# Glutathione-dependent conversion of ferryl leghaemoglobin into the ferric form: a potential protective process in soybean (*Glycine max*) root nodules

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GSH is able to reduce soybean (*Glycine max*) ferryl-leghaemoglobin [Lb(IV)] formed by the reaction of ferric or ferrous Lb with $H_2O_2$; in both cases, ferric Lb is obtained and GSH is incapable of reducing ferric Lb to ferrous Lb. Furthermore, the addition of GSH before $H_2O_2$ to ferric Lb prevents side reactions which lead to a species whose spectrum differs markedly from that of Lb(IV). These reactions are likely to occur *in vivo*, as high GSH concentrations have been detected in soybean nodules. The GSH-dependent reduction of Lb(IV) is associated with the oxidation of GSH to GSSG. E.s.r. experiments show that the glutathione thiyl radical (GS$^\bullet$) is formed during this reaction. In the case of ferric Lb, both ferryl Lb and a globin-derived radical previously described appear to be involved in the formation of GS$^\bullet$. Both of these processes may be protective and can help account for the exclusive presence of ferrous (oxygenated or not) Lb in functioning nodules.

## INTRODUCTION

Leghaemoglobin (Lb), a monomeric protein of $M_r \sim 16000$, is found in large amounts in the $N_2$-fixing root nodules of leguminous plants [1]. The function of this haemoprotein *in vivo* is to facilitate the transport of $O_2$ to the bacteroids at a low, but constant, concentration that is compatible with both bacteroidal nitrogenase activity and respiration [2]. In the root nodules, Lb exists mainly in the reduced ($Fe^{2+}$) form [3] and, because of the low $O_2$ partial pressure, only about 20% of this reduced form is oxygenated [2]. In the oxidizing environment of the plant, Lb is maintained in a reduced state by an active metLb reductase [2]. As with haemoglobin and myoglobin, Lb has been shown to react with $H_2O_2$ to form Lb(IV) [4], which is stable and precludes any further role of this haemoprotein in oxygen transport [4]. In the course of this reaction, a globin-derived radical can be detected [5]. Nodules have a high potential to produce damaging $O_2$-derived species such as $H_2O_2$ due to the strong reducing conditions required for $N_2$ fixation, and the action of several proteins, including ferredoxin [6], uricase, hydrogenase and Lb itself [7]. This haemoprotein is itself subject to an autoxidation process, generating superoxide anion ($O_2^{-}$) and $H_2O_2$ [8].

One of the most important protective mechanisms against $H_2O_2$ in living cells involves GSH [9], and in plant chloroplasts an ascorbate–glutathione cycle has been shown to be operative [10]. $H_2O_2$ is eliminated by an active ascorbate peroxidase [11]; the dehydroascorbate generated in this reaction can be reduced by GSH, whose oxidized form, GSSG, is reduced in turn by an NADPH-dependent glutathione reductase [12]. A similar protective system is present in the root nodules of leguminous plants [13]. Furthermore, Dalton et al. [7] recently showed that the ascorbate–glutathione cycle can adjust to varying physiological conditions in nodules and that there is a key link between $N_2$ fixation and this detoxification process.

In the present study we have investigated the direct interaction between GSH and Lb(IV). Evidence that this molecule can convert Lb(IV) into ferric Lb, in a reaction where a thiyl radical (GS$^\bullet$) is involved, is presented. The potential protective role of this process in functioning nodules is discussed.

## EXPERIMENTAL

### Purification of Lb

Soybeans (*Glycine max*) were grown in a glasshouse, and the Lb components (Lb*a*, $c_1$ and $c_3$) purified from the root nodules as described previously [14]. All the experiments reported here were carried out with Lb*a*. Ferric Lb was prepared by oxidation with ferricyanide, followed by chromatography on a Sephadex G-15 column; ferrous Lb was obtained as described by Aviram et al., [4]. The Lb concentration was determined by the pyridine haemochromogen assay [15].

### Spectrophotometric assays

Reaction of Lb with $H_2O_2$ in the presence of GSH was monitored by recording repetitive scans over the range 450–750 nm; the absorption maxima values for the studied Lb forms were as following: 543 nm for Lb(IV), 625 nm for ferric Lb, 574 and 541 nm for oxyLb. GSH oxidation was measured by coupling it to NADPH oxidation in the presence of GSSG reductase and recording the absorption decrease at 340 nm. Reaction mixtures contained 50 $\mu$M ferric Lb, 1 mM GSH, 0.2 mM NADPH, 0.1 mM diethylenetriaminepenta-acetic acid (DTPA) and 3 U of GSSG reductase in 25 mM $KH_2PO_4$/KOH buffer, pH 7.4.

### E.s.r. experiments

E.s.r. spectra of samples contained in a standard aqueous cell were recorded at room temperature using a Bruker ESP 300 spectrometer equipped with 100 kHz modulation and a Bruker ERO 35M gaussmeter for field calibration. Hyperfine coupling constants were measured directly from the field scan and compared with previously reported values for the GS$^\bullet$ adduct [16]. Signal intensities were determined by measurement of peak-to-peak line heights on spectra recorded with the use of identical spectrometer settings. The commercial samples of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) employed were purified before use as described previously [17].

Abbreviations used: GS$^\bullet$, glutathione thiyl radical; $O_2^{-}$, superoxide anion; Lb, leghaemoglobin; Lb(IV), ferryl-leghaemoglobin; DTPA, diethylenetriaminepenta-acetic acid; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide.

## RESULTS

### Reduction of ferryl Lb by GSH

The addition of $H_2O_2$ to ferrous or ferric Lb is known to generate a ferryl [iron(IV)–oxo] species [4]. However, in the case of ferric Lb, frequent side reactions occur [4]. When the reaction between 50 $\mu$M ferric Lb and 100 $\mu$M $H_2O_2$ was allowed to proceed for at least 2 min, a compound exhibiting absorption maxima at 628 and 575 nm and previously described [18,19] was obtained; its spectrum is represented in Figure 1(b) and differs markedly from that of Lb(IV). This species was not reducible by GSH. When, before $H_2O_2$ addition ferric Lb was mixed with GSH, a typical Lb(IV) spectrum was obtained after 1 min (Figure 1a) without

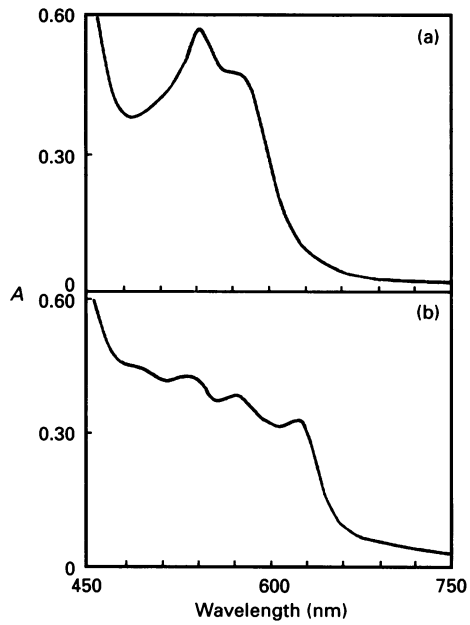


**Figure 1 Action of $H_2O_2$ on ferric Lb from soybean root nodules**

Ferric Lb (50 $\mu$M) was mixed with $H_2O_2$ (100 $\mu$M) in 25 mM $KH_2PO_4$/KOH buffer, pH 7.4, containing 0.1 mM DTPA, in the absence (**b**) or in the presence (**a**) of GSH (1 mM). Spectra were recorded 2 min after $H_2O_2$ addition and reactions were allowed to proceed at 25 °C.

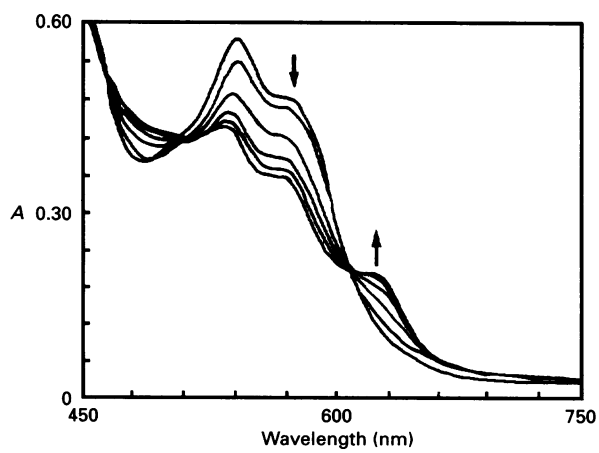


**Figure 2 Spectral changes during the reduction of Lb(IV) by GSH**

Assays conditions were as in Figure 1(a). Repetitive scans were recorded 1 min after addition of $H_2O_2$ and then at 3 min intervals.

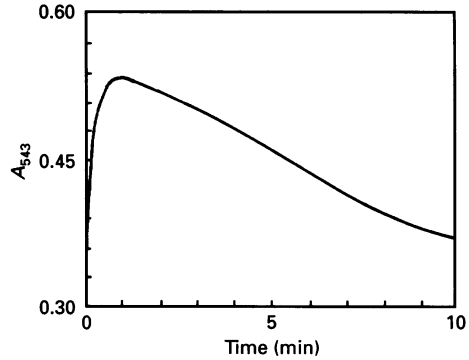


**Figure 3 Time course of absorption variation at 543 nm, the absorption maximum of Lb(IV)**

Assays conditions were as in Figure 1(a).

any further appearance of the compound described above. There was then a conversion of Lb(IV) into ferric Lb, indicating that GSH was able to reduce Lb(IV). Thus, as shown by Figure 2, there was an increase in absorbance at 625 nm with a simultaneous decrease at 543 nm. The excellent isosbestic points clearly indicated that Lb(IV) and ferric Lb were the only two species involved in this reaction. GSH was unable to reduce ferric Lb to ferrous Lb; this process is probably restricted by an unfavourable redox potential. This was clearly shown by the absence of further absorption changes at 541 and 574 nm, the absorption maxima of oxyLb, which should arise from the oxygenation of the ferrous form. In contrast, these spectral modifications were observed upon the addition of NADH to the reaction mixture, pointing to the formation of ferrous Lb. Similar results were observed when Lb(IV) was generated from the reaction between ferrous Lb and $H_2O_2$, except that Lb(IV) was obtained in all cases (i.e. when $H_2O_2$ was added before or after GSH).

The time course of the reaction was monitored at 543 nm, the maximum of absorption of Lb(IV) [4]. After a rapid increase during the first minute [corresponding to the formation of Lb(IV)], there was a decrease due to the reduction of Lb(IV) to ferric Lb (Figure 3). This last phase proceeded almost linearly for 5 min and then slowed markedly (Figure 3).

The GSH-dependent reduction of Lb(IV) was associated with the oxidation of GSH to oxidized glutathione GSSG. This was assayed by the coupled oxidation of NADPH in the presence of GSSG reductase, as illustrated in Figure 4. It should be noted that Lb(IV) oxidized NADPH directly, even in the presence of GSH (Figure 4, curve 1). However, the oxidation rate was significantly higher in the presence of GSSG reductase (curve 2). Furthermore, the oxidation of NADPH in the presence of GSSG reductase proceeded at a higher rate and for longer under aerobic (curve 2) conditions than under anaerobic ones (curve 3). In the latter case, GSH oxidation appeared to cease when the Lb(IV)-into-ferric Lb conversion was complete and a stoichiometry ($H_2O_2$ added/GSSG formed) close to 1 could be calculated. In the presence of $O_2$, continuous oxidation of NADPH resulted in amounts of GSSG in excess of added $H_2O_2$.

### Formation of GS$^{\cdot}$ during the reaction

When similar reaction systems containing 53 $\mu$M ferric Lb, 1 mM GSH, 166 $\mu$M $H_2O_2$ (added last) and the spin trap DMPO (33 mM) were incubated within the cavity of the e.s.r. spec-

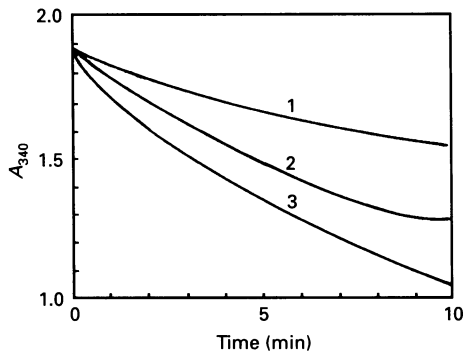


**Figure 4 Oxidation of GSH to GSSG by ferric Lb and $H_2O_2$**

Reaction mixtures contained ferric Lb (50 $\mu$M), GSH (1 mM), NADPH (0.2 mM), DTPA (0.1 mM) and 3 units of GSSG reductase in 25 mM $KH_2PO_4$/KOH buffer, pH 7.4. The reactions were started by the addition of $H_2O_2$ (100 $\mu$M) under aerobic (curves 1 and 2) or anaerobic (curve 3) conditions. Curve 1 was obtained in the absence of GSSG reductase.

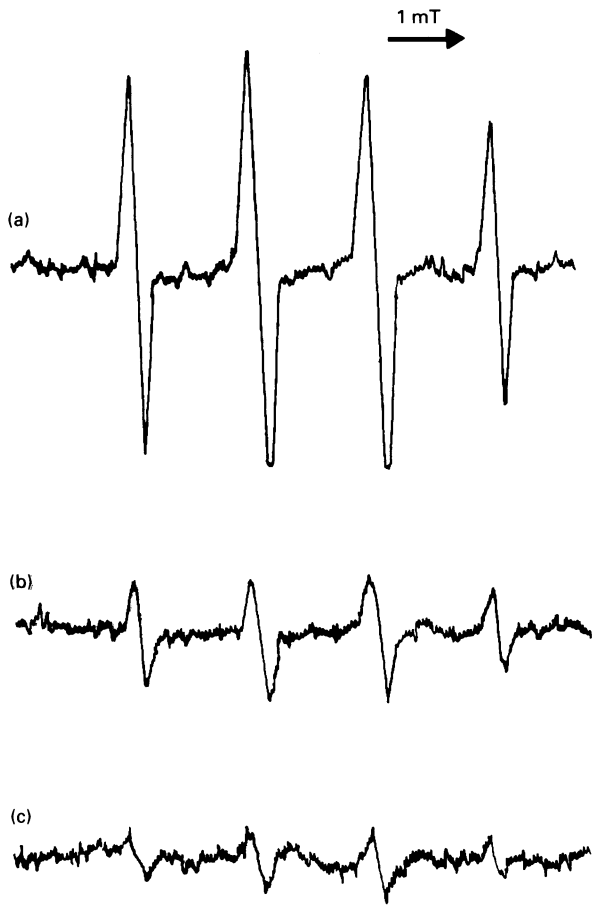


**Figure 5 E.s.r. signals obtained in reaction systems containing 33 mM DMPO and (a) 53 $\mu$M ferric Lb, 1 mM GSH and 166 $\mu$M $H_2O_2$ (added last), (b) 53 $\mu$M ferric Lb and 166 $\mu$M $H_2O_2$ incubated for 5 min and then 1 mM GSH or (c) 93 $\mu$M oxyLb, 1 mM GSH and 330 $\mu$M $H_2O_2$ (added last)**

trometer, a strong signal consisting of four lines was immediately observed (Figure 5a). The parameters of this signal are identical with those previously determined for the GS$^\bullet$ radical adduct to the spin trap [16], suggesting that high concentrations of GS$^\bullet$ are being generated in this system. Omission of any component of the reaction mixture resulted in the loss of this signal, confirming that GSH is incapable of reducing ferric Lb to ferrous Lb, and that molecular oxidation by $H_2O_2$ is not a significant source of GS$^\bullet$ under these conditions.

The exact mechanism of formation of GS$^\bullet$ cannot be determined from these experiments as two possible mechanisms can be envisaged; firstly, reduction of the ferryl species as suggested by the above optical experiments, and secondly, via reaction of GSH with the globin radical as previously reported [5]. In order to obtain further evidence for the former process two further types of experiments were carried out. In the first of these the GSH was added (at concentrations identical with those used above) 5 min after the $H_2O_2$ was added to the ferric Lb; this period of preincubation was chosen as it is known from our previous studies [5] that the globin radical has decayed away by this time. Identical signals were observed (Figure 5b), though at much lower intensities (concentrations), suggesting that though some of the GS$^\bullet$ formation is arising via reaction with the globin radical, significant reaction also occurs at the iron centre. It should however be noted that the optical experiments described above suggest that there are additional unidentified iron species as well as Lb(IV), which are not Lb(III) or Lb(II), present in the reaction system at this time. It is therefore impossible to rule these out as the source of the GS$^\bullet$ radicals.

The second approach employed was to study the reaction of GSH with the ferryl species generated from reaction of oxyLb with $H_2O_2$, as we have shown [5] that the globin radical is not formed to any significant extent under these conditions. Reaction of oxyLb (93 $\mu$M) with GSH (1 mM) and $H_2O_2$ (330 $\mu$M, added last) in the presence of DMPO (33 mM) also results in the immediate detection of the GS$^\bullet$ adduct to DMPO, though at significantly lower concentrations than observed in the ferric Lb system (Figure 5c). Omission of any components of the system resulted in the loss of this signal. These results confirm that the GS$^\bullet$ radical is formed on reduction of the ferryl form to the ferric species by GSH. The low concentrations of the radical observed in this system can be, at least partly, explained by the low rate of formation of the ferryl species in this oxylLb/$H_2O_2$ system, as previously described [4].

## DISCUSSION

The present paper describes the reduction of Lb(IV) to ferric Lb with the concomitant oxidation of GSH. This process can be considered as a peroxidase-like reaction and can be compared with the previously described pseudoperoxidatic activity of Lb [19,20]. This process can, however, also constitute a protection of this haemoprotein, since Lb(IV) is an inactivated form, and the regenerated ferric Lb can, in contrast, be easily re-reduced to the functional ferrous species by the Lb reductase enzymic system present in the nodules [21,22]. It should be noted that a similar process has been described in the case of the GSH-dependent reduction of peroxides during ferryl- and met-myoglobin interconversion [23]. This reaction is likely to occur if any Lb(IV) is formed *in vivo*, as high GSH concentrations have been detected in soybean nodules [7]. Furthermore, provided that GSH and Lb are present in the same cellular compartment (Lb is in the host-cell cytosol and the same localization for GSH appears likely [13]), a molar ratio (GSH/Lb) of 10 can be obtained from the work of Dalton et al. [7]. This is not far from the concentration ratio used in our experiments. On the other hand, GSH is able to prevent the formation of side products during the reaction of ferric Lb with $H_2O_2$. This ability indicates that this hydrophilic molecule can enter the haem pocket; other molecules such as

thiourea, salicylate and desferrioxamine have been previously shown to behave similarly [18]. The penetration of these molecules is probably favoured by the large haem-accessibility of Lb [24].

Thus the essential protective role of GSH against activated oxygen species in nodules is at least 2-fold. This molecule can act (i) via the GSH/ascorbate cycle, or (ii) by directly converting Lb(IV) into the ferric form. It should also be noted that we have recently reported that GSH was able to significantly inhibit (74%) the formation of a globin-derived radical in Lb treated with $H_2O_2$ [5]. The reduction of Lb(IV) involves the formation of GS$^\bullet$ according to the reaction:

$$\text{Fe(IV)-OH} + \text{GSH} \rightarrow \text{Fe(III)} + H_2O + \text{GS}^\bullet$$

The much lower yield of GS$^\bullet$ (as determined by the intensity of the adduct signals in Figure 5c versus Figure 5a) with the 'pure' ferryl system suggests that much of the GS$^\bullet$ formed from the ferric Lb system must be coming from reaction with the globin radical, though some also comes from the ferryl Lb reduction. Both of these processes may be protective in that the further reactions of the globin radical may well result in inactivation of the protein by cross-linking. The latter process could be involved in the formation of the species observed in Figure 1(b) and prevented in the presence of GSH. On the other hand, it can be proposed that the fate of GS$^\bullet$ *in vivo* is to produce GSSG [25].

The differences observed in NADPH oxidation between experiments performed in the presence and in the absence of $O_2$ strongly suggest the involvement of additional sources of $H_2O_2$ formed in the course of the reaction, possibly from the reduction of molecular $O_2$. We failed to detect GSOO$^\bullet$ (peroxysulphenyl radical) and GSSG$^{-\bullet}$ (glutathione disulphide radical anion), which would be expected to have very distinct spectra [26,27] in the e.p.r. experiments and could be involved in the formation of $O_2^-$ in our reaction mixtures. However, these negative results do not preclude the formation of low concentrations of these species.

These direct reactions of Lb with GSH probably result in very low concentrations of Lb(IV) in functioning nodules and can help account for the exclusive presence of ferrous (oxygenated or not) Lb *in vivo* [3], allowing an optimal flux of $O_2$ to the bacteroids for the nitrogen fixation process.

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## REFERENCES


1 Bergersen, F. J. (1980) in Nitrogen Fixation (Stewart, W. D. P. and Gallon, J. R., eds.), pp. 139–160, Academic Press, London and New York
2 Appleby, C. A. (1974) in The Biology of Nitrogen Fixation (Quispel, A., ed.), pp. 521–554, North-Holland Publishing Co., Amsterdam
3 Nash, D. T. and Schulman, H. M. (1976) Biochem. Biophys. Res. Commun. **68**, 781–785
4 Aviram, I., Wittenberg, B. A. and Wittenberg, J. B. (1978) J. Biol. Chem. **253**, 5685–5689
5 Davies, M. J. and Puppo, A. (1992) Biochem. J. **281**, 197–201
6 Misra, H. P. and Fridovich, I. (1971) J. Biol. Chem. **246**, 6886–6890
7 Dalton, D. A., Post, C. J. and Langeberg, L. (1991) Plant Physiol. **96**, 812–818
8 Puppo, A., Rigaud, J. and Job, D. (1981) Plant Sci. Lett. **22**, 353–360
9 Meister, A. and Anderson, M. E. (1983) Annu. Rev. Biochem. **52**, 711–760
10 Foyer, C. H. and Halliwell, B. (1976) Planta **133**, 21–25
11 Nakano, Y. and Asada, K. (1981) Plant Cell Physiol. **22**, 867–880
12 Halliwell, B. (1981) in The Structure and Function of Chloroplasts in Green Leaf Cells, pp. 179–205, Clarendon Press, Oxford
13 Dalton, D. A., Russell, S. A., Hanus, F. J., Pascoe, G. A. and Evans, H. J. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 3811–3815
14 Puppo, A. and Rigaud, J. (1987) Electrophoresis **8**, 212–214
15 Bergersen, F. J., Turner, G. L. and Appleby, C. A. (1973) Biochim. Biophys. Acta **292**, 271–282
16 Davies, M. J., Forni, L. G. and Shuter, S. L. (1987) Chem.–Biol. Interact. **61**, 177–188
17 Buettner, G. R. and Oberley, L. W. (1978) Biochem. Biophys. Res. Commun. **83**, 69–74
18 Puppo, A. and Halliwell, B. (1988) Planta **173**, 405–410
19 Sievers, G. and Rönnberg, M. (1978) Biochim. Biophys. Acta **533**, 293–301
20 Puppo, A., Rigaud, J., Job, D., Ricard, J. and Zeba, B. (1980) Biochim. Biophys. Acta **614**, 303–312
21 Puppo, A., Rigaud, J. and Job, D. (1980) Plant Sci. Lett. **20**, 1–6
22 Saari, L. L. and Klucas, R. V. (1984) Arch. Biochem. Biophys. **231**, 102–113
23 Galaris, D., Cadenas, E. and Hochstein, P. (1989) Free Radical Biol. Med. **6**, 473–478
24 Vuk-Pavlovic, S., Benko, B., Maricic, S., Lahajnar, G., Kuranova, I. P. and Vainshtein, B. K. (1976) Int. J. Peptide Protein Res. **8**, 427–434
25 Ross, D., Norbeck, K. and Moldeus, P. (1985) J. Biol. Chem. **260**, 15028–15032
26 Gilbert, B. C., Laue, H. A. H., Norman, R. O. C. and Sealy, R. C. (1975) J. Chem. Soc., Perkin Trans. II, 892–900
27 Sevilla, M. D., Yan, M. and Becker, D. (1988) Biochem. Biophys. Res. Commun. **155**, 405–410