Effects of oxyradicals on oxymyoglobin

Deoxygenation, haem removal and iron release

M. Renuka PRASAD,* Richard M. ENGELMAN,† Randall M. JONES* and Dipak K. DAS*‡

*Cardiovascular Division, Department of Surgery, Surgical Research Center, University of Connecticut School of Medicine, Farmington, CT 06302, and †Department of Surgery, Baystate Medical Center, Springfield, MA 01107, U.S.A.

We have examined the effects of $O_2^{\cdot-}$ -derived free radicals on oxymyoglobin, the myocardial intracellular protein involved in the storage and transport of O_2 . The oxyradicals generated by the xanthine/xanthine oxidase system decreased the concentration of oxymyoglobin. Based on the decreases in absorbance peaks at 581 nm and 415 nm it is estimated that out of a 10 nmol decrease in oxymyoglobin, 5 nmol appears to be oxidized to ferrimyoglobin (deoxygenation), while haem was removed from the other 5 nmol of haem protein. These processes were inhibited by both catalase alone and superoxide dismutase in combination with catalase, but not by either superoxide dismutase alone or deferoxamine. These results suggest that among H_2O_2 , $OH^{\cdot}$ and $O_2^{\cdot-}$, only H_2O_2 causes the removal of haem and the oxidation of oxymyoglobin. Furthermore, the oxyradicals also released 3 μM free iron from oxymyoglobin, which is at least 5-fold less than the 15 nmol loss of oxymyoglobin. The loss of oxymyoglobin also preceded the release of free iron. These results indicate that oxymyoglobin oxidation and haem removal occur before the removal of free iron. Thus myoglobin appears to be highly susceptible to free radical attack, and this may represent yet another mechanism of free radical-mediated cellular injury.

INTRODUCTION

Recent studies indicate the involvement of oxygen-derived free radicals such as $O_2^{\cdot-}$ and $OH^{\cdot}$, or oxidants such as H_2O_2 , in a variety of diseases (e.g. cancer, stroke and arthritis) and also during the reperfusion of ischaemic myocardium [1–4]. The possible mechanisms by which these radicals cause cellular injury are (1) lipid peroxidation of membranes [1,5], (2) inactivation of membrane-bound enzymes such as $(Na^+ + K^+)$ -ATPase and $(Ca^{2+} + Mg^{2+})$ -ATPase [6,7] and (3) modification of protein structure and degradation of proteins [8,9]. Besides these mechanisms, free radicals are also known to release iron from non-haem proteins such as ferritin. These observations were first made by Blemond *et al.* [10], and subsequently by others [11]. These results suggest the possibility that $O_2^{\cdot-}$ generated during the reperfusion of the ischaemic myocardium may contribute to the formation of free iron required to convert $O_2^{\cdot-}$ and H_2O_2 to $OH^{\cdot}$ [12]. Studies concerning the source of free iron in the heart are important, since recent studies indicated that among $O_2^{\cdot-}$, $OH^{\cdot}$ and H_2O_2 , only $OH^{\cdot}$ causes extensive cellular damage [5,13,14], and $OH^{\cdot}$ can only be formed from $O_2^{\cdot-}$ via a Fenton reaction catalysed by free iron. Although $O_2^{\cdot-}$ and H_2O_2 appear to be less damaging than $OH^{\cdot}$, observations such as the release of iron from ferritin by $O_2^{\cdot-}$ also suggest that $O_2^{\cdot-}$ and H_2O_2 may not be completely harmless.

The abilities of $O_2^{\cdot-}$ to release iron from the non-haem ferritin protein [10,11] and of H_2O_2 to oxidize oxymyoglobin to ferrimyoglobin [15–17] and release iron [18] raise the following questions: (1) whether $O_2^{\cdot-}$ by itself releases iron from other intracellular haem proteins such as myoglobin, and (2) whether $O_2^{\cdot-}$ and $OH^{\cdot}$ also oxidize oxymyoglobin to ferrimyoglobin. The present

results indicate that among $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$, only H_2O_2 causes deoxygenation of oxymyoglobin to ferrimyoglobin as well as the removal of haem from oxymyoglobin, while $O_2^{\cdot-}$ and H_2O_2 release free iron from the haem nucleus.

EXPERIMENTAL

Chemicals

Xanthine, xanthine oxidase (XO), myoglobin (from horse heart), bathophenanthroline disulphonic acid (BPS), ascorbic acid, deferoxamine mesylate, superoxide dismutase (SOD, from bovine erythrocytes) and catalase were all obtained from Sigma (St. Louis, MO, U.S.A.).

Preparation of oxymyoglobin

Oxymyoglobin was prepared chemically by reducing ferrimyoglobin with dithionite on Sephadex G-25 columns, as described by Wittenberg & Wittenberg [19].

Incubation of oxymyoglobin with $O_2^{\cdot-}$

$O_2^{\cdot-}$ was generated by the action of XO on xanthine [20]. The reaction mixture contained 10 mM-Hepes (pH 7.4), 100 μM -xanthine and 30 μM -oxymyoglobin or ferrimyoglobin in a total volume of 1 ml. In order to measure $O_2^{\cdot-}$, 30 μM -myoglobin replaced 100 μM -cytochrome *c*, and the reduction of cytochrome *c* was followed spectrophotometrically at 550 nm [20]. The reaction was started by the addition of 8 munits of XO, and was carried out at either 25 or 37 °C for 20 min. For determination of iron release, the reaction was terminated by the addition of 70 μl of $HClO_4$, while for deoxygenation and haem studies, the absorbance spectra were recorded.

‡ To whom correspondence and reprint requests should be sent.

Estimation of Fe^{3+}

After termination of the reaction by HClO_4 , protein was removed by centrifugation and $5\text{ M-K}_2\text{CO}_3$ was added to bring the pH to 7.4. The total free iron in the supernatant was measured by complexing with BPS in the presence of 10 mM-ascorbate to reduce Fe^{3+} to Fe^{2+} [11]. For measurement of Fe^{2+} , ascorbate was omitted in the assay mixture.

Estimation of deoxygenation and haem loss

The changes in the absorbance at the visible peak at 581 nm and at the Soret peak at 415 nm were used to measure deoxygenation and loss of haem respectively [21]. The ϵ of $124\text{ mM}^{-1}\cdot\text{cm}^{-1}$ at 415 nm [21] was used to calculate the haem concentration of the samples. The absorbance differences at 581 nm between the ($100\text{ }\mu\text{M}$) ferricyanide oxidized and unoxidized oxymyoglobin, and the haem concentration obtained from the measurement at 415 nm were used to calculate the ϵ for oxymyoglobin at 581 nm . In the present studies, a value of $11\text{ mM}^{-1}\cdot\text{cm}^{-1}$ was obtained, and this was used to calculate the loss of oxymyoglobin. The amount of deoxygenation was obtained by subtracting the haem loss from the total loss of oxymyoglobin.

RESULTS

The λ_{max} of the Soret bands at 408 and 415 nm for ferrimyoglobin and oxymyoglobin respectively, and visible peaks at 542 and 581 nm for oxymyoglobin (results not shown), were similar to those observed by others [17,19,21]. The effect of oxyradicals derived from xanthine/XO on the visible absorption peaks of oxymyoglobin is displayed in Fig. 1. It can be seen that the absorption peaks at 581 and 542 nm progressively decreased with the time of incubation, and that after 20 min of incubation, the decreases in the absorbance peaks at 581 and 542 nm were 0.15 and 0.11 units respectively. Based on a value of ϵ of $11.02\text{ mM}^{-1}\cdot\text{cm}^{-1}$ at 581 nm , generation of $\text{O}_2^{\cdot-}$ at a rate of $2\text{--}5\text{ nmol/min}$, along with its dismutation to H_2O_2 could decrease the concentration of oxymyoglobin by about $13.6\text{ }\mu\text{M}$. Since oxyradicals affected oxymyoglobin, we wanted to examine whether oxyradicals also released free iron from

the haem nucleus. The results in Table 1 show that the xanthine/XO system was able to release as much as $4.0\text{ }\mu\text{M-}$ and $3.6\text{ }\mu\text{M-}$ iron from $50\text{ }\mu\text{M-oxymyoglobin}$ and $50\text{ }\mu\text{M-ferrimyoglobin}$ respectively. However, it seems unlikely that $\text{O}_2^{\cdot-}$ derived from the xanthine/XO system was instrumental in releasing iron, since SOD did not prevent the iron release. On the other hand, inhibition of iron release from ferrimyoglobin and oxymyoglobin by the combined action of SOD and catalase suggests that H_2O_2 generated by the dismutation of $\text{O}_2^{\cdot-}$ caused the iron release. The absence of complex formation between

Fig. 1. Changes in absorbance of oxymyoglobin in the presence of $\text{O}_2^{\cdot-}$ as a function of time

The assay mixture contained 10 mM-Hepes buffer ($\text{pH } 7.4$), $30\text{ }\mu\text{M-oxymyoglobin}$ and $100\text{ }\mu\text{M-xanthine}$ in a final volume of 1 ml . The reaction was started by the addition of 8 munits of XO and was carried out at $25\text{ }^\circ\text{C}$. The spectral changes at 0 (—), 5 (---), 10 (···) and 15 min (— · —) were recorded.

Table 1. Effect of oxyradicals and free radical scavengers on the release of iron from oxymyoglobin and ferrimyoglobin

The reaction mixture contained $40\text{ }\mu\text{M-ferri-}$ or oxymyoglobin and 10 mM-Hepes ($\text{pH } 7.2$) in a total volume of 1 ml . The incubation at $37\text{ }^\circ\text{C}$ for 20 min was carried out in the presence or absence of $50\text{ }\mu\text{M-xanthine}$ (X), 8 munits of XO, 50 units of SOD or 50 units of SOD + 100 units of catalase. The reaction was terminated by the addition of $50\text{ }\mu\text{l}$ of 70% HClO_4 . The colour was developed first in the absence of ascorbic acid to measure Fe^{2+} , after which $100\text{ }\mu\text{l}$ of 10 mg of ascorbate/ml was added to reduce Fe^{3+} to Fe^{2+} , and the colour was again measured. The values are means $\pm$ S.E.M. of four independent determinations.

	$\text{Fe}^{2+}(\text{BPS})_3$ formed (pmol)			
	Oxymyoglobin		Ferrimyoglobin	
	— Ascorbate	+ Ascorbate	— Ascorbate	+ Ascorbate
Xanthine (X)	1 ± 0.1	1 ± 0.2	2 ± 0.2	4 ± 0.1
XO	2 ± 0.1	2 ± 0.3	1 ± 0.1	2 ± 0.02
X + XP ($\text{O}_2^{\cdot-}$)	9 ± 1.0	4000 ± 150	9 ± 0.5	3600 ± 300
X + XO + SOD	—	3800 ± 200	—	3000 ± 250
X + XO + SOD + catalase	—	300 ± 30	—	612 ± 100

Fig. 2. Release of iron from myoglobin (a) and loss of oxymyoglobin (b) due to oxyradicals

(a) The reaction mixture contained 10 mM-Hepes (pH 7.2), increasing amounts of xanthine, and 8 munits of XO in the absence (△) or presence of 40 μM -oxymyoglobin (○) or ferrimyoglobin (●) in a total volume of 1 ml. The incubation was carried out at 37 °C for 20 min. The iron released was measured as described in the Experimental section. Production of $\text{O}_2^{\cdot -}$ was also measured (□). (b) The reaction mixture contained 10 mM-Hepes (pH 7.2), 8 munits of XO, 30 μM -oxymyoglobin and the indicated amounts of xanthine. The decrease in the absorbance at 581 nm was recorded spectrophotometrically between 0 and 20 min to measure the disappearance of oxymyoglobin (○). $\text{O}_2^{\cdot -}$ generation (△) was measured spectrophotometrically by recording the reduction of cytochrome *c* at 550 nm as described in the Experimental section. The values are the means of three independent determinations.

Fig. 3. Effect of free radical scavengers on the oxyradical-mediated changes in the absorption spectrum (500–620 nm) of oxymyoglobin

The reaction mixture contained, in a final volume of 1 ml, 10 mM-Hepes buffer (pH 7.2), 8 munits of XO, 30 μM -oxymyoglobin and 100 μM -xanthine in the presence of 50 units of catalase alone, or 50 units of catalase plus 500 units of SOD (D), or 50 units of SOD alone or 1 mM-deferoxamine (C), or none (B). The xanthine oxidase was omitted in a control sample (A). The absorption spectrum was recorded at 0 (a) and 20 min (b) of incubation. Curve E represents the spectrum of 100 μM -ferricyanide-oxidized oxymyoglobin (30 μM).

iron and BPS in the absence of ascorbate suggests that xanthine/XO released ferric iron from both the derivatives of myoglobin.

Figs. 2(a) and 2(b) show that the generation of $\text{O}_2^{\cdot -}$ increased with increasing xanthine concentration, and that at 100 μM -xanthine as much as 2.4 nmol of $\text{O}_2^{\cdot -}$ was generated per min per ml of reaction mixture. The release of iron from either ferrimyoglobin or oxymyoglobin (Fig. 2a) and the loss of oxymyoglobin as judged by the disappearance of the 581 nm peak (Fig. 2b) increased in

parallel with the generation of $\text{O}_2^{\cdot -}$, suggesting that oxyradicals are the cause of the release of iron as well as the loss of oxymyoglobin. However, SOD alone did not significantly inhibit the loss of oxymyoglobin, as judged by the peaks at 581 and 542 nm (Fig. 3). Nevertheless, catalase alone or SOD plus catalase significantly blocked the decreases in absorbance peaks at 542 and 581 nm, suggesting that H_2O_2 formed by the dismutation of $\text{O}_2^{\cdot -}$ is the agent that causes the loss of oxymyoglobin.

Furthermore, deferoxamine, a Fe^{3+} chelator, also could not inhibit the loss of oxymyoglobin, suggesting that $\text{OH}^\cdot$ formed by the reaction between H_2O_2 and the released iron is not causing the loss of oxymyoglobin.

Since the decrease in the absorbance peaks at 542 and 581 nm could arise from either deoxygenation of oxymyoglobin, leading to deoxymyoglobin or ferri-myoglobin, or from the loss of haem itself, we also examined the haem contents of oxymyoglobin during $\text{O}_2^{\cdot-}$ exposure by recording the Soret band at 415 nm (Fig. 4). Thus, at the end of the 20 min incubation period, the absorbances of control and xanthine/XO-treated samples were 0.15 and 0.12 respectively. Thus decrease of

the Soret peak caused by oxyradicals appears to be smaller than that of the visible peak at 581 nm. As with the 581 nm peak, catalase alone or catalase plus SOD, but not SOD alone or deferoxamine, significantly inhibited the decrease of the 415 nm peak. The loss of oxymyoglobin (based on the decrease in the 581 nm peak) and the loss of haem (based on the decrease in the Soret band) as a function of time of $\text{O}_2^{\cdot-}$ exposure in the presence and absence of SOD and SOD plus catalase are shown in Figs. 5(a) and 5(b). After 20 min of incubation, the losses of haem and oxymyoglobin were 5 and 10 nmol respectively. Furthermore, SOD plus catalase or catalase alone, but not SOD alone or

Fig. 4. Effect of free radical scavengers on the oxyradical-mediated changes in the absorbance spectrum (380–600 nm) of oxymyoglobin

The reaction mixture and assay conditions were similar to those described for Fig. 4. At 0 (a) and 20 min (b) of incubation, 50 μl of reaction mixture was diluted into 1 ml of 10 mM-Hepes (pH 7.2), and the spectrum between 380 and 600 nm was recorded. For description of scavengers used, see the legend to Fig. 3.

Fig. 5. Effects of free radical scavengers on the oxyradical-mediated deoxygenation (a) and haem removal (b) of oxymyoglobin

The assay conditions were similar to those described for Fig. 3 and 4. The reaction mixtures have XO omitted ($\circ$), or contain XO plus 1 mM-deferoxamine or 50 units of SOD ($\square$), or 50 units of catalase, or 50 units of catalase plus 50 units of SOD ($\times$), or none ($\triangle$): (a) The absorbance at 581 nm was recorded as a function of time, and the oxymyoglobin remaining was calculated as described in the Experimental section. (b) The absorbance at 415 nm was recorded as a function of time, and the haem remaining was calculated as described in the Experimental section. The values are the means of three independent determinations.

Fig. 6. Generation of oxymyoglobin (Δ), deoxygenation of oxymyoglobin ($\circ$) and release of iron ($\square$) as a function of time

The reaction mixture contained, in a total volume of 1 ml, 10 mM-Hepes (pH 7.2), 100 μ M-xanthine, 8 munits of XO and 30 μ M-oxymyoglobin. At the times indicated, one set of incubations was processed for measurement of free iron, while for another set absorbances at 581 nm (undiluted samples) and 415 nm (20 times diluted) were measured. Deoxygenation was calculated by subtracting the haem loss from the loss of oxymyoglobin as described in the Experimental section. Generation of $O_2^{\cdot-}$ was calculated by measuring the reduction of cytochrome *c* at 550 nm. The values are the means of three independent determinations.

deferoxamine, prevented the loss of haem as well as preventing deoxygenation at all times of incubation.

Generation of $O_2^{\cdot-}$, deoxygenation of oxymyoglobin and iron release from oxymyoglobin as a function of time are displayed in Fig. 6. The times required to reach 50% of the maximum values of $O_2^{\cdot-}$ generation, deoxygenation and iron release were 2, 4 and 10 min respectively, suggesting that deoxygenation and haem loss occur before the release of iron.

DISCUSSION

Several recent studies suggest that H_2O_2 is not only capable of oxidizing oxymyoglobin to ferrimyoglobin [15–17], but also releases iron from haemoglobin and produces $OH^{\cdot}$ [18]. However, it is not known whether oxyradicals like $O_2^{\cdot-}$ and $OH^{\cdot}$ also release iron from myoglobin and oxidize oxymyoglobin to ferrimyoglobin. Therefore in this study we used xanthine/XO system and free radical scavengers such as SOD, SOD plus catalase and desferoxamine, a ferric iron chelator, to investigate the effects of $O_2^{\cdot-}$ and $OH^{\cdot}$ on oxymyoglobin. Generation of $O_2^{\cdot-}$ at about 2.5 nmol/min per ml of reaction mixture appears to be within the physiological range since submitochondrial particles from heart produced 3–5 nmol $O_2^{\cdot-}$ /min per mg of protein [22]. However, it has been suggested that $O_2^{\cdot-}$ can undergo spontaneous dismutation to H_2O_2 . It is generally estimated that 70 and 30% of the free radical/oxidant products are H_2O_2 and $O_2^{\cdot-}$ respectively. Therefore in the present study, H_2O_2 and $O_2^{\cdot-}$ were produced at rates of up to 5 nmol and 2.5 nmol/min, or 100 μ M and 50 μ M/20 min of reaction period respectively. Thus, the present results showing that $O_2^{\cdot-}$ -derived free radicals/oxidants produced in micromolar concentrations cause a loss of

13 μ M-oxymyoglobin in 20 min suggest that oxymyoglobin is a free radical-susceptible myocardial cytosolic protein. These results are similar to those of Yusa & Shikama [16], Whitburn [15,17] and Puppo & Halliwell [23,24] in which pure H_2O_2 alone at micromolar concentrations caused significant oxidation of oxymyoglobin to ferrimyoglobin, or of oxyhaemoglobin to ferrihaemoglobin. However, the spectral properties at 415 nm were not studied to see whether conversion of oxymyoglobin to ferrimyoglobin was also accompanied by the loss of haem from the haem protein [15–17,23,24]. The present results showing that $O_2^{\cdot-}$ and H_2O_2 generated by xanthine/XO cause not only a decrease in the 581 nm peak, but also in the 415 nm peak, suggest haem loss from oxymyoglobin as well as oxidation of oxymyoglobin to ferrimyoglobin (deoxygenation). Therefore, the oxyradical-mediated loss of oxymyoglobin involves 50% each of both haem removal and deoxygenation. Inhibition of oxyradical-mediated loss of oxymyoglobin by catalase, but not by SOD, suggests that H_2O_2 , but not $O_2^{\cdot-}$, produced in the xanthine/XO system caused haem loss as well as deoxygenation. Oxyradicals produced by the xanthine/XO system also released 3 μ M-iron from oxymyoglobin, suggesting the formation of $OH^{\cdot}$ from H_2O_2 by the Fenton reaction, as given below [12]:

Indeed, the formation of $OH^{\cdot}$ from oxymyoglobin and H_2O_2 has been reported by Puppo & Halliwell [24]. However, the inability of deferoxamine, a Fe^{3+} chelator, to inhibit the oxidation of oxymyoglobin as well as haem loss suggests that $OH^{\cdot}$ produced by oxyradicals of the xanthine/XO system and myoglobin is also not capable of attacking oxymyoglobin. Therefore, our present results indicate that among $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$, only H_2O_2 is capable of deoxygenating oxymyoglobin as well as removing haem from it.

It is interesting to note that 3 μ M-iron was released from each of the 40 μ M-myoglobin derivatives as compared with about 5 μ M-iron released from 500 μ M-ferritin by the same xanthine/XO system [10,11]. Therefore it is possible to suggest that myoglobins are more susceptible to free radical attack in releasing iron as compared with ferritin during myocardial ischaemia/reperfusion [1,2,4]. The release of 4 μ M-iron from oxymyoglobin by 100 μ M- H_2O_2 and 50 μ M- $O_2^{\cdot-}$ produced by the xanthine/XO system in 20 min, and release of about 2 μ M-iron from haemoglobin by 100 μ M- H_2O_2 in 2 h [18], suggest that $O_2^{\cdot-}$ by itself can also release iron from oxymyoglobin. This suggestion is also consistent with our observation that the xanthine/XO system releases mainly Fe^{3+} as compared with release of the Fe^{2+} form by pure 1 mM- H_2O_2 (results not shown). Based on the present results, the following scheme can be proposed for the overall effects of oxyradicals on oxymyoglobin (My):

This suggestion receives support from two sets of observations: (1) the times required to reach 50% of the maximum values for $O_2^{\cdot-}$ generation, deoxygenation and iron release are 2, 4 and 10 min respectively; and (2) for every 1 nmol of $O_2^{\cdot-}$ or 2 nmol of H_2O_2 generated, deoxygenation of 0.5 nmol of oxymyoglobin and release of 0.12 nmol of iron took place. The release of iron from oxymyoglobin by $O_2^{\cdot-}$ may represent a potential source and mechanism of free iron formation required for the conversion of H_2O_2 to $OH^{\cdot}$ in the reperfused myocardium. However, $OH^{\cdot}$ formed by $O_2^{\cdot-}$ or H_2O_2 with myoglobin or haemoglobin may not be the only cellular toxic agent that is produced *in vivo*. Thus, Whitburn and his coworkers [15,17,25,26] have observed that H_2O_2 also produces ferryl myoglobin from either oxymyoglobin or ferri-myoglobin. The irreversible conversion of oxymyoglobin to ferryl myoglobin and formation of ferri-peroxide derivatives by oxygen-derived free radicals may also contribute significantly to the damage of ischaemic reperfused myocardium [1,2,4]. Thus, free radicals produced during ischaemia and reperfusion may decrease the cellular O_2 level and the transport of O_2 to the mitochondria [27–29]. This impaired function of myoglobin could be the principal mechanism by which cellular ATP and phosphocreatine are reduced during ischaemia and reperfusion of the myocardium [29]. Furthermore, removal of haem from oxymyoglobin by $O_2^{\cdot-}$ can be regarded as yet another pathway for the catabolism of myoglobin in addition to the cytochrome P-450 haem-oxygenase pathway that has been recently described by Abraham *et al.* [30].

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