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OXIDATION OF DIMETHYLSULPHOXIDE TO FORMALDEHYDE BY OXYHAEMOGLOBIN AND OXYLEGHAEMOGLOBIN IN THE PRESENCE OF HYDROGEN PEROXIDE IS NOT MEDIATED BY "FREE" HYDROXYL RADICALS

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In the presence of excess hydrogen peroxide, human oxyhaemoglobin and oxyleghaemoglobin from soybean root nodules cause oxidation of dimethylsulphoxide to formaldehyde. This reaction is inhibited by thiourea but not by phenylalanine, HEPES, mannitol or arginine. It is concluded that dimethylsulphoxide oxidation is not mediated by "free" hydroxyl radicals, consistent with previous conclusions that intact haemoglobin, leghaemoglobin or myoglobin molecules do not react with H_2O_2 to form hydroxyl radicals detectable outside the protein.

KEY WORDS: Haemoglobin, leghaemoglobin, dimethylsulphoxide, hydroxyl radical, haem proteins.

INTRODUCTION

Formation of hydroxyl radical (·OH), or a similar highly-oxidizing species, accounts for much of the damage done to biological systems by increased generation of superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2).[1] Hydroxyl radical formation in systems generating O_2^- and H_2O_2 requires the presence of a suitable metal ion catalyst; particular attention has been paid to low-molecular mass iron complexes[2] and to iron proteins (ferritin,[3] haemosiderin,[4] lactoferrin[5] and transferrin[5]) as catalysts of ·OH production *in vivo*. Recently, detailed studies on the ability of human haemoglobin,[6,7] soybean leghaemoglobin[8] and horse-heart myoglobin[9] to promote ·OH formation from H_2O_2 have been described. All these studies concluded that H_2O_2 did not react with the intact haem proteins to give ·OH radical that could be detected outside the protein by such ·OH-reactive molecules as deoxyribose or aromatic compounds.[6-9] Rather, excess H_2O_2 caused degradation of the haem ring, liberating iron ions that could react with H_2O_2 outside the protein to form ·OH.[6]

However, Sadrzadeh *et al.*[10] reported that a mixture of oxyhaemoglobin and H_2O_2 was able to oxidize dimethylsulphoxide into formaldehyde, a reaction that can be mediated by ·OH radicals.[11] Formaldehyde production was inhibited by thiourea, a powerful scavenger of ·OH radicals[12] (k_2 4.7 × $10^9 M^{-1}s^{-1}$[12]). Sadrzadeh *et al.*[10]

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therefore suggested that the intact oxyhaemoglobin molecule can react with H_2O_2 to form $\cdot$OH. Unfortunately, thiourea is far from specific as a scavenger of $\cdot$OH[8,13,14] and experiments with other $\cdot$OH scavengers were not reported.

In the present paper, we have further investigated the conversion of dimethylsulphoxide to formaldehyde using both human oxyhaemoglobin and soybean (*Glycine max*) root nodule oxyleghaemoglobin.

MATERIALS AND METHODS

Human haemoglobin and soybean root nodule leghaemoglobin *a* were purified, and other reagents obtained, as described in previous papers.[7,8] Assays of the ability of oxyhaem proteins to oxidize dimethylsulphoxide into formaldehyde were carried out essentially as described in [10]; full details are given in the lengend to Table 1.

RESULTS

In agreement with the results of Sadrzadeh *et al.*,[10] incubation of human oxyhaemoglobin with excess H_2O_2 and 50 mM dimethylsulphoxide at 37°C for 30 min caused

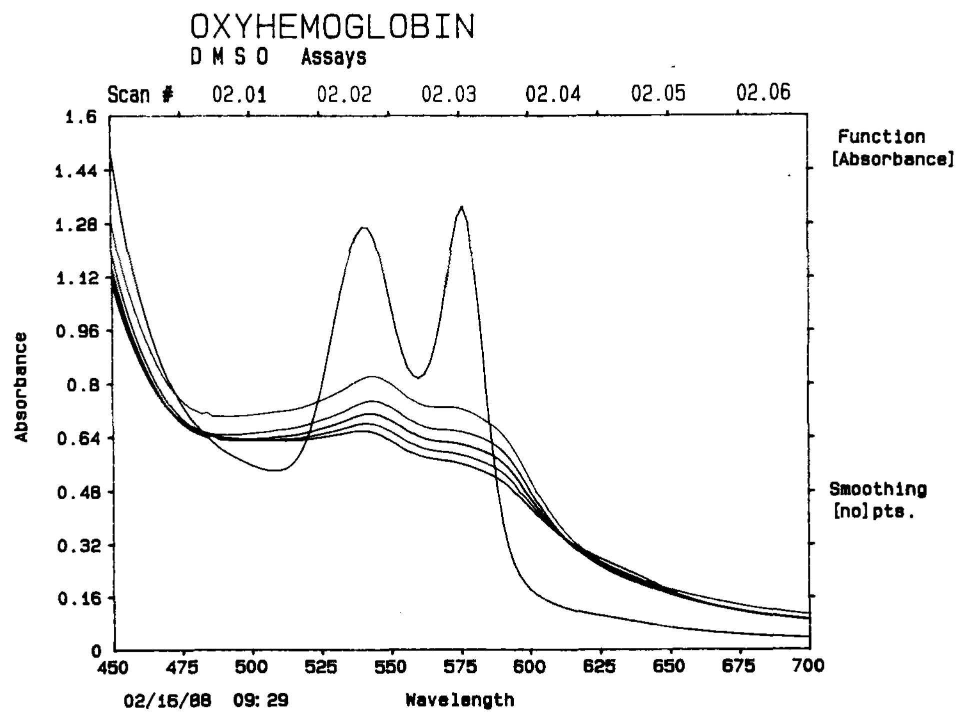

FIGURE 1 *Spectral changes during the experiment with oxyhaemoglobin and H_2O_2 listed in Table 1.* The spectra of mixtures of oxyhaemoglobin and excess H_2O_2 (described in the legend to Table 1) were run at 5-minute intervals. The slow rate of haem degradation may be seen. Results were identical whether or not 50 mM dimethylsulphoxide was included in the reaction mixtures.

TABLE 1

Oxidation of dimethylsulphoxide to formaldehyde by oxyhaemoglobin and oxyleghaemoglobin plus H_2O_2. Experiments were performed at 37°C (haemoglobin) or 25°C (leghaemoglobin) for 30 min as described using the following reagents at the final concentrations stated: oxyhaemoglobin (89.6 μM haem), methaemoglobin (92.3 μM haem), oxyleghaemoglobin a (94.4 μM haem) or metleghaemoglobin (81.8 μM haem), dimethylsulphoxide (50 mM), H_2O_2 (0.44 mM), desferrioxamine (20 μM) and KH_2PO_4 – KOH buffer pH 7.4 (40 mM in phosphate). Formaldehyde was assayed by the Nash method, which produces a yellow colour measured at 412 nm.[10] Scavengers were added to give the final cocnentrations stated. Results varied by 5% or less in five different experiments.

	Oxyhaemoglobin		Oxyleghaemoglobin	
Scavenger added to reaction mixture	A_{412}	% inhibition of formaldehyde generation	A_{412}	% inhibition of formaldehyde generation
None (omit H_2O_2)	0.000	–	0.000	–
None (methaemoglobin or metleghaemoglobin used instead of oxyprotein)	0.020	81	0.026	78
None	0.107	0	0.121	0
10 mM thiourea	0.035	67	0.047	61
100 mM Hepes	0.110	0	0.123	0
100 mM mannitol	0.101	6	0.118	3
100 mM arginine	0.110	0	0.129	0

formaldehyde formation (Table 1). The oxy form of soybean root nodule leghaemoglobin *a* also promoted formaldehyde production. Very much less formaldehyde production was seen with methaemoglobin or metleghaemoglobin plus H_2O_2, or with the oxy-proteins without added H_2O_2 (Table 1). Figure 1 shows that, as expected,[6,7] incubation of oxyhaemoglobin with excess H_2O_2 leads to some haem degradation. The presence of 50 mM dimethylsulphoxide did not affect the rate or extent of haem degradation. Degradation of haem would be expected to lead to release of iron ions capable of catalyzing $^{\cdot}OH$ formation outside the protein.[7–9] Desferrioxamine was included in the reaction mixture (as it was in[10]) to prevent $^{\cdot}OH$ generation involving released iron ions, and hence to stop any dimethylsulphoxide oxidation by $^{\cdot}OH$[13] generated by reaction of released iron ions with H_2O_2.

The question then arises as to whether the oxidation of dimethylsulphoxide by oxyhaem proteins plus H_2O_2 is truly mediated by $^{\cdot}OH$ radicals, as claimed in.[10]

Dimethylsulphoxide reacts with $^{\cdot}OH$ with a second-order rate constant of $7 \times 10^9\,M^{-1}s^{-1}$.[15] The buffer Hepes reacts with $^{\cdot}OH$ with k_2 $5.1 \times 10^9\,M^{-1}s^{-1}$,[16] yet 100 mM Hepes (twice the concentration of dimethylsulphoxide) had no effect on formaldehyde production from the dimethylsulphoxide-H_2O_2-oxyhaemoglobin (or oxyleghaemoglobin) system (Table 1). Over a 50% inhibition would be expected if the formaldehyde production was due to free $^{\cdot}OH$. Mannitol reacts with $^{\cdot}OH$ with $k_2 (2.70 \pm 0.46) \times 10^9\,M^{-1}s^{-1}$,[17] yet 100 mM mannitol had little effect on formaldehyde production; about a 30–40% inhibition would be expected if free $^{\cdot}OH$ mediated the formaldehyde production. 5 mM phenylalanine, which reacts with $^{\cdot}OH$,[7] had no effect on dimethylsulphoxide oxidation. Finally, arginine reacts with $^{\cdot}OH$ with k_2 of $2.1 \times 10^9\,M^{-1}s^{-1}$,[12] yet 100 mM arginine did not affect formaldehyde production. None of these scavengers altered the rate of haem degradation (Figure 1). Comparable results were obtained with oxyleghaemoglobin (Table 1). It follows that conversion of dimethylsulphoxide to formaldehyde by oxy(leg)haemoglobin-H_2O_2 mixtures is not mediated by $^{\cdot}OH$ radicals that are "free", in the sense of being accessible to the scavengers tested.

In agreement with,[10] thiourea was found to inhibit formaldehyde production in both the oxyleghaemoglobin- and the oxyhaemoglobin-H_2O_2 systems. However, this might well be due to properties other than the ability of thiourea to scavenge $^{\cdot}OH$.[13,14] Indeed, thiourea produced striking alterations in the spectrum of the protein. It appeared to prevent the haem degradation, but solutions also became slightly turbid. Thus the inhibition by thiourea is probably related to a direct effect on the protein.

DISCUSSION

Our results confirm the experimental observations reported in[10] and extend them to oxyleghaemoglobin-H_2O_2 mixtures. However, they show that conversion of dimethylsulphoxide into formaldehyde by oxyhaem proteins in the presence of H_2O_2 is not inhibitable by Hepes, arginine, phenylalanine or mannitol, and therefore does not seem to be mediated by free $^{\cdot}OH$.

It might be argued that dimethylsulphoxide could penetrate into the haem binding site and scavenge $^{\cdot}OH$ formed by a reaction between H_2O_2 and iron located within the haem ring; any $^{\cdot}OH$ formed in such reactions would not be expected to escape from the protein and would probably attack the haem ring. This could account for the inability of hydrophilic $^{\cdot}OH$ scavengers such as mannitol to inhibit the dimethylsulphoxide oxidation. Suppose, however, that $^{\cdot}OH$ was generated in this way and attacked the haem ring. Dimethylsulphoxide would then be expected to decrease the haem degradation, yet no evidence for this was found (Figure 1).

Oxyhaem proteins have well-established oxidase and peroxidase activities (reviewed in [20]). It may simply be that dimethylsulphoxide is oxidized by this mechanism rather than by attack of $^{\cdot}OH$ upon it. If this is so, it follows that dimethylsulphoxide is not specific as a scavenger of $^{\cdot}OH$, despite its wide use in *in vivo* experiments (reviewed in [2,14]). Several other papers have already commented on the lack of specificity of dimethylsulphoxide as a $^{\cdot}OH$ scavenger. For example, it suppresses O_2^- formation by activated phagocytes,[21,22] depresses histamine release from mast cells,[23] may be a substrate for the peroxidase activity associated with cyclooxygenase[24] and has been reported to stimulate lipid peroxidation in hepatocytes.[25] Thus inhibition of a physiological or pathological process by dimethylsulphoxide cannot be taken as evidence that the process is mediated by $^{\cdot}OH$ radicals.

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