Pergamon

0031-9422(95)00161-1

Phytochemistry, Vol. 39, No. 6, pp. 1281–1286, 1995

0031-9422/95 $9.50 + 0.00

THE REACTIVITY OF ASCORBATE WITH DIFFERENT REDOX STATES OF LEGHAEMOGLOBIN

SOPHIE MOREAU, ALAIN PUPPO* and MICHAEL J. DAVIES†

Laboratoire de Biologie Végétale et Microbiologie, CNRS URA 1114, Université de Nice-Sophia Antipolis, 06108 Nice Cédex 2, France; †Department of Chemistry, University of York, York YO1 5DD, U.K.

(*Received in revised form* 19 *December* 1994)

Key Word Index—Leghaemoglobin; ascorbate; hydrogen peroxide; ferryl; radicals; electron paramagnetic resonance spectroscopy; oxidative damage; nitrogen fixation; root nodules.

Abstract—Ascorbate has been previously shown to reduce the short-lived and reactive ferryl [Fe(IV) = O] states of myoglobin and haemoglobin. In this study it is shown that ascorbate is also able to reduce the otherwise long-lived and stable ferryl species [Lb(IV)] formed by the reaction of ferric or ferrous soybean leghaemoglobin with H_2O_2. The conversion of the ferryl species to ferric Lb, which appears to be moderately fast, is followed by a slow conversion of ferric Lb to oxyLb, by way of deoxy ferrous Lb. No reaction between ascorbate and oxyLb has been observed, in contrast to the previously reported pro-oxidant effect of ascorbate on oxymyoglobin. The addition of ascorbate prior to H_2O_2 to ferric Lb also prevents the formation of an additional species (which is not observed with myoglobin or haemoglobin); the optical spectra and migration on isoelectric focusing gels of this compound differ markedly from that of Lb(IV). When added after H_2O_2, ascorbate is able to reduce slowly this additional compound to oxyLb. These reactions are likely to occur *in vivo* as high ascorbate concentrations have been detected in soybean nodules. The reduction of Lb(IV) to ferric Lb is accompanied by oxidation of ascorbate, which has been detected by loss of the parent molecule in optical experiments and by the direct detection of ascorbyl radicals by electron paramagnetic resonance (EPR) spectroscopy. Analysis of the kinetics and stoichiometry of ascorbate oxidation suggests that the Lb(IV) species, the globin-derived radical(s) (which are situated on different residues and are in markedly different environments to those present in myoglobin and haemoglobin), the additional species and Lb(III) all appear to be involved in the formation of the ascorbyl radical. All of these processes are protective and may contribute to the maintenance of Lb in its functional ferrous form.

INTRODUCTION

Nitrogen fixation in legume root nodules requires, among other nodule-specific mechanisms, the presence of leghaemoglobin (Lb), a monomeric protein of M_r *ca* 16000. The major function of this haemoprotein *in vivo* appears to be to transport oxygen to the bacteroids at a low, but constant, concentration that is compatible with both bacteroidal nitrogenase activity and respiration [1]. In the root nodules, Lb exists mainly in the reduced form but, because of the low O_2 partial pressure *in vivo*, only *ca* 20% is oxygenated under steady-state conditions to give oxyleghaemoglobin (oxyLb) [2]. OxyLb can undergo a slow autoxidation to the ferric form with the concomitant production of superoxide radical ($O_2^{\cdot-}$) and hence H_2O_2 by disproportionation [3]. As with haemoglobin and myoglobin, Lb has been shown to react with H_2O_2 to form Lb(IV); with the met (ferric) form of the protein a globin-derived radical can also be detected during the course of this reaction [4]. The formation of potentially damaging O_2-derived species, such as H_2O_2, in root nodules, may also occur (in addition to Lb autoxidation) as a result of the strong reducing conditions required for N_2-fixation and the action of several proteins including ferredoxin, uricase and hydrogenase.

It is well known that ascorbic acid is involved in important protective mechanisms against H_2O_2 in living cells and in plant chloroplasts, where an ascorbate–glutathione cycle is operative [5]. A similar protective system is present in the root nodules of leguminous plants [6]. Furthermore, Dalton *et al.* [7] have recently shown that the effectiveness of these systems is highly correlated with both the enzymatic activities and concentrations of most of the components of the ascorbate-glutathione pathway. In addition to its action via this cycle, glutathione (GSH) has also been shown to be able to directly convert Lb(IV) into the ferric form, thus contributing to the maintainance of Lb in its functional form [8].

In the present study, we have investigated the direct interaction between ascorbate and various redox states

*Author to whom correspondence should be addressed.

of Lb. Evidence that this molecule can convert Lb(IV) into both the ferric and (functional) ferrous Lb forms is presented. The role of this process *in vivo* is discussed.

RESULTS

Reduction of ferryl Lb and the additional compound by ascorbate

It has previously been shown [8, 9] that beside the generation of a ferryl [iron(IV)-oxo] species, the addition of H_2O_2 to ferric Lb gives rise to a compound with spectral characteristics differing markedly from those of Lb(IV). Furthermore, when submitted to isoelectric focusing, this species migrates differently from authentic Lb(IV) obtained by reaction of ferrous Lb with H_2O_2 (unpublished results). In this report, this species will be referred to as the additional species. When ferric Lb was mixed with ascorbate immediately before H_2O_2 addition, a typical Lb(IV) spectrum was obtained after 1 min without the appearence of the unidentified compound described above. Lb(IV) was subsequently converted into ferric Lb, indicating that ascorbate was able to reduce Lb(IV). This spectral change, which is characterized by an increase in absorbance at 625 nm with a simultaneous decrease at 543 nm, is illustrated by Fig. 1A. The excellent isosbestic points obtained clearly indicate that Lb(IV) and ferric Lb are the only two major species involved in this reaction. The conversion to ferric Lb, which appeared moderately fast under our experimental conditions, was complete 2.5 min after addition of H_2O_2. This rapid interconversion is followed by a further slow process which is clearly shown by the presence of further absorbance changes at 541 and 574 nm; these correspond to the absorption maxima of oxyLb (Fig. 1B). This conversion of ferric Lb to oxyLb, is presumed to occur via the formation of the deoxy (ferrous) form and subsequent rapid oxygenation. The excellent isosbestic points obtained during this second process rule out the possibility of the formation of other species in addition to ferric and oxyLb. The observation of this second process is in good agreement with a previous report on the reduction of (untreated) ferric Lb by ascorbate [10].

The oxidation of ferric Lb to Lb(IV) by H_2O_2 in reaction mixtures which contained ascorbate was only transitory and the rate of re-reduction of Lb(IV) to ferric Lb, was found to be dependent upon the amount of ascorbate added, as measured by the rate of decrease in absorbance at 543 nm (the absorbance maximum of Lb(IV); Fig. 2). With 50 μM Lb and 100 μM H_2O_2, the Lb(IV) reduction was complete after 2.5 min with 1 mM ascorbate, and after 5 min with 250 μM ascorbate. In all of these cases, no absorbance changes were observed at 611 nm, the wavelength of one of the isosbestic points corresponding to the conversion of Lb(IV) to ferric Lb. A small decrease in absorbance at this wavelength was however observed in the absence of ascorbate.

The reduction of Lb(IV) to ferric Lb was accompanied by the oxidation of ascorbate. H_2O_2 alone was found to be unable to oxidize ascorbate, and ferric Lb catalysed this oxidation very poorly (see above). In contrast, oxidation occurred rapidly when both ferric Lb and H_2O_2 were present in the reaction mixture (Fig. 3), and the rate of oxidation was found to be dependent upon the amount of H_2O_2 added. When the ratio H_2O_2/Lb was <1 little ascorbate oxidation was observed, in good agreement

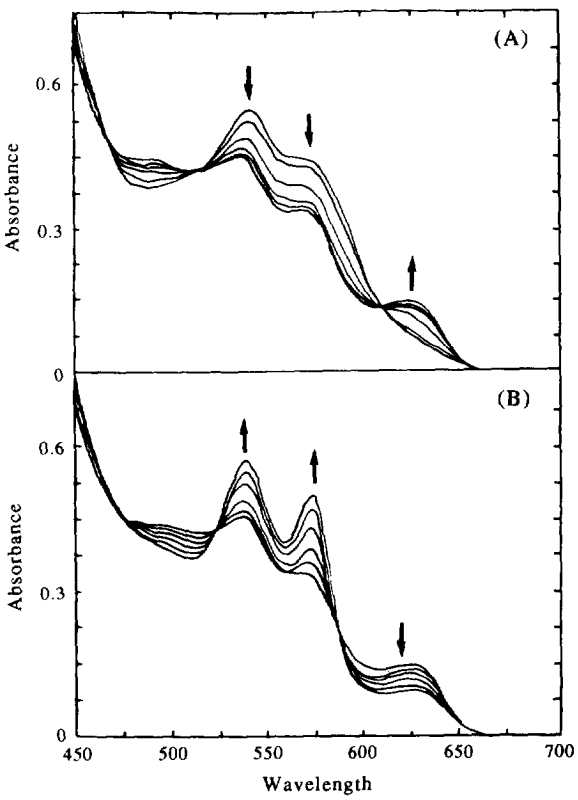

Fig. 1. Spectral changes during the reduction of Lb(IV) by ascorbate. Ferric Lb (50 μM) was mixed with H_2O_2 (100 μM) immediately after the addition of 1 mM ascorbate, in 25 mM KH_2PO_4–KOH buffer containing 0.1 mM DTPA. Reactions were run at 25° and repetitive scans were recorded at 30 sec-intervals (A) and then at 30 min-intervals (B). Spectral changes are indicated by arrows. Data are representative of experiments made in triplicate.

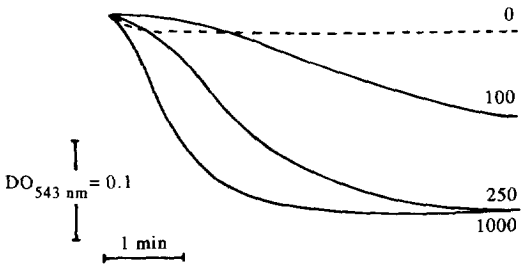

Fig. 2. Dependence of Lb(IV) reduction on ascorbate concentration. Assays conditions were as in Fig. 1 except that the concentrations of ascorbate were 0, 100, 250 and 1000 μM. Data are representative of experiments made in triplicate.

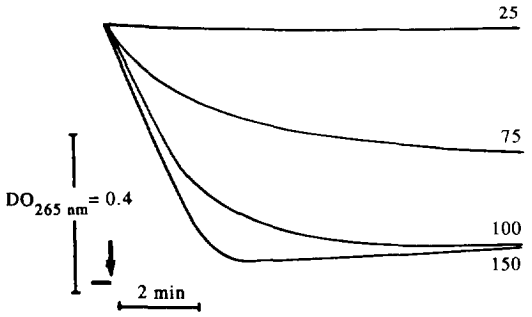

Fig. 3. Oxidation of ascorbate by ferric Lb and H_2O_2. Assays conditions were as in Fig. 1 except that the concentration of ascorbate was 100 μM. H_2O_2 was added to the reaction mixtures at 25, 75, 100 and 150 μM final concentrations. Data are representative of experiments made in triplicate.

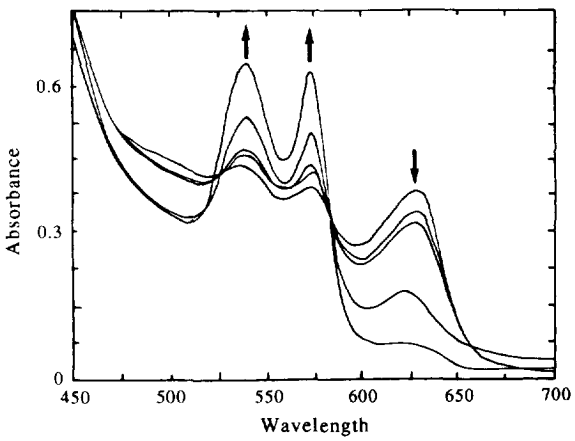

Fig. 4. Spectral changes during the reduction of the additional species by ascorbate. Assays conditions were as in Fig. 1 except that H_2O_2 was mixed with Lb before the addition of 1 mM ascorbate. Scans were recorded 0, 1, 2, 6 and 18 hr after the addition of ascorbate; spectral changes are indicated by arrows. Data are representative of experiments made in triplicate.

with previous reports which indicate that ratios >1 are necessary for Lb(IV) formation [11]. The amount of ascorbate oxidized in the presence of ferric Lb was found to be stoichiometrically related to the amount of H_2O_2 added and was *ca* 1:1 (Fig. 3): ascorbate (100 μM) was fully oxidized in the presence of 100 μM H_2O_2. A similar ratio has been reported for the corresponding myoglobin reaction [12]. This is somewhat surprising given the dramatically different accessibilities of the haem centres in these two proteins. However, in contrast, to the myoglobin system [13], no reaction was observed between ascorbate and oxyLb.

When ascorbate was added after pre-incubation of Lb and H_2O_2 for 5 min, the additional compound described previously [8, 9] appeared and was subsequently slowly reduced (Fig. 4). After 18 hr, a typical spectrum of oxyLb was obtained. No evidence for the intermediacy of Lb(III) was obtained.

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 ascorbate).

Direct detection of ascorbyl radical formation

Similar reaction systems were incubated within the cavity of an EPR spectrometer, to investigate the possible formation of ascorbyl radicals during these processes. In all cases, under our experimental conditions, it proved impossible to eliminate completely ascorbate oxidation in the presence of buffer alone; even after treatment of all solutions with Chelex 100, small background signals from the ascorbyl radical were still present, though at a low level.

In all the systems described in the above paragraph, EPR spectra consisting of a pair of sharp lines, characteristic of the ascorbyl radical [14] were detected. The concentration of this radical was however found to vary dramatically depending on the order in which the reagents were added (Fig. 5). Omission of Lb gave some ascorbyl radical (presumably by oxidation with H_2O_2) which was in excess of that seen with ascorbate alone.

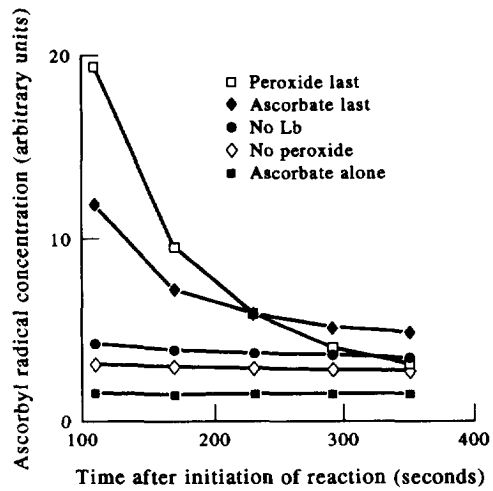

Fig. 5. Direct measurement of ascorbyl radical formation by EPR spectroscopy. Variation of the ascorbyl radical concentration (measured in arbitrary units), determined by EPR spectroscopy, with time during the reaction of ferric Lb (50 μM) with H_2O_2 (100 μM) in the presence of 1 mM ascorbate in 10 mM KH_2PO_4–KOH buffer, pH 7.4. Data from a single experiment out of three separate determinations which differed in value by less than 15%.

Similarly, omission of H_2O_2 gave significantly greater yields of ascorbyl radical than controls with ascorbate alone.

Much greater yields of ascorbyl radicals were seen with all the reagents present but the amount depended on whether the peroxide or ascorbate were added last; a lower concentration of ascorbyl radicals was generated when ascorbate was added after H_2O_2 (Fig. 5). The yield of ascorbyl radicals was even lower when ascorbate was

added after 5 min of preincubation of Lb with H_2O_2, instead of immediately after H_2O_2 addition. It is also worth noting that ascorbyl radical generation occurred for a lot longer (and hence gave higher levels of ascorbyl radicals) when the ascorbate was added at increasingly long times after H_2O_2. As shown in Fig. 6, the lines for ascorbate added immediately after H_2O_2 and after 5 or 10 min of preincubation cross over.

DISCUSSION

The results described above are consistent with the reduction of the species obtained by reaction of Lb(III) with H_2O_2, by ascorbate. By comparison with the previously described reactivity of ascorbate towards different redox states of myoglobin and haemoglobin, several important differences are worthy of note: (i) unlike myoglobin, Lb forms the additional compound [8, 9] that appears also reducible by ascorbate; (ii) in contrast with the ferryl state of myoglobin, the ferryl form of Lb is very long-lived and it has already been shown that the radical on Lb behaves in a different manner to the corresponding radical on myoglobin in that it is less accessible to the surface, is located on a different site, and does not react to give a spin adduct with DMPO [4]; nevertheless, both ferryl Lb and the radical form appear reducible by ascorbate; (iii) the absence of any observable reaction between ascorbate and oxyLb differs markedly from the recently reported oxidation of oxymyoglobin by ascorbate [13]. This difference may be due to the presence of an electrostatic gate in Lb [15]; this restricts the escape of O_2^-· from the haem pocket, and may also inhibit the entrance, and hence oxidation, of ascorbate.

Thus, in contrast with myoglobin [13], there is no pro-oxidant action of ascorbate on Lb. Taken together, these results are consistent with a very efficient role of ascorbate in reducing the different oxidized forms of Lb.

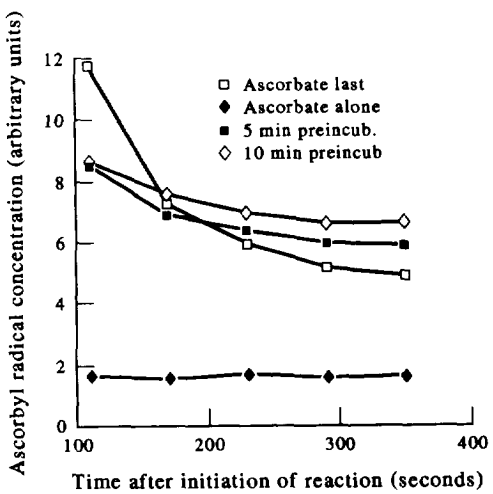

Fig. 6. Effect of preincubation of ferric Lb with H_2O_2 on ascorbyl radical formation. Reaction system and experimental design as in Fig. 5 except that H_2O_2 was mixed with the Lb for zero (ascorbate last line), 5 or 10 min before addition of 1 mM ascorbate. Data from a single experiment out of three separate determinations which differed in value by less than 10%.

The reduction process described here can be compared with the previously described pseudoperoxidatic activity of Lb [16] and with similar reactions obtained with reduced glutathione [8]. In all cases, Lb is reduced from Lb(IV) to Lb(III) and/or from Lb(III) to Lb(II) in oxidizing molecules. It may also constitute an important protective role in nitrogen-fixing nodules, since inactivated forms of Lb can be reduced to the functional ferrous species. It should be noted that, in contrast with reduced glutathione, ascorbate is able to reduce both the ferryl and the additional species of Lb to oxyLb. In the case of the additional species, the lack of evidence for the intermediacy of Lb(III) suggests that the limiting step is the conversion of the additional compound to the ferric form. These reactions are likely to occur if any Lb(IV) is formed *in vivo*, as high ascorbate concentrations have been detected in soybean nodules [17]. Furthermore, the molar ratio of ascorbate to Lb (of 8) believed to be present in the host cell cytosol [17], is not far from the concentration ratio used in our experiments. In addition, ascorbate has been shown to be able to prevent the formation of the additional compound during the reaction of ferric Lb with H_2O_2. This is believed to arise because this hydrophilic molecule can enter the haem pocket; similar behaviour has been observed with other compounds such as thiourea, salicylate and desferrioxamine [9]. The penetration of these molecules is probably favoured by the large haem pocket accessibility of Lb [18]. On the other hand, the formation of the additional species appears responsible for the small absorbance decrease observed at 611 nm in the absence of ascorbate.

In the processes described here, the formation of ascorbyl radicals could arise from different routes (in addition to that involving ascorbate plus H_2O_2). The first one involves the slow reduction of ferric Lb by ascorbate (see Fig. 1B):

$$\text{Lb(III)} + \text{ascorbate}^- \rightarrow \text{Lb(II)} + \text{ascorbyl radical}^{\cdot} \quad (1)$$

The second—and probably the most important quantitatively—involves the globin radical:

$$\text{Globin radical}^{\cdot} + \text{ascorbate}^- \rightarrow \text{globin-H} + \text{ascorbyl radical}^{\cdot} \quad (2)$$

This reaction has previously been demonstrated to be rapid, on the basis of the extremely fast loss of the protein radical on addition of ascorbate [4]; this process is presumably the cause of the higher yield of ascorbyl radicals obtained when the ascorbate is added immediately after the H_2O_2, as opposed to 5 min later, by which time the protein radical will have decayed away [4].

The increased levels of ascorbyl radicals seen above control values after 5 min preincubation is probably due to the reduction of the ferryl species, as observed in the optical experiments (Fig. 1A):

$$\text{Lb(IV)} + \text{ascorbate}^- \rightarrow \text{Lb(III)} + \text{ascorbyl radical}^{\cdot} \quad (3)$$

The possible role of the additional species—evidenced both spectrophotometrically and by isoelectric focusing — in generating ascorbyl radicals cannot be ruled out. This possibility is strengthened by the crossing over of the lines for ascorbate added immediately after H_2O_2 and after 5 or 10 min of preincubation (see Fig. 6). This can be rationalized in terms of a further slow reaction—the reduction of the additional compound seen in the optical experiments by ascorbate:

$$\text{Additional species} + \text{ascorbate}^- \rightarrow \text{Lb(III) and/or}$$
$$\text{Lb(II)} + \text{ascorbyl radical} \quad (4)$$

The occurrence of this last reaction may also explain why the amount of ascorbyl radical was always found to be higher when ascorbate was added before H_2O_2. In this case, the prevention of the formation of the additional compound—as shown by the spectrophotometric experiments—would be expected to favour the very fast reaction of the globin radical with ascorbate. The reactivity of ascorbate with different redox states of leghaemoglobin is summarized in Fig. 7.

The observed stoichiometry of one molecule of ascorbate oxidized per H_2O_2 reacting with the protein (see Fig. 3) is consistent with the occurrence of all of the reactions (2)–(4), as subsequent decay of the ascorbyl radical via the well-characterized disproportionation reaction to give ascorbate and dehydroascorbate will result in the regeneration of one mole of ascorbate per two moles oxidized, and hence the consumption of one mole of ascorbate per two oxidizing equivalents (i.e. one mole of H_2O_2). The reactions of ascorbate with different redox states of Lb are summarized in Fig. 7.

Thus, ascorbate appears to be effective in protecting against activated oxygen species in nodules by two different ways: (i) via the GSH/ascorbate cycle, and (ii) by reducing the species formed during the reaction of Lb with H_2O_2 to the functional ferrous form. These direct reactions of Lb with ascorbate may contribute to the maintainance of Lb exclusively in its ferrous (oxygenated or not) form *in vivo* [19], allowing an optimal functioning of the nitrogen-fixing bacteroids.

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 [20]. All the experiments reported here were carried out with Lbc_1 and c_3. Ferric Lb was prepared by oxidation with ferricyanide, followed by chromatography on a Sephadex G-15 column; ferrous Lb was obtained as described in ref. [11]. The Lb concentration was determined by the pyridine haemochromogen assay [21].

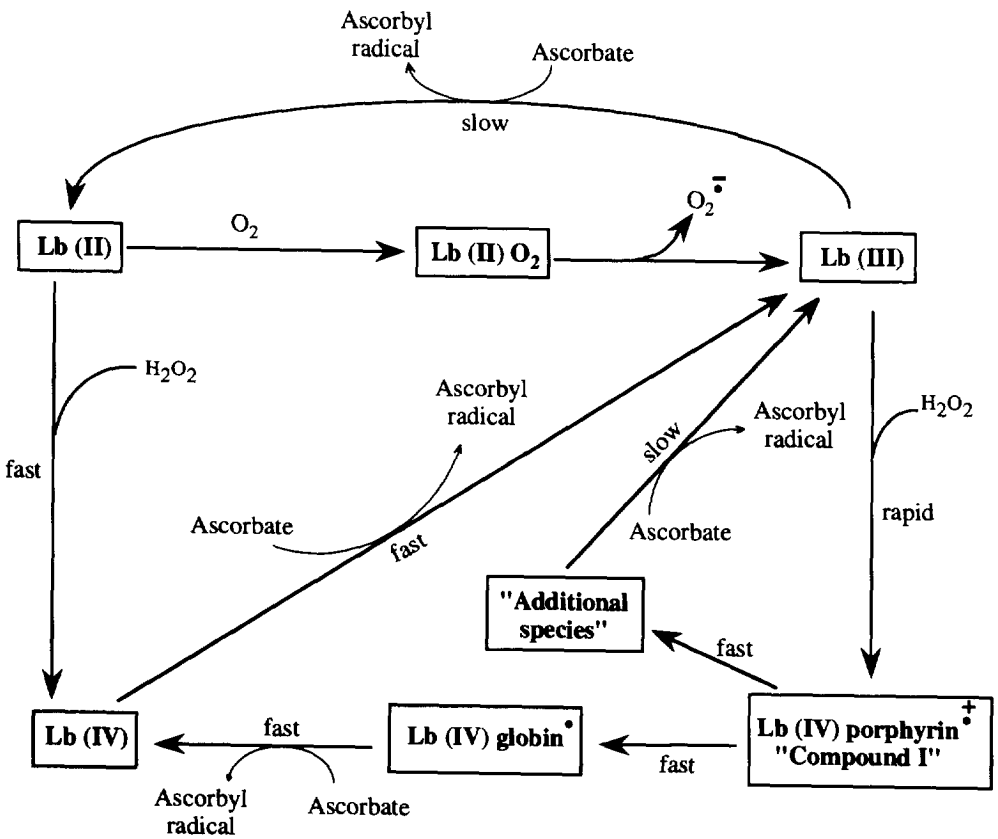

Fig. 7. Reactivity of ascorbate with different redox states of Lb.

Spectrophotometric assays. Reaction of Lb with H_2O_2 in the presence of ascorbate was monitored by recording repetitive scans over the range 450–750 nm; the absorption maxima values for the studied Lb forms were as follows: 543 nm for Lb(IV), 625 nm for ferric Lb, 574 and 541 nm for oxyLb. The oxidation of ascorbate was followed by recording the A at 265 nm [12]; although Lb(IV) and Lb(III) absorb at this wavelength, the spectral difference between these species is minimal and does not interfere with ascorbate determination.

EPR spectra. EPR spectra of samples contained in a standard aqueous cell were recorded at room temp. using either a Bruker ESP 300 spectrometer equipped with a Bruker ER035M gaussmeter for field calibration, or a Jeol RELX spectrometer, both of which employed 100 kHz field modulation. Hyperfine coupling constants were measured directly from the field scan. Signal intensities, which are directly proportional to the absolute radical concentration, were determined by measurement of peak-to-trough line heights on spectra recorded at fixed times after the initiation of the reaction, with identical spectrometer settings.

Acknowledgements—We thank Mr A. Gilabert for his help in growing the plants. The help of Mrs M. C. Poggi and Miss H. Le Bris in preparing this manuscript is also gratefully acknowledged.

REFERENCES

1. Bergersen, F. J. (1982) *Root Nodules of Legumes: Structure and Function*, pp. 23–50. Research Studies Press, Chichester.
2. Appleby, C. A. (1984) *Ann. Rev. Plant Physiol.* **35**, 443.
3. Puppo, A., Rigaud, J. and Job, D. (1981) *Plant Sci. Lett.* **22**, 353.
4. Davies, M. J. and Puppo, A. (1992) *Biochem. J.* **281**, 197.
5. Foyer, C. H. and Halliwell, B. (1976) *Planta* **133**, 21.
6. 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.
7. Dalton, D. A., Langeberg, L. and Treneman, N. C. (1993) *Physiol. Plant.* **87**, 365.
8. Puppo, A., Monny, C. and Davies, M.J. (1993) *Biochem. J.* **289**, 435.
9. Puppo, A. and Halliwell, B. (1988) *Planta* **173**, 405.
10. Becana, M. and Klucas, R.V. (1990) *Proc. Natl Acad. Sci. U.S.A.* **87**, 7295.
11. Aviram, I., Wittenberg, B. A. and Wittenberg, J. B. (1978) *J. Biol. Chem.* **253**, 5685.
12. Galaris, D., Cadenas, E. and Hochstein, P. (1989) *Arch. Biochem. Biophys.* **273**, 497.
13. Giulivi, C. and Cadenas, E. (1993) *FEBS Lett.* **332**, 287.
14. Yamazaki, I. and Piette, L. H. (1961) *Biochim. Biophys. Acta* **50**, 62.
15. Job, D., Zeba, B., Puppo, A. and Rigaud, J. (1980) *Eur. J. Biochem.* **107**, 491.
16. Puppo, A., Rigaud, J., Job, D., Ricard, J. and Zeba, B. (1980) *Biochim. Biophys. Acta.* **614**, 303.
17. Dalton, D. A., Post, C. J. and Langeberg, L. (1991) *Plant Physiol.* **96**, 812.
18. Vuk-Pavlovic, S., Benko, B., Maricic, S., Lahajnar, G., Kuranova, I. P. and Vainshtein, B. K. (1976) *Int. J. Peptide Protein Res.* **8**, 427.
19. Nash, D. T. and Schulman, H. M. (1976) *Biochem. Biophys. Res. Commun.* **68**, 781.
20. Puppo, A. and Rigaud, J. (1987) *Electrophoresis* **8**, 212.
21. Bergersen, F. J., Turner, G. L. and Appleby, C. A. (1973) *Biochim. Biophys. Acta* **292**, 271.