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FORMATION OF HYDROXYL RADICALS IN BIOLOGICAL SYSTEMS. DOES MYOGLOBIN STIMULATE HYDROXYL RADICAL FORMATION FROM HYDROGEN PEROXIDE?

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Incubation of horse-heart oxymyoglobin or metmyoglobin with excess H_2O_2 causes formation of myoglobin(IV), followed by haem degradation. At the time when haem degradation is observed, hydroxyl radicals (.OH) can be detected in the reaction mixture by their ability to degrade the sugar deoxyribose. Detection of hydroxyl radicals can be decreased by transferrin or by .OH scavengers (mannitol, arginine, phenylalanine) but not by urea. Neither transferrin nor any of these scavengers inhibit the haem degradation. It is concluded that intact oxymyoglobin or metmyoglobin molecules do not react with H_2O_2 to form .OH detectable by deoxyribose, but that H_2O_2 eventually leads to release of iron ions from the proteins. These released iron ions can react to form .OH outside the protein or close to its surface. Salicylate and the iron chelator desferrioxamine stabilize myoglobin and prevent haem degradation. The biological importance of .OH generated using iron ions released from myoglobin by H_2O_2 is discussed in relation to myocardial reoxygenation injury.

KEY WORDS: Myoglobin, haem, hydroxyl radical, hydrogen peroxide, ischaemia/reperfusion.

INTRODUCTION

It now seems to be established (for reviews see[1,2]) that formation of hydroxyl radical (.OH), or a similar highly-oxidizing species, accounts for much of the damage done to living systems by increased intracellular generation of superoxide radicals (O_2^-) and H_2O_2. Formation of hydroxyl radicals from H_2O_2 requires the presence of a transition metal ion; particular attention has been paid to low-molecular-mass iron ion complexes and to iron proteins as potential promoters of .OH formation *in vivo*.[1,2] The abilities of iron bound to ferritin,[3] haemosiderin,[4] lactoferrin[5] and transferrin[5] to catalyze .OH formation have been studied. Detailed studies upon haemoglobin have also been performed.[6,7] From these, it has been concluded that neither oxyhaemoglobin nor methaemoglobin can react with H_2O_2 to form .OH that is detectable "in free solution". Rather, the H_2O_2 damages the haem ring of the protein, releasing iron ions that can react with H_2O_2 outside the protein, or on its surface, to form .OH.[6,7] However, it was also found that the intact oxyhaemoglobin molecule can apparently react with H_2O_2 to form an oxidant that is not identical to .OH.[7] Formation of this additional oxidant was observed in reaction mixtures containing mammalian oxyhaemoglobin and H_2O_2, but not when soybean oxyleghaemoglobin, a monomeric protein, was incubated with H_2O_2.[8]

Myoglobin is also a potential candidate for .OH formation *in vivo*. For example, it can be released into the circulation from muscle cells after prolonged exercise.[9] Reperfusion of heart after ischaemia causes a "reoxygenation damage" that appears to involve O_2^-, H_2O_2 and .OH.[10] The source of the iron catalyst of .OH generation is unclear, and it is possible that cardiac myoglobin might provide it. Myoglobin has long been known to accelerate the peroxidation of membrane lipids,[1,11–13] although there is some debate as to whether it might act by forming .OH or by producing other reactive iron/oxygen complexes.[12,13] Several studies of the reaction between myoglobin and H_2O_2 have been reported[14–16,33] but none has addressed the question of .OH formation by using direct experimental measurements of this radical.

Because of the potential biological importance of free radical generation promoted by myoglobin,[1,2,11] particularly in relation to cardiac reperfusion injury,[10] we have carried out a detailed study of the ability of both oxymyoglobin and metmyoglobin to form .OH from H_2O_2. Hydroxyl radical was measured by its ability to degrade the sugar deoxyribose; this is an accurate and sensitive assay for .OH formation[17–19] and has been made the basis of a simple and convenient method for determining the rate constants for reaction of "scavengers" with .OH.[20,21]

MATERIALS AND METHODS

Pure porcine transferrin was a kind gift from Speywood Laboratories Ltd. Desferrioxamine (desferal) was a gift from CIBA Geigy. All other reagents were of the highest quality available from Sigma or BDH Chemicals. Cardiac myoglobin (type III) from Sigma was oxidised with ferricyanide or reduced with a small excess of dithionite under aerobic conditions, and then purified by gel filtration on a 30 × 2.5 cm column of Sephadex G-15. Deoxyribose degradation was measured essentially as described in;[4] full details are given in Table and figure legends. Breakdown of myoglobin haem was determined by measurements at 490, 560 and 580 *nm*, and calculation of the amounts of oxymyoglobin, metmyoglobin (myoglobin[III]) and myoglobin(IV) was according to the equations provided by Whitburn (refs 22, 23 and personal communication).

$$[MbO_2] = 2.8\,A_{490} - 127\,A_{560} + 153\,A_{580}$$

$$[Mb^{III}] = 146\,A_{490} - 108\,A_{560} + 2.1\,A_{580}$$

$$[Mb^{IV}] = -62\,A_{490} + 242\,A_{560} - 123\,A_{580}$$

Spectrophotometric measurements were performed on a Beckman DU-7 spectrophotometer.

RESULTS

Studies upon oxymyoglobin

Figure 1 shows the spectral changes observed when oxymyoglobin was treated with an approximately ten-fold molar excess of H_2O_2. As expected,[33] the spectrum of myoglobin(IV) rapidly appeared (changes complete after 10 min) and was stable for

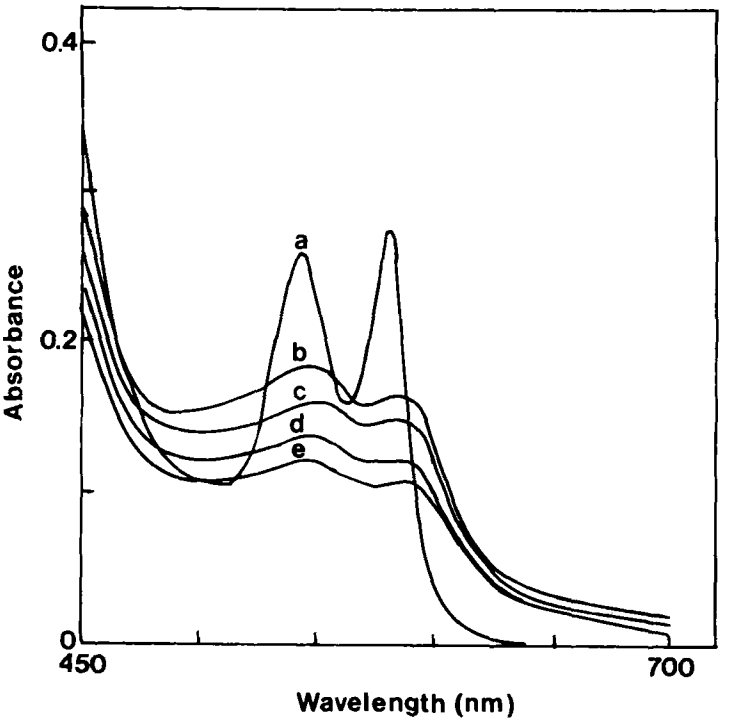

FIGURE 1 *Degradation of oxymyoglobin by* H_2O_2. Oxymyoglobin (20.2 μM) was incubated with H_2O_2 (200 μM) in 25 mM KH_2PO_4-KOH buffer pH 7.4. Spectra were recorded after 1 (line a), 30 (line b), 60 (line c), 90 (line d) and 120 (line e) min of incubation.

a further 20 min. Analysis of the reaction mixtures by the Whitburn equations (as explained in the Materials and Methods section) showed that a small percentage (about 10%) of the myoglobin in the reaction mixture was present as metmyoglobin (myoglobin[III]), but the rest was present entirely as myoglobin(IV) between 10 and 30 min. No loss of haem could be detected at 30 min. However, at 60 min some haem loss was observed and this continued for at least another hour (Figure 1). After 60 minutes incubation both myoglobin(IV) and metmyoglobin were present in the reaction mixture at about a 4:1 molar ratio. At later times this ratio declined, i.e. the haem loss was largely due to a drop in myoglobin(IV). For example, between 30 and 120 min metmyoglobin decreased from 4.5 to 4.1 μM, whereas myoglobin(IV) decreased from 15.4 to 10.0 μM.

The sugar deoxyribose is attacked by .OH to form a product that, upon heating with thiobarbituric acid at low pH, yields a pink chromogen.[17,18] Addition of deoxyribose to the reaction mixtures at concentrations sufficient to trap any .OH formed had no effect on the spectral changes. However the deoxyribose was degraded. Deoxyribose degradation was inhibited by adding scavengers of .OH such as mannitol,[1] arginine,[7] and phenylalanine[19] but not by adding urea, which reacts only slowly with .OH [24] (Table I). Thus the deoxyribose degradation can be attributed to .OH formation in the reaction mixtures. Deoxyribose degradation was also inhibited by adding a low concentration of the iron transport protein transferrin, which binds iron

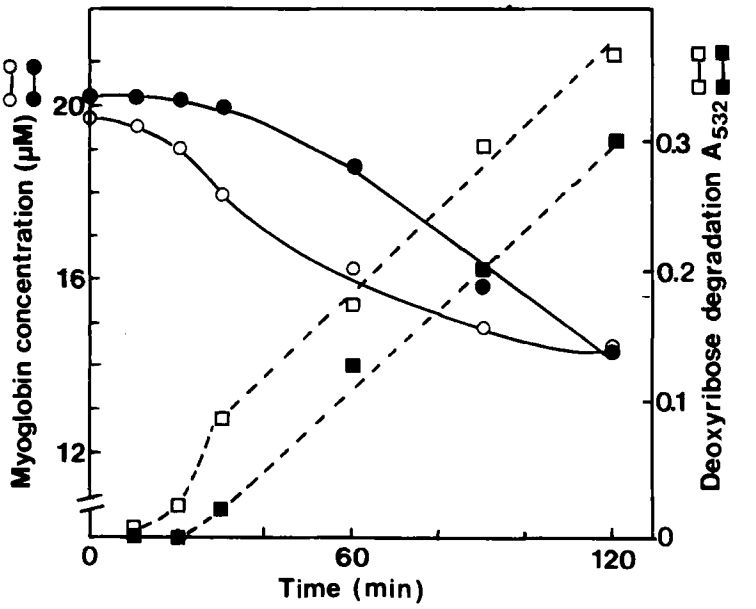

FIGURE 2 *Time course of deoxyribose degradation and breakdown of oxymyoglobin or metmyoglobin in the presence of* H_2O_2. Oxymyoglobin (20.2 μM) or metmyoglobin (19.7 μM) were incubated with H_2O_2 (200 μM) as described in the legend to Figure 1, in the presence of 6.7 mM deoxyribose. Deoxyribose degradation in the presence of oxymyoglobin (■) or metmyoglobin (□) was measured by the thiobarbituric acid method, which yields a pink chromogen absorbing at 532 nm [17]. Extent of haem degradation in the case of oxymyoglobin (●) or metmyoglobin (○) was determined by measurements at 490, 560 and 580 nm as described in Materials and Methods.

ions and prevents them from participating in .OH generation.[5] Neither transferrin nor the .OH scavengers (mannitol, phenylalanine, arginine) had any effect on the spectral changes observed in the reaction mixture (Figure 1). Figure 2 shows the time course of deoxyribose degradation; also plotted is the extent of haem loss from the myo-

TABLE I

Deoxyribose degradation as a measure of .OH formation by myoglobin. Reaction mixtures were as described in the legend to Fig. 2 and were incubated for 90 min. Concentrations stated are the final concentrations in the reaction mixtures

Addition to reaction mixture	Extent of deoxyribose degradation (A_{532})			
	Oxymyoglobin		*Metmyoglobin*	
	A_{532}	% inhibition	A_{532}	% inhibition
None	0.358	0	0.401	0
None (omit H_2O_2 or myoglobin)	0.000	100	0.000	100
Mannitol (20 mM)	0.088	75	0.122	70
Urea (10 mM)	0.384	0	0.424	0
Phenylalanine (10 mM)	0.160	55	0.248	38
Transferrin (10 μg/ml)	0.023	94	0.018	96
Salicylate (5 mM)	0.079	78	0.129	68
Desferrioxamine (100 μM)	0.054	85	0.061	85
Arginine (50 mM)	0.104	71	0.156	61

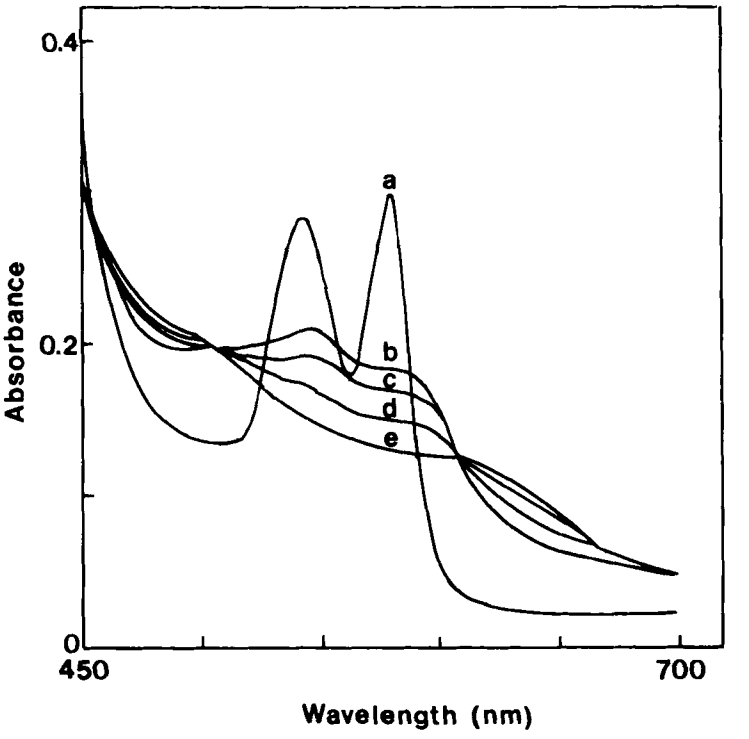

FIGURE 3 *Action of desferrioxamine on the breakdown of oxymyoglobin by* H_2O_2. Oxymyoglobin (21.8 μM) was incubated with H_2O_2 (200 μM) in the presence of 100 μM desferrioxamine in 25 mM KH_2PO_4-KOH buffer pH 7.4. Spectra were recorded after 1 (line a), 10 (line b), 20 (line c), 30 (line d) and 60 (line e) min of incubation.

globin calculated from the data in Figure 1. It may be seen that no significant deoxyribose degradation was observed until haem degradation had begun.

The ability of transferrin to inhibit the .OH generation prompted us to test the action of desferrioxamine, a powerful iron chelating agent that is known to inhibit iron-dependent generation of .OH[25,26] Desferrioxamine did indeed inhibit the .OH generation (Table I). However, it also had striking effects on the spectral changes observed in the reaction mixture (Figure 3). The myoglobin (IV) formed after 10 min slowly converted to another compound, as indicated by the presence of isobestic points at 507 and 610 nm. The compound obtained after 60 min was stable on further incubation for at least one hour, i.e there was no haem degradation. It should be noted that incubation of oxymyoglobin with desferrioxamine in the absence of H_2O_2 under our reaction conditions produced no changes in the spectrum of the protein. Iron-loaded desferrioxamine (ferroxamine)[26] did not inhibit ·OH generation and did not produce the spectral changes shown in Fig. 3.

Salicylate, a powerful scavenger of .OH[27,28] was also examined. It decreased .OH formation (Table I), but again had striking effects on the behaviour of the protein (Figure 4). The myglobin(IV) produced after 10 min remained stable up to 60 min; no haem loss was observed. After 60 min slow spectral changes were observed, corresponding to formation of an unknown product different from that formed with

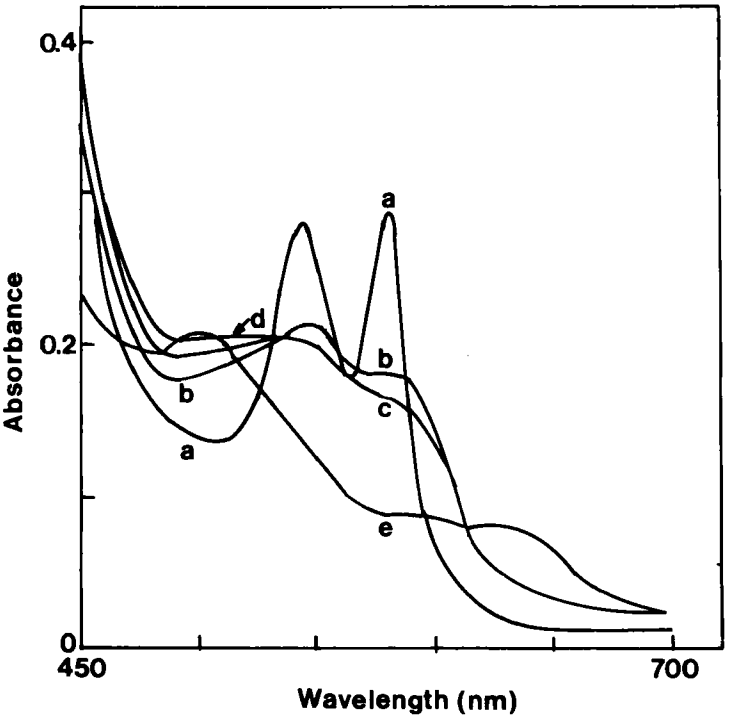

FIGURE 4 *Action of salicylate on the breakdown of oxymyoglobin by* H_2O_2. Oxymyoglobin (21.2 μM) was incubated as described in the legend to Figure 3 except that desferrioxamine was replaced by 5 mM salicylate. Spectra were recorded after 1 (line a), 10 (line b), 90 (line c), 120 (line d) and 180 (line e) min of incubation.

desferrioxamine. If reaction was allowed to continue for 180 min (Figure 4), a spectrum resembling that of leghaemoglobin (III)[16] was observed.

Incubation of oxymyoglobin and salicylate in the absence of H_2O_2 produced no changes in the spectrum of the protein under our reaction conditions.

Studies upon metmyoglobin

When metmyoglobin was treated with a ten-fold molar excess of H_2O_2, both the formation of myoglobin (IV) and its subsequent degradation were faster than with oxymyoglobin. Thus even after 30 min there was already a significant loss of haem (data not shown). Again, deoxyribose added to the reaction mixture was degraded and the extent of degradation paralleled the loss of haem (Figure 2). Deoxyribose degradation was again inhibited by transferrin and by the .OH scavengers mannitol, arginine and phenylalanine, but not by urea (Table I); none of these reagents affected the spectral changes. Salicylate and desferrioxamine also inhibited the .OH generation, but altered the spectra in a way very similar to that observed with oxymyoglobin (data not shown).

DISCUSSION

Muscle myoglobin (especially cardiac myoglobin) exists almost entirely in the oxymyoglobin form under physiological conditions, and this will be the form present when ischaemic tissues are reoxygenated.[10] Hence we have concentrated our studies upon oxymyoglobin. Addition of H_2O_2 to this protein causes formation of myoglobin(IV)[33] with small amounts of metmyoglobin and then loss of haem. Addition of deoxyribose to the reaction mixture showed that .OH radicals are produced (as confirmed by the inhibition by phenylalanine, mannitol and arginine, plus the lack of inhibition by urea). Deoxyribose is a hydrophilic molecule with extensive hydrogen bonds to water and is unlikely to enter the haem binding site of myglobin. It will thus dectect .OH radicals formed outside the protein, or close to its surface. Hydroxyl radical production was only detected by deoxyribose when haem degradation had begun (Figure 2), suggesting that it was mediated by iron ions released from the protein. Further evidence for this is the inhibition of .OH formation by transferrin (Table I). Neither transferrin nor the above scavengers inhibited haem degradation. We conclude that mixtures of intact oxymyoglobin molecules and H_2O_2 do not lead to formation of .OH that can be detected in free solution, but that H_2O_2 can cause iron release from the protein and the released iron can then react with H_2O_2 to form .OH. Our studies upon metmyoglobin led to an identical conclusion.

We cannot exclude the possibility that reaction of the haem iron in the protein with H_2O_2 leads to .OH and/or other reactive radicals (eg ferryl) that cause the observed haem degradation and iron release. However, any .OH so formed would have to be inaccessible to deoxyribose, arginine, mannitol and phenylalanine, since these compounds do not inhibit the haem degradation.

Both salicylate and desferrioxamine appear to stabilize myoglobin, preventing haem degradation. Similar effects have been observed when leghaemoglobin is treated with these compounds.[8] The fact that desferrioxamine prevents degradation of myoglobin(IV) by H_2O_2 could be of clinical importance, in view of the widespread use of the chelating agent.[29] Oxymyoglobin itself (no H_2O_2 added) was not affected by incubation with desferrioxamine under our reaction conditions. It is possible that effects might be seen after more prolonged incubations, since incubation of erythrocyte haemolysates with desferrioxamine for 24 h was reported to cause partial conversion of oxyhaemoglobin to methaemoglobin.[30] It is also possible that such haemolysates generate H_2O_2.

In the presence of H_2O_2, both oxymyoglobin and metmyoglobin can stimulate damaging radical reactions such as lipid peroxidation.[11-13, 31] Additional damage can be caused by H_2O_2-mediated release of iron ions from the proteins and subsequent .OH formation, which might well contribute to the lipid peroxidation observed. For example, when ischaemic hearts are reperfused, H_2O_2 is generated and .OH is formed.[10] It is certainly possible that the iron necessary for .OH production is produced by degradation of cardiac myoglobin. No evidence was obtained in our studies for the formation in myoglobin-H_2O_2 mixtures of a reactive deoxyribose-degrading species other than .OH, such as has been described in the oxyhaemoglobin-H_2O_2 system.[7]

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