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Generation of hydroxyl radicals by soybean nodule leghaemoglobin

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Abstract. Leghaemoglobin, a protein present in root nodules of soybean (*Glycine max* (L.) Merr.), generates the highly reactive hydroxyl radical (·OH) upon incubation with hydrogen peroxide (H_2O_2). The H_2O_2 appears to cause breakdown of the haem, releasing iron ions that convert H_2O_2 into ·OH outside the protein. Oxyleghaemoglobin (oxygenated ferrous protein) is more sensitive to attack by H_2O_2 than is metleghaemoglobin (ferric protein). The possibility of oxyleghaemoglobin breakdown by H_2O_2 and formation of damaging ·OH may explain why the root nodule is equipped with iron-storage proteins and enzymes that can remove H_2O_2.

Key words: Fenton reaction – *Glycine* (nodule) – Hydrogen peroxide – Hydroxyl radical – Iron (root nodule) – Leghaemoglobin – Root nodule.

Introduction

Leghaemoglobin, a monomeric protein of relative molecular mass about 16000, is found in the nitrogen-fixing root nodules of leguminous plants (Appleby 1974). Its main biological function seems to be that of ensuring an adequate supply of O_2 to the bacteroids (Bergersen 1980). In the root nodules, leghaemoglobin exists mainly in the reduced (Fe^{2+}) form (Nash and Schulman 1976) and, because of the low O_2 tension, only about 20% of this reduced form is oxygenated (Appleby 1974).

Oxyleghaemoglobin (the oxygenated ferrous form) can undergo a slow breakdown to produce superoxide radical (O_2^-), which disproportionates to give H_2O_2 (Puppo et al. 1981). In the presence of suitable transition-metal catalysts, especially those of iron, H_2O_2 can generate highly reactive and damaging species such as the hydroxyl radical, ·OH (reviewed in Halliwell and Gutteridge 1985, 1986). The hydroxyl radical can attack all types of biomolecules, including enzymes. For example, Brunori and Rotilio (1984) suggested that inhibition of nitrogenase by O_2 could be the consequence of a metal-catalysed formation of ·OH at the active site of this enzyme.

Since leghaemoglobin can lead to formation of H_2O_2, it is interesting to know if it can also act as a promoter of ·OH formation. Previous studies on the ability of mammalian haemoglobin to catalyse formation of ·OH have given conflicting results. For example, Sadrzadeh et al. (1984) claimed that the intact haemoglobin molecule could convert H_2O_2 into ·OH, whereas Halliwell (1978) reported that it did not. Gutteridge (1986) suggested that intact human haemoglobin is incapable of forming ·OH, but that it is easily broken down by exposure to H_2O_2, liberating iron ions that then convert H_2O_2 into ·OH outside the protein. Puppo and Halliwell (1987) reported that human oxyhaemoglobin reacts with H_2O_2 to form an oxidizing radical that is not identical to ·OH.

In view of the generation of H_2O_2 in the root nodule, and the sensitivity of the nodule to oxidant species, it is important to investigate whether or not leghaemoglobin is likely to promote ·OH formation within root nodules. In the present paper, we report a detailed investigation of this point.

Material and methods

Assays of deoxyribose degradation and studies of the aromatic hydroxylation of phenylalanine were as described by Puppo

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Abbreviation: Hepes = 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid

Table 1. Generation of hydroxyl radicals from H_2O_2 and oxyleghaemoglobin from soybean root nodules. Oxyleghaemoglobin (24 μM) was incubated with H_2O_2 (240 μM) in 25 mM KH_2PO_4-KOH buffer pH 7.4 for 60 min in a final reaction volume of 1 ml. For the deoxyribose studies, 6.7 mM deoxyribose was included in the reaction mixture and its breakdown measured by the thiobarbituric-acid method, which yields a pink chromogen absorbing at 532 nm (Gutteridge 1981). For the aromatic hydroxylation studies, phenylalanine (10 mM) was included in the reaction mixture and the formation of three isomeric tyrosines (*o*-, *p*- and *m*-tyrosines) measured by high-performance liquid chromatography with electrochemical detection (Puppo and Halliwell 1987; Halliwell and Grootveld 1987). Results are expressed in terms of total hydroxylated products formed, i.e. the sum of the concentrations of all three tyrosines. All concentrations stated are final concentrations in the reaction mixtures

Reagent added to reaction mixture	Extent of deoxyribose degradation (A_{532})	Extent of aromatic hydroxylation (nmol tyrosines formed)	Percentage inhibition of	
			Deoxyribose degradation	Aromatic hydroxylation
None (complete reaction mixture)	0.215	2.03	0	0
None (H_2O_2 omitted)	0.000	0.00	100	100
100 μM desferrioxamine	0.028	0.31	87	85
5 μM apotransferrin	0.009	–	96	–
20 mM mannitol	0.057	0.51	73	75
10 mM thiourea	0.014	–	93	–
10 mM urea	0.228	1.92	0	5
20 mM Hepes	0.123	–	43	–
5 mM salicylate	0.007	0.04	97	98
50 mM arginine	0.062	0.69	71	66
5 mM phenylalanine	0.123	–	43	–

and Halliwell (1987). Desferrioxamine B methanesulphonate (Desferal®) was a gift from CIBA-Geigy, Basel, Switzerland and porcine apotransferrin was a gift from Speywood laboratories Wrexham, Clwyd, UK. Leghaemoglobin (as metleghaemoglobin) was prepared from soybean (*Glycine max* (L.) Merr.) as described by Puppo et al. (1982) and the leghaemoglobin *a* was separated from the other components according to Appleby et al. (1975). Oxyleghaemoglobin was obtained by reduction with sodium dithionite, followed by gel filtration on Sephadex G-15 (Wittenberg et al. 1974).

Results

Unoxygenated ferrous leghaemoglobin has been reported (Aviram et al. 1978) to react with H_2O_2 to form a stable product, with a characteristic absorption spectrum, leghaemoglobin(IV). This observation was confirmed in the present study. We therefore concentrated our studies on oxyleghaemoglobin (O_2-Fe^{2+}-protein), and also investigated metleghaemoglobin (Fe^{3+}-protein). Leghaemoglobin *a* was used in all our experiments.

Studies with oxyleghaemoglobin. When oxyleghaemoglobin was incubated with a 10-fold molar excess of H_2O_2 at pH 7.4, ·OH was generated. The ·OH was measured by its ability to attack the sugar deoxyribose, degrading it to form a product that can eventually be measured as an absorbance at 532 nm (Gutteridge 1981; Halliwell and Gutteridge 1981). Addition of several compounds known to react with and remove ·OH decreased the observed rate of deoxyribose degradation; these compounds ("scavengers" of ·OH) were mannitol, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (Hepes), thiourea, arginine, salicylate and phenylalanine. Urea, which is a very poor scavenger of ·OH, had little effect (Table 1). Generation of ·OH was confirmed by a completely different assay technique, based on the ability of ·OH to attack aromatic compounds, with eventual generation of hydroxylated products (Halliwell 1978; Richmond et al. 1981). Thus ·OH converts phenylalanine into three isomeric tyrosines (*o*-, *m*- and *p*-tyrosines), which may be separated by high-performance liquid chromatography and measured using an electrochemical detector (Halliwell and Grootveld 1987; Puppo and Halliwell 1987). Formation of these three tyrosines from phenylalanine was again inhibited by scavengers of ·OH, but not by the non-scavenger urea (Table 1).

Figure 1 shows what happens to the protein when oxyleghaemoglobin is incubated with H_2O_2 at pH 7.4. Line a shows the spectrum of the protein alone. Incubation with equimolar H_2O_2 for 60 min produced only a slight spectral change, but increasing concentrations of H_2O_2 led to a progressive loss of the haem absorbance, indicating degradation of the haem (Fig. 1, lines c, d). No shoulder around 630 nm was seen in any of these experiments, indicating that metleghaemoglobin is not formed. Figure 2 shows that, when oxyleghaemoglobin was incubated with different concentrations of H_2O_2 for 60 min, the extent of haem degradation (measured as a fall in absorbance at 575 nm) was linearly related to the amount of ·OH gener-

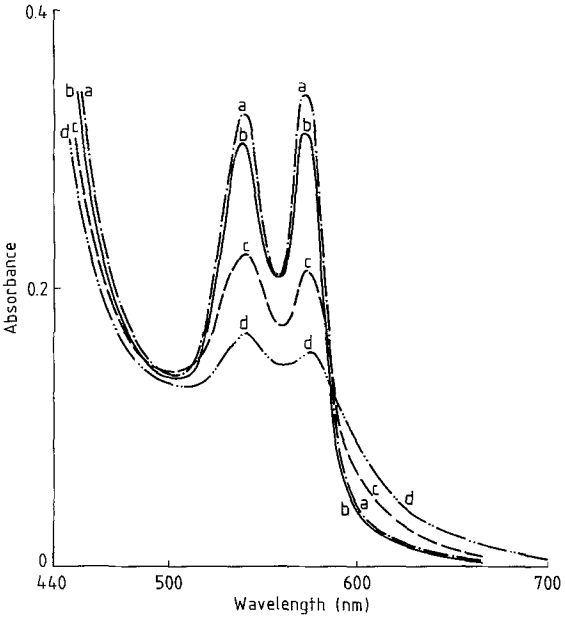

Fig. 1. Degradation by H_2O_2 of oxyleghaemoglobin derived from soybean root nodules. Oxyleghaemoglobin (22 μM) was incubated with various concentrations of H_2O_2 for 60 min in 25 mM KH_2PO_4 – KOH buffer pH 7.4 before running the spectrum. *Line a*, no H_2O_2 (spectrum of oxyleghaemoglobin); *line b*, 22 μM H_2O_2; *line c*, 110 μM H_2O_2; *line d*, 220 μM H_2O_2. All concentrations are those present in the final reaction mixture

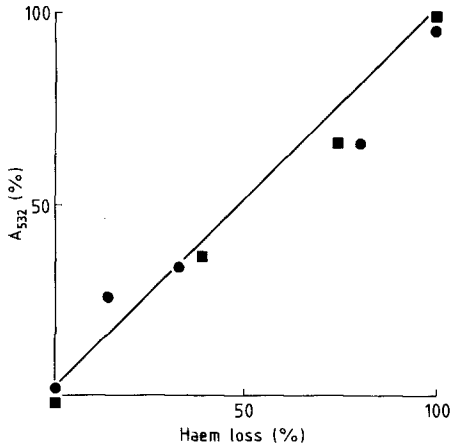

Fig. 2. Correlation of deoxyribose degradation and breakdown of soybean root nodule oxyleghaemoglobin or metleghaemoglobin in the presence of H_2O_2. Oxyleghaemoglobin (●—●) or metleghaemoglobin (■—■) were incubated with various concentrations of H_2O_2 for 60 min, as described in the legend to Fig. 1, in the presence of 6.7 mM deoxyribose. Deoxyribose degradation was measured as described in Table 1, breakdown of oxyleghaemoglobin as a fall in absorbance at 575 nm and breakdown of metleghaemoglobin as a fall in absorbance at 588 nm. 100% is the protein breakdown seen in the presence of 220 and 250 μM H_2O_2, respectively, after 60 min (lines d in Figs. 1 and 4)

ated (measured as deoxyribose degradation). There are two possible explanations of this observation. One is that H_2O_2 reacts with the Fe^{2+} at the haem site of oxyleghaemoglobin, and the highly reactive ·OH produced attacks the haem. A second explanation is that the haem is degraded by H_2O_2, liberating iron ions that react with H_2O_2 to generate ·OH outside the protein (Gutteridge 1986). The experiment shown in Fig. 1 was repeated in reaction mixtures containing the ·OH scavengers mannitol (20 mM), Hepes (20 mM), arginine (50 mM) or phenylalanine (5 mM) and spectral changes identical to those of Fig. 1 (lines b, c, d) were observed (data not shown). Hence these reagents, at concentrations sufficient to remove ·OH (Table 1), had no effect on the rate of haem degradation, even though it is likely that they can enter the fairly "open" haem pocket of oxyleghaemoglobin (see *Discussion*). This tends to support the second explanation. Further evidence supporting this explanation is that the iron-binding protein apotransferrin, which binds iron ions in a form unable to generate ·OH (Aruoma and Halliwell 1987) and is too large to enter the haem pocket, almost completely prevented ·OH formation (Table 1) but did not affect the haem degradation, as shown by the spectral changes identified in Fig. 1.

However, anomalous results were obtained with two other ·OH scavengers, salicylate and thiourea. Inclusion of 10 mM thiourea in reaction mixtures containing oxyleghaemoglobin and H_2O_2 produced a spectrum containing a "shoulder" at 630 nm (Fig. 3, line c), indicative of the formation of metleghaemoglobin. This shoulder was lowered, but not abolished, by adding sodium dithionite, indicating that not only metleghaemoglobin but also another species (perhaps choleglobin) is formed. Salicylate at 5 mM caused a stable spectrum (Fig. 3, line b), identical to that of leghaemoglobin(IV), to appear. The iron-chelating agent desferrioxamine, which inhibits generation of ·OH from H_2O_2 by iron ions (Gutteridge et al. 1979; Halliwell 1985), also appeared to cause some metleghaemoglobin formation (based on the appearance of a shoulder at 630 nm) when it was included in the reaction mixture at 100 μM concentration (Fig. 3, line c). Cautious addition of sodium dithionite caused the shoulder to disappear, which further indicates that metleghaemoglobin is formed. Hence the effects of thiourea, salicylate and desferrioxamine in stopping ·OH detection (Table 1) may not only be due to their ability to scavenge ·OH (thiourea, salicylate) or prevent its formation (desferrioxamine), but also to a direct interaction

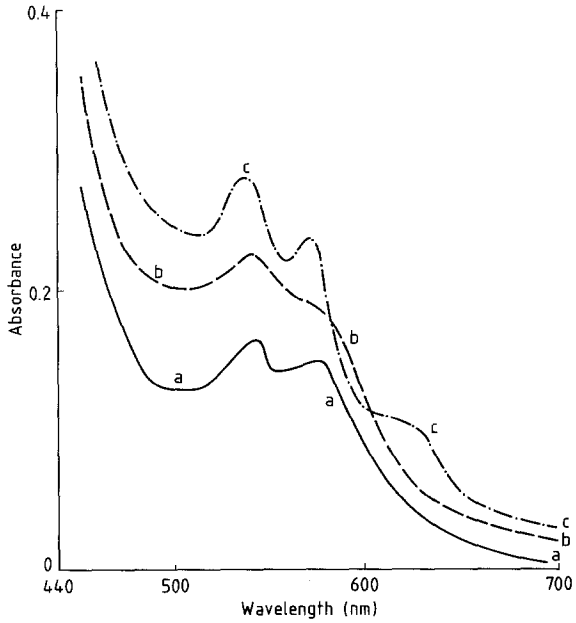

Fig. 3. Action of salicylate, thiourea and desferrioxamine on the breakdown of oxyleghaemoglobin by H_2O_2. Oxyleghaemoglobin (22 μM) was incubated with 220 μM H_2O_2 for 60 min as described in the legend to Fig. 1. *Line a*, protein plus H_2O_2; *line b*, 5 mM salicylate present; *line c*, 100 μM desferrioxamine or 10 mM thiourea present

with the protein, leading to a decrease in haem breakdown.

Studies with metleghaemoglobin. Incubation of metleghaemoglobin with excess H_2O_2 also caused formation of ·OH and damage of the haem, although higher molar H_2O_2:protein ratios were required than in the case of oxyleghaemoglobin. For example, even a 1:1 molar ratio of H_2O_2 to the latter protein produced some haem loss (Fig. 1) and ·OH generation, whereas 1:1 or 2:1 molar ratios of H_2O_2 to metleghaemoglobin did not generate ·OH or degrade the haem. Addition of a 1:1 molar ratio of H_2O_2 to metleghaemoglobin produced a stable absorption spectrum (Fig. 4, line b), as reported by Sievers and Rönnberg (1978). Molar ratios for H_2O_2:metleghaemoglobin of 5 or greater produced a spectrum with a broad absorbance maximum around 588 nm, which was gradually lost on incubation, indicating haem degradation (Fig. 4, lines c, d). If the extent of haem loss was measured by this fall in absorbance at 588 nm, it was again linearly correlated to the amount of ·OH generated (Fig. 2).

The ·OH scavengers mannitol, Hepes, phenylalanine and arginine prevented ·OH detection (Table 2), but had no effect on the spectral changes. However, salicylate addition (5 mM) produced a stable leghaemoglobin(IV) spectrum. Addition of

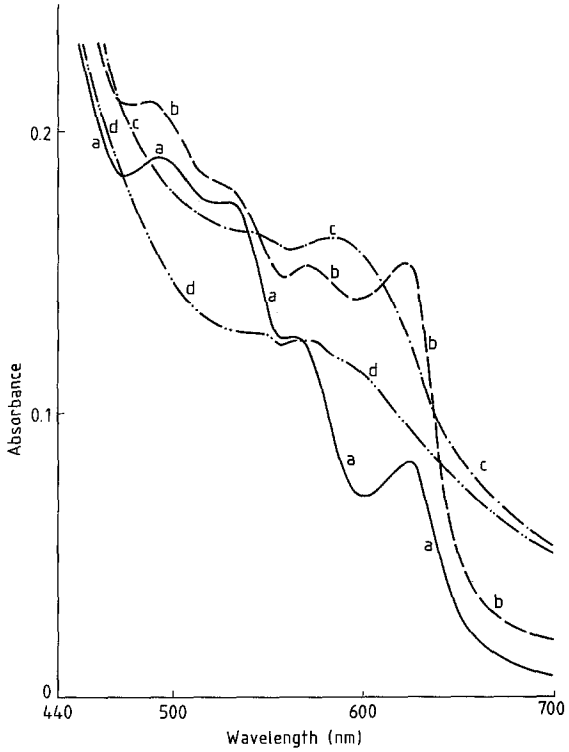

Fig. 4. Action of H_2O_2 on metleghaemoglobin (ferric leghaemoglobin) from soybean root nodules. Metleghaemoglobin (25 μM) was incubated with various concentrations of H_2O_2 in 25 mM KH_2PO_4–KOH buffer pH 7.4. *Line a*, no H_2O_2 (spectrum of metleghaemoglobin); *line b*, spectrum 2 min *or* 60 min after adding 25 μM H_2O_2 (no change on incubation); *line c*, spectrum 2 min after adding 250 μM H_2O_2; *line d*, spectrum 60 min after adding 250 μM H_2O_2

Table 2. Generation of hydroxyl radicals from H_2O_2 and metleghaemoglobin from soybean root nodules. Experiments were carried out exactly as described in the legend to Table 1, except that metleghaemoglobin (25 μM) was used instead of oxyleghaemoglobin

Reagent added to reaction mixture	Extent of deoxyribose degradation (A_{532})	% inhibition of deoxyribose degradation
None (complete reaction mixture)	0.193	0
None (H_2O_2 omitted)	0	100
100 μM desferrioxamine	0.005	97
5 μM transferrin	0.003	98
20 mM mannitol	0.062	68
10 mM thiourea	0.027	86
10 mM urea	0.206	0
20 mM Hepes	0.058	70
5 mM phenylalanine	0.122	37
5 mM salicylate	0	100
50 mM arginine	0.068	65

thiourea or desferrioxamine also produced a leghaemoglobin(IV) spectrum, which degraded very slowly during 60 min incubation (data not shown).

Discussion

Incubation of human oxyhaemoglobin with H_2O_2 causes, among other reactions, production of methaemoglobin and generation of a species not identical to ·OH (Puppo and Halliwell 1987). There was no evidence for either of these processes when oxyleghaemoglobin derived from soybean root nodules was incubated with H_2O_2: the only reactive species detected was ·OH, confirmed by two very different assay methods and by the use of ·OH scavengers.

The simplest explanation of our results is that H_2O_2 enters the leghaemoglobin haem site and attacks the haem to release iron ions, which then react with H_2O_2 outside the protein, or on its surface, to form ·OH (Gutteridge 1986). Hence the observed ·OH formation is prevented by apotransferrin and ·OH scavengers, and the amount of ·OH generated correlates with the extent of haem degradation (Fig. 2). An alternative explanation, that ·OH is formed by reaction of H_2O_2 with the haem iron and then leads to haem degradation, seems less likely, although impossible to rule out completely. The ability of thiourea, salicylate and desferrioxamine to form characteristic spectral complexes with oxy- and metleghaemoglobin (e.g. Fig. 3) indicates that these hydrophilic molecules can enter the haem pocket. Hence the ·OH scavengers mannitol, Hepes, phenylalanine and arginine ought to be able to do so as well, yet they had no effect on the haem degradation, although they prevented ·OH detection (Tables 1, 2). Indeed, iron porphyrins are known to be sensitive to oxidative breakdown by H_2O_2 (Gutteridge 1986; Pasternack and Halliwell 1979). The actions of thiourea, salicylate and desferrioxamine on the protein mean that their effectiveness in stopping ·OH detection (Tables 1, 2) is not necessarily only due to their scavenging (thiourea, salicylate) or metal-binding (desferrioxamine) activities.

The ability of H_2O_2 to release iron ions, leading to ·OH generation, from oxyleghaemoglobin may be relevant in the root nodule. Although only about 20% of the ferrous leghaemoglobin present is oxygenated (Appleby 1974), this is still a substantial amount because the total leghaemoglobin concentration in root nodules approaches 1.5 mM. Also, oxyleghaemoglobin is more sensitive to attack by H_2O_2 than is metleghaemoglobin (which is, in any case, rarely present in root nodules except possibly during senescence). Our studies may explain why the root nodule is equipped with an iron-storage protein, phytoferritin (Ko et al. 1987), and enzymes that degrade H_2O_2 such as peroxidase (Puppo et al. 1980), catalase (Puppo et al. 1982) and the ascorbate-glutathione cycle (Dalton et al. 1986), a mechanism for removing H_2O_2 that is frequently found in plant tissues (Foyer and Halliwell 1976; Halliwell 1987).

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