.

Methionine oxidation by the "Iron Redox" system and the inhibition by metmyoglobin were not affected by pH between 6.5 and 8.0 (results not shown).

The peroxidative activities of metmyoglobin, methaemoglobin and ferric-cytochrome c were determined and compared with horseradish peroxidase. It was found that peroxidase activity was almost 10^5-fold higher than that of metmyoglobin or haemoglobin (Figure 4). and fit the results reported by others.[33]

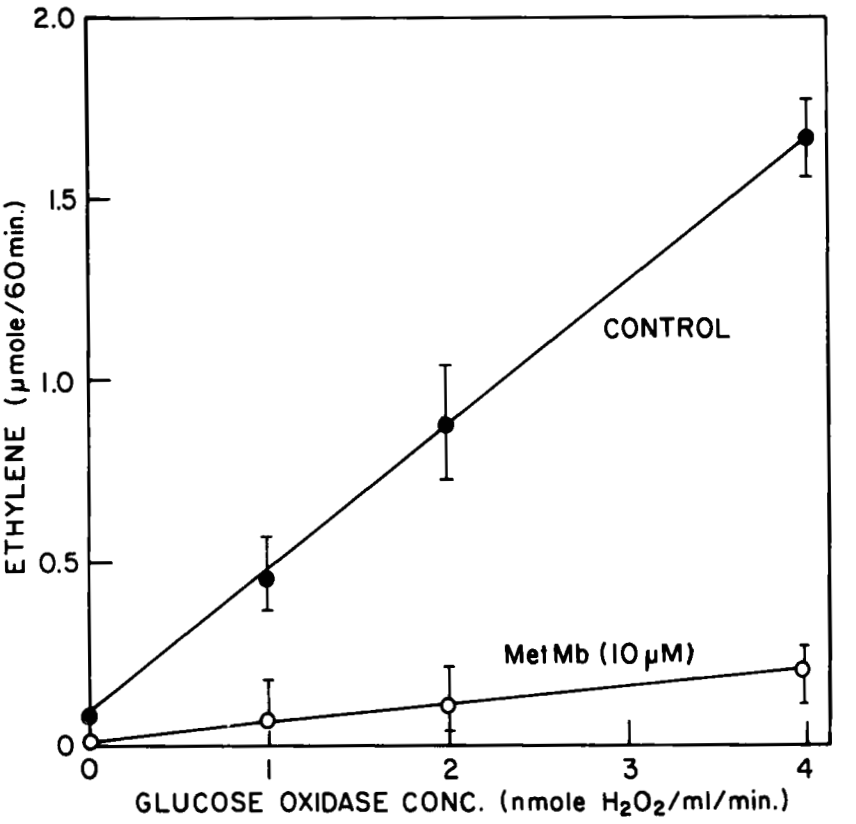

FIGURE 1 The effect of glucose oxidase concentration on methionine oxidation by the "Iron Radox" system with and without metmyoglobin. control: EDTA (33 μM), $FeCl_3$ (30 μM), ascorbic acid (200 μM). ●; as control but in the presence of 10 μM MetMb, ○; for other conditions see Table 1.

TABLE I
Inhibition of methionine oxidation in an "Iron Redox" system by oxyhaemoglobin and albumin

	Oxyhaemoglobin concentration (μM)	Ethylene evolution (μmole/60 min)
Control	0	0.94
	1.5	0.40
	3.0	0.25
	6.0	0.15
	12.0	0.05
Albumin (0.5 mg/ml)		0.85

The reaction mixture contained, in a final volume of 10 ml, the following reagents added in that order to give the final concentration: acetate buffer (50 mM), pH 6.5; glucose (4 mM); methionine (10 mM); EDTA (33 μM); ascorbic acid (200 μM); $FeCl_3$ (30 μM); glucose oxidase injected for generation of H_2O_2 at a rate of 2 nmole/ml/min, at 25°C.

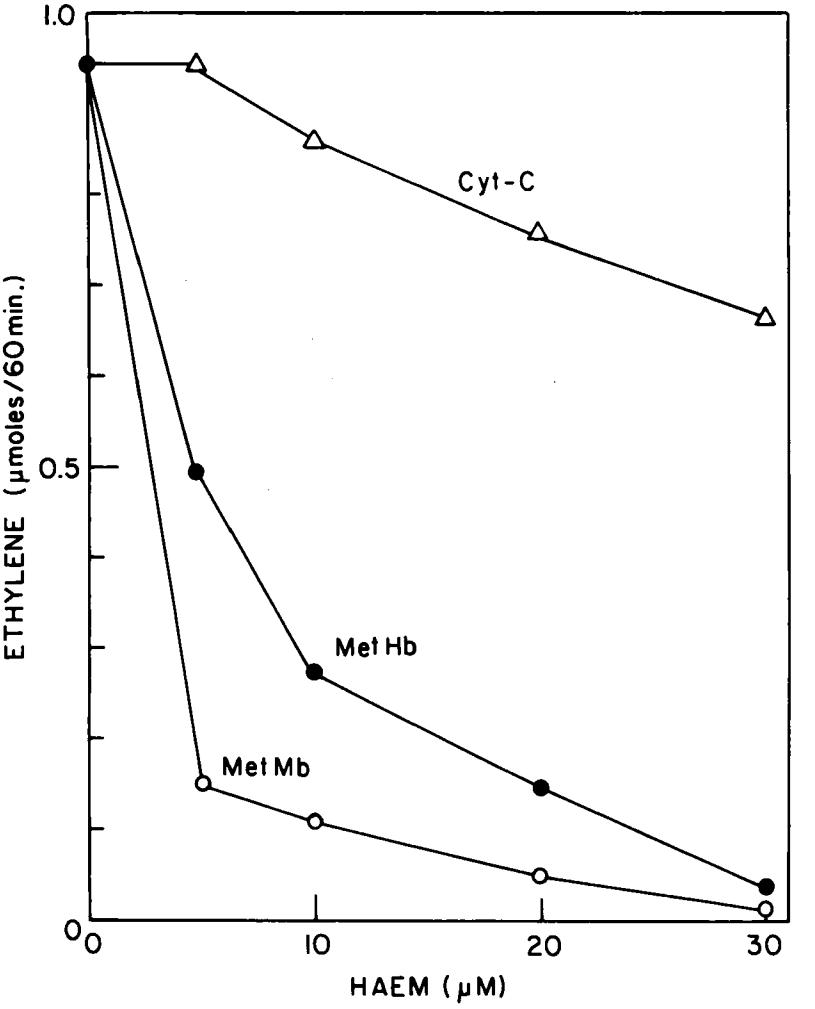

FIGURE 2 The effect of haem concentration on methionine oxidation by the "Iron Redox" system (for conditions, see Table 1).

DISCUSSION

Hydrogen peroxide at a low level is formed in all aerobic cells.[28] In response to special stimuli, several leukocytes (neutrophils, macrophages) undergo an oxidative burst and produce superoxide and, indirectly, hydrogen peroxide.[3] In contrast to superoxide, which diffuses out of cells only through anion channels, hydrogen peroxide can easily cross the cellular membrane and is likely to be responsible, in part, for intra- and intercellular damages. In the presence of H_2O_2, certain transition metals will catalyze the formation of highly reactive species such as hydroxyl radicals[6,7] or species of similar reactivity .[29,30] These highly reactive species are implicated in the

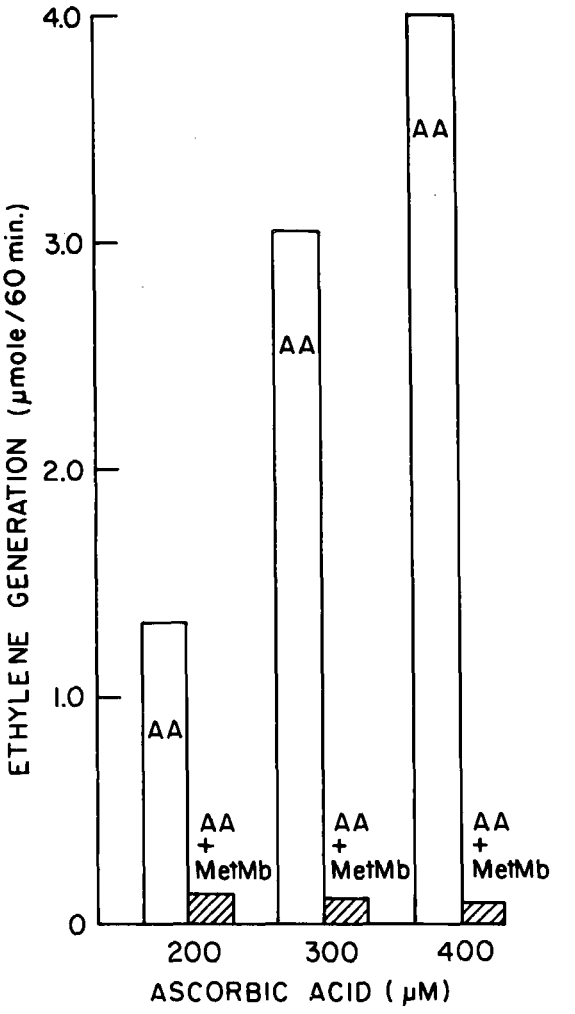

FIGURE 3 The effect of ascorbic acid concentration on methionine oxidation by the "Iron Redox" system (for conditions, see Table 1: metmyoglobin concentration was 10 μM).

oxidative stress of many biological systems and in the etiology of several degenerative diseases, aging, and cancer.[31,32]

In our system, hydroxyl radicals or another species of similar reactivity were generated by reactions [2] and [3]. In this system, however, other reactions are involved.[4–6]

$$Fe^{+3}EDTA + AH_2 \longrightarrow Fe^{+2}EDTA + AH\cdot + H^+ \quad [2]$$

$$Fe^{+2}EDTA + H_2O_2 \longrightarrow Fe^{+3}EDTA + HO^- + HO\cdot \quad [3]$$

$$Fe^{+2}EDTA + O_2 \rightleftharpoons Fe^{+3}EDTA + O_2^{\cdot-} \quad [4]$$

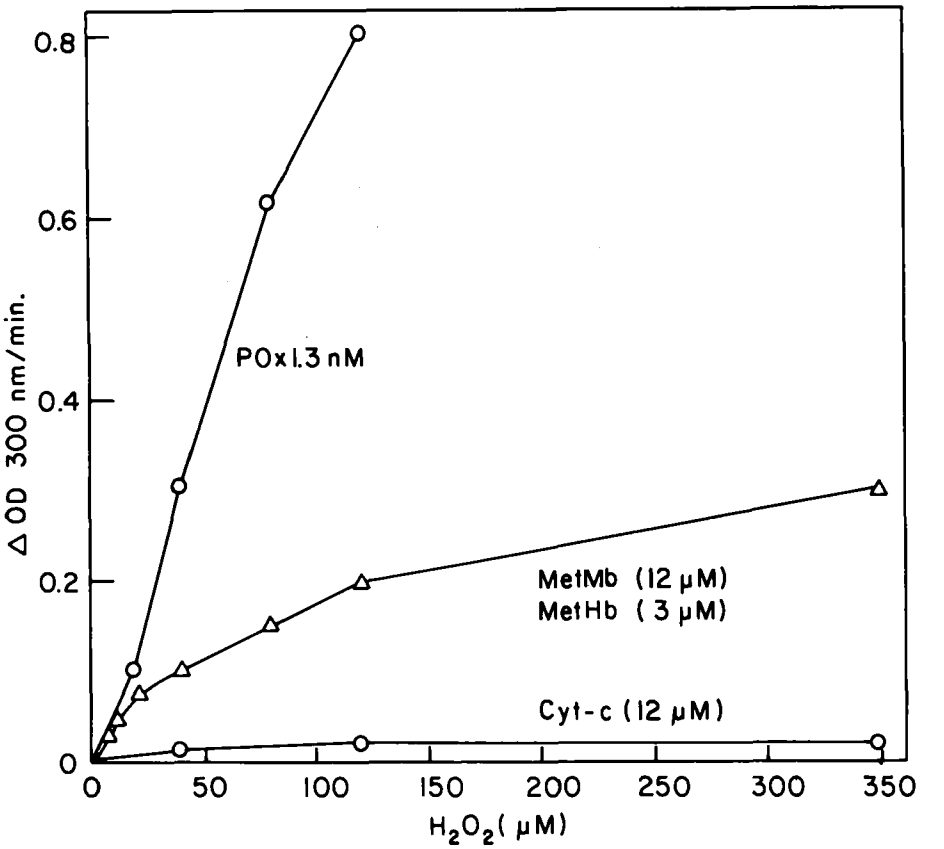

FIGURE 4 Pyrogallol oxidation by horseradish peroxidase and haemproteins as affected by H_2O_2 concentration. The reaction mixture contained pyrogallol (3.2 mM) in 2.5 ml of acetate buffer (50 mM), pH 6.5, at 25°C.

$$AH\cdot + O_2 \longrightarrow A + H^+ + O_2^- \quad [5]$$

$$AH\cdot + O_2^- - H+ \longrightarrow A + H_2O_2 \quad [6]$$

Reactions [2]–[6] represent the system which efficiently oxidizes methionine to ethylene. Introducing oxy or met form of myoglobin and haemoglobin at a concentration of one-sixth of that of Fe^{+3}EDTA efficiently inhibited methionine oxidation to ethylene. This was done by the sum reaction [7]–[10]. The rate constant of several reactions are known,[33] others were measured at a condition different from ours, or are not known.

$$\text{Fe}^{+3} + H_2O_2 \longrightarrow \text{Fe}^{+4} = O + H_2O \quad [7]$$

$$\text{Fe}^{+4} = O + AH_2 \longrightarrow \text{Fe}^{+3} + A + H_2O \quad [8]$$

$$\text{Fe}^{+4} = 0 + Fe^{+2}EDTA \xrightarrow{H^+} \text{Fe}^{+3} + Fe^{+3}EDTA + HO^- \quad [9]$$

$$\text{Fe}^{+3} + \text{Fe}^{+2}\text{EDTA} \rightleftharpoons \text{Fe}^{+2} + \text{Fe}^{+3}\text{EDTA} \quad [10]$$

For this reason, it was not possible to predict a specific reaction which especially works to inhibit hydroxyl radicals generation.

We suggest that haemoglobin and myoglobin in the presence of ascorbic acid work efficiently to eliminate H_2O_2, as a peroxidase (reactions [7] and [8]), thus preventing the generation of hydroxyl radicals in a system adopted by many researchers to simulate in vivo cytotoxicity.[34,35,36]

The effective inhibitory reaction of oxymyoglobin or oxyhaemoglobin seems to derive from reactions described in equations [11]–[13]. It seems

$$\text{Fe}^{+2}\text{-}O_2 \xrightarrow[2H+]{H_2O_2} \text{Fe}^{+4} + 2H_2O \quad [11]$$

$$\text{Fe}^{+2}\text{-}O_2 \rightleftharpoons \text{Fe}^{+2} + O_2 \quad [12]$$

$$\text{Fe}^{+2} + O_2 \rightleftharpoons \text{Fe}^{+3} + O_2^{\cdot-} \quad [13]$$

that H_2O_2 catalyzes the oxidation of oxyhaem to ferryl compound[12]; however, during a long incubation period a part of oxyhaem could also auto-oxidize to met form which interacts with H_2O_2, as described (reactions [7] and [8]).

It is also possible that the high efficiency of haemoglobin or myoglobin to prevent HO· radical formation derived also from the reaction between ferrous-EDTA and ferryl haemproteins or ferric haemproteins (reactions [9] and [10]).

Reaction [10] seems to be very inefficient, since ferricytochrome c inhibits only slightly methionine oxidation to ethylene.

Our results could explain several observations by the Rice-Evans group[36] who came to the conclusion that under iron-mediated oxidative stress in erythrocytes, methaemoglobin plays a role in decreasing the susceptibility of the membranes to oxidation.

Hydrogen peroxide was used by many researchers to induce haemolysis and membrane lipid peroxidation of erythrocytes.[36,37,38] Recently, it was found that a high concentration of ascorbic acid could prevent this phenomenon.[38] Several hypotheses were postulated by the researchers to explain the antioxidant action of ascorbic acid, such as direct free radical scavenging or regeneration of α-tocopherol, but it is also possible that haemglobin in the presence of large amounts of ascorbic acid works catalytically to decompose hydrogen peroxide, thus preventing the generation of hydroxyl radicals and membrane destruction.

Hydrogen peroxide reacts with metmyoglobin with a rate constant of $1.4 \times 10^2 M^{-1}S^{-1}$, compared with rate constants of $(1 - 5) \times 10^7 M^{-1}S^{-1}$ for peroxidases.[33] These data fit our results very well (Figure 4). However, the amount of the peroxidases in biological systems is in the range of pM–nM, and that of myoglobin and haemoglobin in the range of μM–mM.

As haemoglibin and myoglobin in several cells are present in relatively high concentrations, they seem to work efficiently as H_2O_2 decomposers. Several other studies also implicate haemproteins, especially at high concentration, as inhibitors of lipid peroxidation.[39–43]

Previously, we had considered that haemoglobin and myoglobin could be activated by H_2O_2 to a species which stimulates several harmful oxidative reactions.[14,16] This work was supported recently by other researchers.[15,44] Several studies show that haemoglobin and myoglobin in the presence of H_2O_2 release iron,[23,24] which could

further interact with H_2O_2 to produce hydroxyl radicals. Our recent data show that reducing agents decrease the process of iron release from haemproteins by H_2O_2.[25] This work demonstrated that in the presence of reducing agents, haemoglobin and myoglobin act to inhibit pro-oxidative effects of an H_2O_2-iron-redox system.

The data presented by us previously and herein have implicated haemproteins, such as haemoglobin and myoglobin, in several oxidative reactions as pro-oxidants or antioxidants. The special nature of their activities seems to be induced by compartmentalization and the concentration of the haemproteins, H_2O_2 and reducing agents.

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