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THE SUPPRESSION OF IRON RELEASE FROM ACTIVATED MYOGLOBIN BY PHYSIOLOGICAL ELECTRON DONORS AND BY DESFERRIOXAMINE

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Interactions between ferrimyoglobin and hydrogen peroxide have been postulated to lead to the formation of activated ferryl myoglobin. In such systems hydroxyl radical formation has also been demonstrated by its ability to degrade deoxyribose subsequent to the release of iron from the porphyrin ring of the myoglobin. We have investigated the potential for ferrylmyoglobin formation and for iron release from ferrylmyoglobin exposed to hydrogen peroxide; the modulation of the stability of the haem group by membranes and in the presence of desferrioxamine and ascorbate have also been assessed. The results show that iron release from ferrimyoglobin activated by hydrogen peroxide is suppressed in the presence of membranes, apparently by the reduction of the ferryl myoglobin species, and lipid peroxidation occurs. In the presence of desferrioxamine, formation of the ferrylmyoglobin species is suppressed by the electron donating properties of the trihydroxamate moiety, which also functions as a chain-breaking antioxidant when added to peroxidising membranes. The physiological antioxidant ascorbate not only suppresses the formation of the ferryl myoglobin species under the conditions described here, but also reduces the myoglobin iron to the iron II state.

KEY WORDS: Ferrimyoglobin, ferrylmyoglobin, iron, desferrioxamine, ascorbate, membranes.

INTRODUCTION

It has been suggested that myoglobin is a potential candidate for hydroxyl radical formation *in vivo*.[1] Situations may occur *in vivo* in which myoglobin is released from its normal cellular location, for example, the release into the circulation from muscle cells after prolonged exercise[2] and the release into the blood immediately after an acute myocardial infarction.[3] Studies by Halliwell *et al.* have shown that exposure of myoglobin to excess hydrogen peroxide generates hydroxyl radical[1] as measured by its ability to degrade deoxyribose,[4] whereas when oxyhaemoglobin is reacted with excess hydrogen peroxide two oxidising species are formed, one of which is hydroxyl radical and the other is not.[5,6]

The potential involvement of myoglobin in the initiation and propagation of free radical damage is of considerable interest in relation to myocardial post-ischaemic reperfusion injury. Studies on isolated heart preparations with coronary artery ligation have shown that incorporation of hydroxyl radical scavengers and the iron

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chelator desferrioxamine prior to reperfusion decreased the incidence of heart arrhythmias[7] and other markers of radical-mediated damage on reperfusion.[8]

These observations have been interpreted as implicating chelatable iron in the generation of hydroxyl radicals in reperfusion injury. However, this does not take into account the location and origins of the iron, the site of the formation of the putative hydroxyl radical and the access of the applied antioxidants and inhibitors to the site of radical generation.

In the cardiac myocyte there is no catalase and little glutathione peroxidase.[9] Furthermore ischaemia causes decreased levels of reduced glutathione and modified antioxidant enzyme activities,[10] thus decreasing the ability of the cell to protect itself against oxygen radicals.

Our interest is in the potential involvement of myoglobin in the initiation and propagation of membrane damage. In the work presented here we have investigated the interaction between activated myoglobin and membranes and the relationship between ferryl radical formation and iron release from myoglobin. The effects of the iron chelator desferrioxamine and the antioxidant ascorbate in these systems have also been studied and reveal their role in the suppression of the formation and reactivity of ferryl myoglobin radicals, apparently by electron donation.

MATERIALS AND METHODS

Myoglobin (ferric form, horse heart, type III), ferrozine, thiourea, thiobarbituric acid, malonaldehyde bis-(dimethylacetal) 1,1,3,3-methoxypropane derivative, and ferrous sulphate heptahydrate were all purchased from Sigma Chemical Co. Desferrioxamine mesylate was from CIBA; all other chemicals were of Analar grade and were supplied by BDH Chemicals.

Incubation of myoglobin (20 μM or 50 μM final concentrations) with hydrogen peroxide was carried out in 5 mM phosphate buffered saline, pH 7.4. Final concentrations of ascorbate and desferrioxamine were 1 mM and 400 μM respectively. For assessing the reactivity towards membranes of the radicals formed, human haemoglobin-free erythrocyte membranes were utilised as the most relevant membrane system for lack of iron-containing proteins and minimal iron contamination. Membranes were prepared from normal fresh erythrocytes according to the procedure of Dodge *et al.*[11] in phosphate buffer at pH 7.4. Membrane concentration was assessed by assaying protein.[12] Final concentration of membranes was 0.5 mg/ml. For studying the effects of membranes, desferrioxamine and ascorbate on the myoglobin/H_2O_2 system in terms of the stability of the haem ring, high concentrations of H_2O_2 and prolonged incubation times were chosen to create conditions favouring extensive iron release in the absence of the additives.

Non-haem iron released from myoglobin was estimated utilising a modification of the ferrozine assay of Ceriotti and Ceriotti,[13] with ferrous sulphate as a standard and incorporating appropriate blanks. Absorbance of the iron-ferrozine complex was monitored at 562 nm. For assessing overall lipid peroxidation, the thiobarbituric acid assay was applied[14] as described in.[15] In these experiments the absorbance of the chromophore was measured at 532 nm and the background absorbance at 580 nm due to possible contributions from haem protein absorption was subtracted. Standards were run simultaneously under the same conditions utilising malondialdehyde prepared by acid hydrolysis of the acetal.[15]

RESULTS

The spectral characteristics of ferrimyoglobin exposed to varying concentrations of hydrogen peroxide are shown in Figure 1. At the 1:1.25 molar ratio of ferrimyoglobin to hydrogen peroxide the development of the characteristic maximum of ferrylmyoglobin at 580 nm is notable with a decrease in the shoulder at 630 nm (characteristic of the protein in the iron III state). At a 5-fold molar excess of hydrogen peroxide all the ferrimyoglobin is converted to the ferryl state. The amount of iron released from myoglobin as a function of hydrogen peroxide concentration is shown in Table 1. After one hour no non-haem iron release is detectable up to 1.25 molar excess of

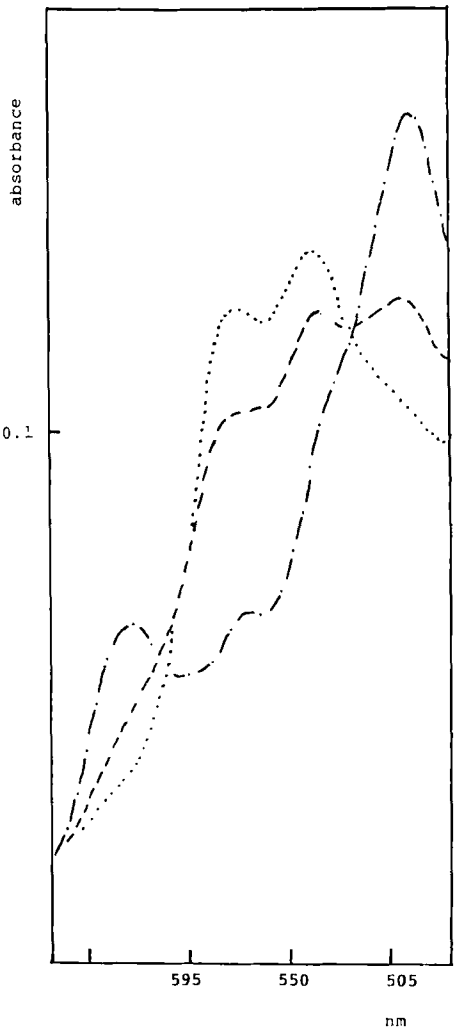

FIGURE 1 Spectra showing the effects of hydrogen peroxide on ferrimyoglobin [ferrimyoglobin (20 μM) –.–.–.; Mb: H_2O_2 mole ratio 1:1.25 – – –; 1:5 . . . . . , 1 hr incubation]

TABLE I
INFLUENCE OF HYDROGEN PEROXIDE ON IRON RELEASE FROM MYOGLOBIN III (50 μM)

Mb:H_2O_2 mole ratio	% iron released	
	1 hr	3 hr
1:1	0	3.2 (2)
1:1.25	0	3.5 ± 2 (4)
1:5	14.3 ± 2	21.8 ± 1 (4)
1:10	22.0 ± 3	41.7 ± 1 (4)
1:20	32.1 ± 4 (4)	55.9 ± 5 (11)

Values are mean ± SD for (N) determinations

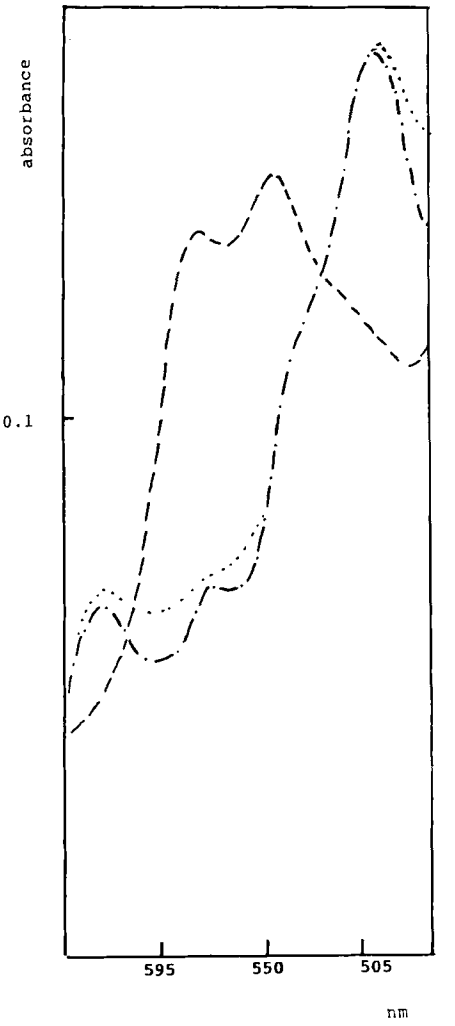

FIGURE 2 Effects of desferrioxamine (400 μM) on hydrogen peroxide-activated myoglobin [ferrimyoglobin (20 μM) –.–.–.; Mb: H_2O_2 mole ratio 1:5 – – – –, with desferrioxamine . . . . .]

H_2O_2. As the H_2O_2 concentration is increased disruption of the porphyrin ring occurs and nonhaem iron relase becomes measurable. Prolonging the exposure to peroxide considerably enhances the loss of iron from the haem protein.

The iron chelator desferrioxamine was incorporated into the assay mixture to prevent the released iron becoming available for reaction with hydrogen peroxide to generate reactive oxygen radicals. The effects on the myoglobin were examined spectroscopically. As depicted in Figure 2 activation of ferrimyoglobin by hydrogen peroxide (5-fold molar excess) to the ferryl form of the haem protein was totally suppressed by desferrioxamine (continuously present in the reaction mixture) and the release of iron inhibited (Table II). At a 20-fold molar excess of peroxide the stability of the haem group was affected only after prolonged exposure when desferrioxamine was present, 3 hour incubation releasing 14% iron; this a considerable inhibition compared with 55% iron release in the absence of the iron chelator. That desferrioxamine did not merely prevent the released iron from becoming available for assay by binding to it, is confirmed by measurements of the total myoglobin concentration and of Mb(II), Mb(III) and Mb(IV), from the spectra according to the Whitburn equations.[16,1] Desferrioxamine had no effect on metmyoglobin in the absence of hydrogen peroxide.

Ascorbate was effective in preventing the breakdown of the haem group in ferrimyoglobin. As shown in Table II, only 5% released iron was detectable on prolonged exposure (3 hr) to a 20 fold excess of hydrogen peroxide. Spectroscopic investigation revealed that conversion to oxymyoglobin takes place in the presence of ascorbate and this is essentially maintained in the presence of excess hydrogen peroxide with prolonged incubation, although a small amount of oxidised myoglobin was detected, probably due to autoxidation of ascorbate under these conditions (Figure 3).

The formation of the ferryl myoglobin radical species from ferrimyoglobin activated by hydrogen peroxide was assessed by its ability to peroxidise erythrocyte membranes. Table III shows the effects of membranes in the myoglobin III/excess H_2O_2 system and the consequences for the peroxidation of the lipids and the release of iron. It is clear from this data that, in the presence of membranes from the time of initiation of the myoglobin $-$ H_2O_2 interaction, iron release from oxidised myoglobin

TABLE II

Influence of ascorbate and of desferrioxamine on iron release from 50 μM myoglobin (III) in the presence of hydrogen peroxide

Mb:H_2O_2 mole ratio	% iron released	
1:1	3.2	(2)
1:5	21.8 ± 1	(4)
1:20	55.9 ± 5	(11)
+ desferrioxamine 400 μM		
1:	0	
1:5	0	
1:20	14.2 ± 3	(3)
+ ascorbate 1 mM		
1:1	0	
1:5	2 ± 2	
1:20	5 ± 2	(3)

Results are means ± SD of (N) determinations.

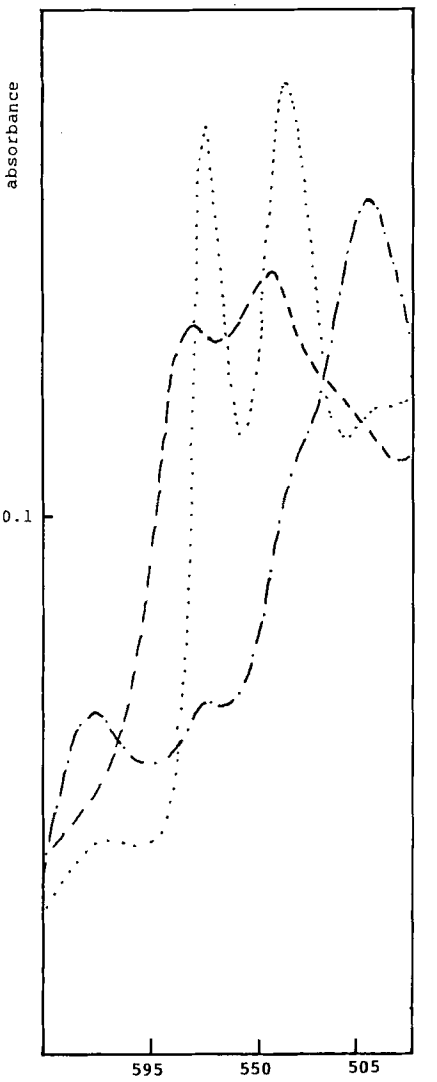

FIGURE 3 Effects of ascorbate (1 mM) on hydrogen peroxide-activated myoglobin [ferrimyoglobin (20 μM) –.–.–.; Mb: H_2O_2 mole ratio 1:5 – – –, with ascorbate . . . . . .]

is considerably suppressed after 1.5 hr incubation compared to that detectable in the absence of membranes, but correspondingly lipid peroxidation occurs.

Exposure of membranes to myoglobin/hydrogen peroxide mixtures in the presence of desferrioxamine, or ascorbate, added at the beginning of the incubation (Table III), totally inhibits the observed peroxidation of the membrane lipids over and above the background response from membranes alone. Furthermore, the amount of available non-haem iron is similar to that released in the absence of membranes and considerably inhibited in relation to the amount observed in the absence of desferrioxamine or ascorbate. This latter observation is in contrast with the data of Kanner and Harel[17] who observed increased lipid peroxidation in the presence of ascorbate.

TABLE III

The interactions between membranes and hydrogen peroxide-activated ferrimyoglobin and the consequences for iron release and lipid peroxidation. [Ferrimyoglobin: H_2O_2 ratio 1:20; membrane concentration 0.5 mg/ml; desferrioxamine 400 μM, ascorbate 1 mM, ferrimyoglobin 50 μM].

Additions	Iron Release (%) at total incubation time		Thiobarbituric Acid Reactive Compounds (nmoles/mg membrane protein)	
	3 hr	1.5 hr	3 hr	1.5 hr
reaction mixture, no membranes	55.9 ± 5 (11)	41.9 ± 2 (5)		
+ membranes added at t = 0	11.6 ± 4 (9)	12.9 ± 3 (6)	3.2 ± 1 (9)	2.2 ± 0.7 (6)
+ membranes added at t = 1.5 hr	44.9 ± 4 (9)		0.79 ± 0.1 (9)	
+ DFO + membranes	10.5 ± 2 (4)		0.40 ± 0.05 (4)	
+ membranes + DFO added at t = 1.5 hr	11.6 ± 4 (4)		1.29 ± 0.08 (4)	
+ asc + membranes	5.4 ± 1 (3)		0.43 ± 0.03 (3)	
+ membranes + asc added at t = 1.5 hr	18.3 ± 5 (4)		8.2 ± 1 (4)	
[Mb + membranes only]	0 (9)		0.47 ± 0.05 (9)	
[Membrane only]ø	0 (9)		0.40 ± 0.05 (9)	
			{A_{532} = 0.039}	

The incubation of membranes with the myoglobin/hydrogen peroxide system for $1\frac{1}{2}$ hr with addition of desferrioxamine or ascorbate at this time point induced contrasting responses (Table III). This later incorporation of desferrioxamine produced a similar extent of iron release as in its absence but the amount of lipid peroxidation was considerably decreased (1.29 nmoles/mg protein) compared to the level observed at $1\frac{1}{2}$ hr after the start of the experiment, prior to the addition of desferrioxamine (2.2 nmoles/mg protein). In an identical system incorporating ascorbate (rather than desferrioxamine) $1\frac{1}{2}$ hours after exposure of the membranes to the ferryl myoglobin radical, the extent of lipid peroxidation was markedly enhanced (8.2 nmoles/mg protein) and the inhibition of iron release less pronounced (18.3%), compared with ascorbate being added at the start of the experiment.

DISCUSSION

In the presence of hydrogen peroxide, myoglobin stimulates lipid peroxidation.[17–20] Several workers have shown that the radical species generated is akin to that designated as ferryl myoglobin[21–24] in which the haem iron is one oxidising equivalent above that of MbIII and one oxidising equivalent is on the globin moiety. Furthermore, it has been suggested that additional peroxidative damage may result from hydrogen

peroxide-mediated release of iron from the haem protein and subsequent generation of the hydroxyl radical.[1]

The studies presented in this paper demonstrate that the high propensity for hydrogen peroxide-mediated iron ion release from ferryl myoglobin is dramatically decreased in the presence of membranes, which presumably donate an electron from a susceptible site on the polyunsaturated fatty acyl lipid chain, initiating lipid peroxidation and reducing the myoglobin to MbIII.

The suppression of the formation of ferryl myoglobin radical and iron release from the haem group by desferrioxamine, forming ferrimyoglobin, may proceed via the mechanism proposed by King *et al.*[21] whereby the radical transferred from the vicinity of the haem to a tyrosine residue on the globin surface is accessible to external reducing agents. This is consistent with the ability of this trihydroxamate to act as an electron donor in these systems. It also fits in with the suggestion of Kanner[25] that desferrioxamine acts as an electron donor, as a substrate for peroxidases, and with the ESR studies of Morehouse *et al.*[26] and Davies *et al.*[27] demonstrating the formation of a desferrioxamine nitroxide free radical on interaction with horseradish peroxidase/H_2O_2 mixtures and superoxide radicals respectively. The potential of desferrioxamine to act as an electron donor is further exphasised in the observed suppression of the generation of breakdown products of lipid peroxidation when added to peroxidising membrane lipids. Under these conditions desferrioxamine is acting as a chain breaking antioxidant.

Ascorbate was more effective than desferrioxamine in inhibiting membrane damage and iron release from activated myoglobin when present continuously. The effect of ascorbate in such systems[28] may be a reflection of a balance between the oxidation of Mb^{III} by H_2O_2 and the reduction of ferryl myoglobin species by ascorbate. Only when all the ascorbate is oxidised can H_2O_2 oxidise the remaining ferrimyoglobin. In the experiments described here the H_2O_2 and ascorbate were in equimolar concentrations at the highest H_2O_2 levels applied. The action of ascorbate can be accounted for by the suppression of the generation of ferryl myoglobin through an electron donating mechanism ultimately reducing myoglobin to its functional ferrous Mb^{II} form. When ascorbate is incorporated into already peroxidising membranes initiated by ferryl myoglobin, lipid peroxidation is considerably enhanced due to the ability of ascorbate to reduce the already released iron and increase the rate of propagation of the peroxidation process.[1] Hence where ascorbate is concerned, the timescale of its exposure to the ferryl myoglobin system is crucial. If continuously present, at the appropriate concentrations its effects are beneficial, when added to preformed activated Mb^{IV} its effects are damaging.

In each instance cited here it seems that modulation of iron release from myoglobin is due to the suppression of the formation of ferryl myoglobin, mediated by polyunsaturated fatty acyl chains of membrane lipids, desferrioxamine or ascorbate added at the appropriate time point. This implies that the release of iron from myoglobin occurs through the intermediacy of ferryl myoglobin.

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