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LEGHEMOGLOBIN: PROPERTIES AND REACTIONS

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I. Introduction

Plants release oxygen during photosynthesis and use it as the terminal electron acceptor in the respiratory chain of mitochondria. There is now evidence that they contain a monomeric oxygen-binding protein to coordinate and transport oxygen (*1*). The occurrence of these myoglobin-like proteins in plants was first described by Kubo in 1939, who demonstrated that the nitrogen-fixing root nodules of soy-

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bean and other plants contained a red-colored soluble heme protein that could reversibly bind oxygen (*2*). The protein was named leghemoglobin (Lb) at an early stage by analogy to the mammalian protein. The nodules from which the protein is extracted arise from the interaction between *Rhizobium* spp., a soil bacteria, and leguminous plants (Fig. 1). More recent studies have demonstrated the presence of similar structures in the nonleguminous plant *Parasponia* spp. (*3*). The origin of the symbiosis in legumes has been reviewed (*4*), and the processes that occur during the development of the relationship are reasonably well characterized (*5–7*). Hemoglobins and their parent genes have also been recently identified in a number of nonnodulating plants and in phylogenetically diverse plant genera including monocots (*8*, *9*). Moreover, a new hemoglobin gene has been isolated recently from soybean (*10*) that is expressed in different parts of the plant (including the nodules). This contrasts with the expression of Lbs, which are active only in the infected cells of the nodule. Thus, two different types of hemoglobin are known to be present in plants, (i) a nonsymbiotic type that is widely distributed among species and (ii) a symbiotic type that is induced only on nodulation in (at least) two plant families (*1*). The genes that code for these two types of hemoglobins are separated into four exons by three introns: the first and third introns are in positions homologous to those of the two introns found in vertebrate α-globin and β-globin and myoglobin genes. It has been proposed, and seems likely, that there is a common ancestral gene for plant and animal hemoglobins (*1*, *11–13*). This review will concentrate on the properties and reactions of the symbiotic type of plant protein, the leghemoglobins.

Until now, Lb has been the most extensively studied oxygen-carrying hemoprotein from plants. The main role of Lb *in vivo* is now widely established (though for many years this was hotly disputed) as being to facilitate oxygen transport to the vigorously respiring, phosphorylating, nitrogen-fixing *Rhizobium* microsymbionts, at a low and stable oxygen tension (around 10 nM in soybean nodules) (*7*, *12*, *14*, *15*). Terminal oxidases having a K_m for oxygen as low as 7 nM are present in the microsymbionts and are able to use the Lb-delivered oxygen to support their respiration under the oxygen-limiting conditions of the nodule (*16*). This low oxygen tension appears to be a compromise between the requirement for oxygen by the respiring bacteria and the damage that oxygen can inflict on the nitrogen-fixing complex; the latter is very readily inactivated at high oxygen levels (*12*, *15*).

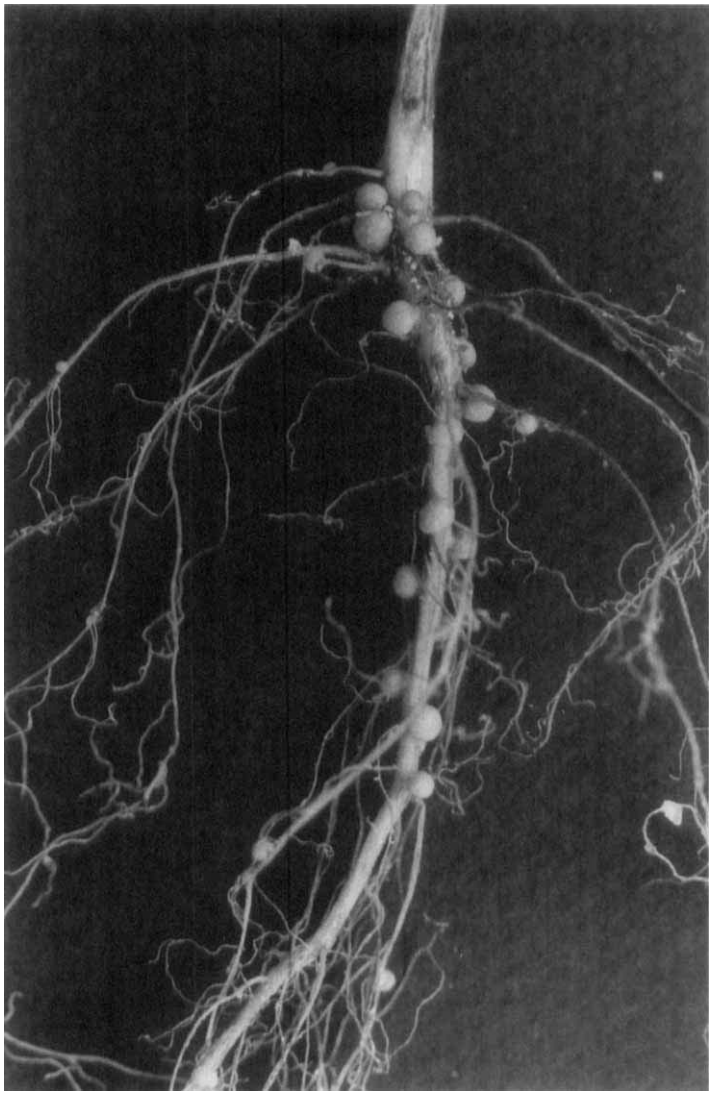

FIG. 1. Nitrogen-fixing root nodules on a soybean plant.

II. Structure

Early studies by Ellfolk demonstrated that there were several Lb isomers in soybean nodules (*17*). Ammonium sulfate fractionation of nodule extracts followed by anion exchange chromatography in ace-

tate buffers allowed the separation and purification of two major and two minor species of soybean Lb. The major species were named Lb_a and Lb_c, and the minor components Lb_b and Lb_d. Subsequent work using isoelectric focusing, which has a greater resolving power, has shown that there are four major components (subscripts *a*, *c1*, *c2*, and *c3*) rather than the two originally separated, and four minor ones (*b*, *d1*, *d2*, and *d3*) (*18*). The latter minor components are N-terminal acetylation products of the major components (*19*). All of these species are present in single nodules, with the relative concentrations of the different components varying with nodule development (*20*). This pattern of multiple gene products is widely established in other legume and nonlegume symbioses (*12*, *18*, *21*). All of the multiple Lb proteins from soybean have been sequenced, and it is now known that the differences in amino acid sequences between different Lb components from a particular plant species are relatively slight. Despite discrepancies between the published sequences obtained in early studies, it has been shown in a large study of both 69 domesticated cultivars of soybean and 18 wild species that there are few (if any) differences between the same type of leghemoglobin obtained from a number of different cultivars of a single species. The published discrepancies have been ascribed to problems in the sequencing procedures (*22*). In all cases the proteins (molecular weight *ca.* 16,000) consist of a single polypeptide chain of 144–155 amino acids, and the iron protoporphyrin IX prosthetic group. The proteins are acidic with pI values in the range 5.0–5.5.

The amino acid sequences of the soybean proteins, as well as many others, have a very high degree of amino acid sequence homology with vertebrate myoglobins and hemoglobins, and an even higher degree of homology of amino acid type. The sequence of soybean Lb_a, which is one of the most extensively studied species, contains two histidine residues (His61 and His92) (*17*), which are analogous to the proximal and distal histidines of mammalian hemoglobins and myoglobins. The spacing of these residues in the sequence is also very similar. Crystal structure work on both the soybean [(*23*); Ellis and Freeman, unpublished data] and lupin (*24–30*) forms of the protein have emphasized the similar key features of the three-dimensional protein structures of the plant and mammalian proteins. There are also a number of key differences, which appear to have quite dramatic functional consequences and determine both the very high oxygen-binding affinity of this protein (discussed later) and the ability of the plant proteins to bind larger ligands at the heme site. The evolution of the plant globin gene family has been the subject of a number of studies [see, for ex-

ample, (*31*)] and it is now believed that the plant and animal gene families diverged at a very early stage. This is estimated to be 900 to 1.4 billion years ago, though the derivation of these numbers requires certain assumptions about the rate of evolutionary change (*9*, *13*, *31*).

A number of studies have shown that the iron protoporphyrin IX group of Lb can be replaced, *in vitro,* by a variety of other species including mesohemin IX, deuterohemin IX, hematohemin IX, diacetyldeuterohemin IX, and other analogs. As expected, a number of these substitutions cause significant changes in the heme environment, and this is reflected in both the optical absorption and EPR spectra of these species (*32*, *33*). The apoprotein can also be reconstituted with a number of noniron porphyrins. Thus, holoproteins have been produced with both cobalt (disulfophthalocyanine and protoporphyrin IX) and zinc (protoporphyrin IX) among others (*18*, *34–36*); in the case of the cobalt protoporphyrin protein, information has also been obtained on the oxygen-binding capabilities of this species (*18*).

III. Biological Localization

After considerable controversy in the literature over the last thirty years it is now widely accepted that Lb only occurs in the cytoplasm of the host cell, and not in the peribacteroid space that surrounds the bacteroid (bounded by the peribacteroid membrane, Fig. 2) or the bacteroid itself (*7*, *15*, *37*). The nature and development of the membrane that surrounds the bacteroids have been examined in some detail because this membrane regulates the transport of materials to and from the bacteroids (e.g., dicarboxylates, metal ions, etc.) (*38–41*). The local concentration of Lb can be high (up to 2–3 mM) in very active nodules, with an average concentration over the entire nodule of *ca.* 300 μM (*42*). Although it is well established [see reviews (*12*, *18*)] that the apoprotein is produced by the plant (*43*) and that the Lb assembly must occur in the host cell or on the outside the peribacteroid membrane, the origin of the protoporphyrin moiety remains unclear. It is generally proposed that heme is made by the *Rhizobium* symbiont (*44*, *45*), but the possibility remains that plant organelles are at least partly responsible for this process (*46*). The mechanism of control of the synthesis of the apoprotein is disputed. The observation that heme synthesis in isolated *Bradyrhizobium japonicum* is greatly stimulated by microaerobic conditions (*45*) suggests that heme export from the nascent bacteroids to the plant cell cytoplasm might be a major stimulus for apoprotein synthesis. However, the observa-

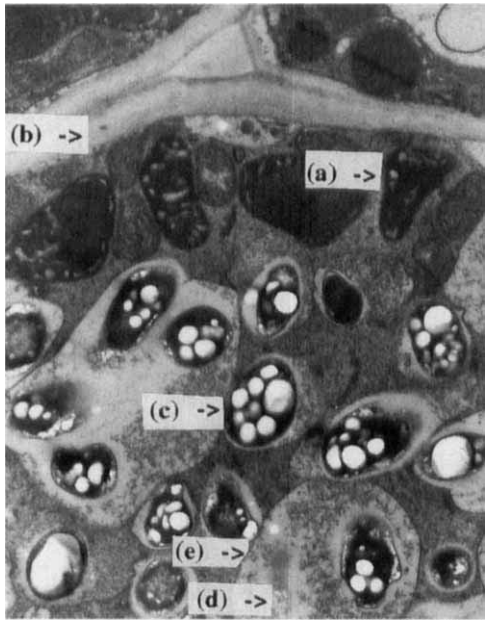

FIG. 2. Transmission electron micrograph (16,000× enlargement) through an infected root cell of a soybean plant. The subcellular organelles of the host cell [mitochondria and plasts; (a)] are present at the periphery of the cell adjacent to the host cell wall (b). The nitrogen-fixing bacteroids (c) are kept apart from the host cell cytoplasm (the location of leghemoglobin) by the peribacteroid space (d) and the peribacteroid membrane (e), which regulates transport of materials to and from the bacteroids.

tion that ineffective nodules in some mutant forms of *Bradyrhizobium japonicum* deficient in heme synthesis can contain almost normal levels of apoprotein (*47*, *48*) suggests that synthesis of the protein may not be wholly heme regulated. The assembly of holoprotein appears to precede and be independent of the presence of functional nitrogenase (*37*, *43*, *49*). However, the synthesis of the latter appears to depend on the former, possibly as a result of the control exerted by intact Lb on oxygen tension, which prevents damage to the nitrogenase (*37*).

The turnover time of intact Lb is relatively short [reviewed in (*37*)], *cf.* the long half-lives of myoglobin and hemoglobin in mammals, with the major Lb components of pea nodules having turnover times of about two days (*49*). This is considerably less than the lifetime of the nitrogenase (*50*). There must therefore be continual synthesis and degradation of Lb within the host cell cytoplasm, and there is evidence for dramatic changes (as mentioned earlier) in relative concen-

trations of different Lb isoforms during this period. Thus, it is known that Lb I predominates over Lb IV in young but not old pea nodules (*51*), and Lb_{c2} and Lb_{c3} predominate in young soybean nodules (*20*). The destruction of Lb is believed to occur by multiple pathways (*12*, *37*), with the relative contributions of these different routes strongly dependent on the environmental situation; some of these processes are discussed further later. Whatever the process, there is a strong correlation between nitrogen-fixing ability and the presence of intact functional Lb, and considerable work is under way to both understand and control these factors.

IV. Reactions with Different Molecules

A. Diatomic Gases

The extremely high oxygen-binding activity of the Fe(II) Lb is critical for its biological function, and considerable work has been undertaken to understand the factors that determine this feature of the protein. It is now well established that the high affinity arises from larger "on" (association) rate constants and smaller "off" (dissociation) rate constants (see Table I), which results in K_D values in the range of 36 to 78 nM depending on the source of the Lb and the pH (*15*, *21*, *37*, *51*, *52*). The O_2 off rate constants for soybean Lb decrease by about

TABLE I

Kinetics and Equilibrium Constants for Binding of Gaseous Ligands to Leghemoglobins and Other Monomeric Hemoglobins (*21*, *55*, *56*, *166*, *167*)

Protein	Oxygen			Carbon monoxide			Nitric oxide		
	k'[a]	k[b]	K[c]	k'[a]	k[b]	K[c]	k'[a]	k[b]	K[c]
Spermwhale Mb	14	12	860	0.51	0.019	37	17	0.00012	0.007
Spermwhale Mb[d]	140	1600	11400	5.8	0.038	6.6	150	0.00015	0.001
Human α-chain Hb	50	28	560	4.0	0.013	3.25	30	0.000046	0.0015
Soybean Lb	120	5.6	48	13	0.0078	0.6	120	0.00002	0.0001
Lupin Lb I[e]	540	20	36	42	0.014	0.33	—	—	—
Lupin Lb II[e]	320	25	78	52	0.015	0.015	—	—	—

[a] Association rate constant; $\mu M^{-1}s^{-1}$.
[b] Dissociation rate constant; s^{-1}.
[c] Equilibrium constant, k/k'; nM.
[d] Mutant spermwhale myoglobin with E7 His → Gly mutation.
[e] Major component of heterogeneous reaction.

fivefold as the pH is lowered from 7 to 4, with a pK_a of 5.46. This has been ascribed to the formation of a hydrogen bond between the bound oxygen and distal histidine (*52*). The K_D values are 11 to 24 times smaller than those for sperm whale myoglobin, which is mainly due to the higher on rate constant (*21*, *29*). Thermodynamic quantities (ΔG, ΔH, ΔS) have been determined for the binding of a number of ligands including O_2 and CO to soybean Lb (*53*), and the K_D values for all eight components of soybean Lb have been determined. Though there are significant differences in these values, the variation in terms of free O_2 concentration within nodules has been calculated to be a maximum of 3% (*53*). Thus, an earlier suggestion (*54*) that the large number of different forms of Lb in particular species is due to differing oxygen requirements, hence requiring Lb species of different oxygen affinities, is not strongly supported by this data.

High on rate constants have also been determined in studies on a sperm whale myoglobin mutant in which the E7 distal His was mutated to a Gly. The on rate constant for this mutant is approximately the same as that of soybean Lb, though it is still smaller than for two lupin forms (*21*, *55*, *56*). This type of alteration to the heme pocket is not, however, responsible for the high affinity of Lb, as the off rate constant of this mutant is orders of magnitude greater than those for the soybean and lupin forms (*55*, *56*). There must therefore be other factors that contribute to the very high affinity of Lb molecules for oxygen.

Detailed X-ray crystallographic studies of a number of different states of lupin Lb have been reported (*24–30*, *57*), and similar studies have been undertaken on the soybean protein (*23*). The structures of the oxy, deoxy, CO, and NO forms of Fe(II) lupin Lb, determined to 1.7- to 1.8-Å resolution (*29*, *30*), have allowed Fe–ligand geometries and structural changes on ligand binding to be examined. It has been found that the Lb protein has a unique feature as compared to other hemoglobins in that the proximal histidine has a remarkable rotational freedom. Thus, in the deoxyform of the protein, the imidazole is able to oscillate between two different orientations, eclipsing either a line between the N-1 and N-3 pyrrole nitrogens of the porphyrin or a line between N-2 and N-4 (*29*). In the oxy form, however, it is fixed in a staggered orientation. As with other heme proteins, the iron atom moves (by *ca.* 0.3 Å) from an out-of-plane position to an in-plane position on oxygen binding, with the Fe–N bond distances remaining constant (*29*). The Fe–O–O bond angle is 152°, very close to that seen in the human hemoglobin α-chain (153°), and the oxygen is hydrogen bonded to the distal histidine. In both forms of Lb the heme is ruffled

due to rotation of the pyrrole groups around the N–Fe–N bonds, in accord with earlier suggestions based on resonance Raman studies (*58*), though this suggestion was later disputed (*59*). This ruffling results in the methine bridges being out of plane by up to 0.32 Å (*29*).

It has been suggested from the crystal structures that the reason for the high on rate constant is the mobility of both the proximal and distal histidines in the deoxy form of the protein (*29*). The eclipsed nature of the proximal histidine would maximize steric hindrance with the pyrrole nitrogens in the deoxy form and hence minimizer $\pi \rightarrow$ p electron donation, whereas the staggered position in the oxy form would result in the opposite effect. These two factors in combination might result in a reduction of the activation energy for oxygen binding (*29*). Furthermore, though the distal histidine appears fixed in the crystal structure, it has been postulated that this can swing out of the heme pocket at a rapid rate to allow ready access of the oxygen molecule (*15*, *23*, *60*).

The kinetics of CO and NO binding to Lb have been studied, and rate constants for both association and dissociation determined under a number of different conditions (e.g., pH). The values obtained are compared with those for Mb and Hb in Table I (*61*). The off rate constants for CO are, unlike those for O_2, independent of pH (*52*). In particular, such studies have been directed to determine the mode of binding of the ligand and whether this has a bent geometry as has been observed for some hemoglobins. Such an orientation is believed to result from a steric interaction with the globin and is a major factor in reducing the affinity for these poisons (*30*). The large flexible heme site of Lb and the rapid movement of the distal histidine out of the heme pocket might be expected to result in large on rate constants for reaction with both NO and CO. The crystal data at 1.8-Å resolution has been interpreted in terms of a bent orientation of both CO and NO with an Fe–CO angle of 160° and an Fe–NO angle of 147° (*30*). The former angle is in conflict with spectroscopic data obtained with myoglobin, where a linear geometry was determined (*62*, *63*), but in accord with the angles obtained from myoglobin X-ray crystal structures (*64*, *65*) and picosecond time-resolved infrared measurements (*66*). The NO conformation is in accord with the expected bent geometry, with a strong Fe–NO bond (and hence low off rate constant), and the (predicted) lengthened (2.2-Å) iron-proximal histidine bond (*30*).

The binding of NO to the Fe(II) form of Lb (LbNO) may be of considerable biological relevance as a result of the slow off rate constant (*21*, *30*). This will result in the formation of Lb, which cannot fulfill its

role as an oxygen carrier. Such reactions have long been recognized as having a potential role in nitrate-induced stress (*67*). It has been demonstrated that Lb extracted from plants grown in the presence of nitrate contains significant amounts of LbNO; thus, an early study reported that up to 27% of the Lb present in soybean nodules was in this form of the protein (*68*). The species can be readily recognized on the basis of its characteristic UV-vis absorption features (*69*, *70*) or its intense EPR signal in the *g ca.* 2 region, which has highly distinctive hyperfine couplings arising from the nitrogen nucleus (*68*). Although it is possible that some of the NO may arise as an artifact of the isolation procedure, it may also arise from the reduction of nitrate to NO by either enzymatic or nonenzymic reactions (*67*, *68*, *71*). The former process may involve a nitrate reductase from either the plant or bacteroid. Such reactions have been characterized previously (*71*).

It is known that a variety of reducing agents such as ascorbate or reduced flavins can also bring about this reduction. Such reactions are believed to be responsible for the formation of nitrosylmyoglobin in mammalian systems (*72*). Recent careful studies have confirmed that the root nodules of nitrate-fed soybean plants do indeed contain LbNO and that this species is not an artifact of the extraction procedure (*69*, *70*). Thus, extraction of Lb from root nodules in the presence of high concentrations (100 nmol/mg protein) of nitrate added to the nodules at the time of harvest did not result in amounts of LbNO higher than those seen in plants grown in the absence of nitrate. It has also been demonstrated that the formation of LbNO requires both the presence of the Fe(II) protein and a reducing agent. Incubation with monoiodoacetic acid, a thiol (−SH) blocking agent, repressed the formation of this species, suggesting that an enzymatic process may be involved (*69*). The levels of LbNO in root nodules appear to be enhanced by culturing the plants in higher concentrations of nitrate [*cf.* the detection of 27% LbNO by Maskell *et al.* for plants grown with 0.5 mM nitrate, and of 60% by Kanayama and Yamamoto using 10 mM nitrate (*68*, *69*)] or for increasing lengths of time [*cf.* 60% and 86% LbNO for culture with 10 mM nitrate for 12 and 24 h respectively (*69*)]. The formation of such LbNO complexes may have profound physiological significance, particularly at these very high nitrate levels, as the levels of LbNO in root nodules have been shown to be inversely correlated with the acetylene (ethylene)–reducing activity of the nodules (a commonly used marker of their reductive capacity), and hence their ability to fix nitrogen (*69*).

Although these experiments have determined that LbNO can arise from the growth of plants in the presence of nitrate, the situation

with plants grown in the absence of nitrate is less well established. In the work of Kanayama and Yamamoto, levels of LbNO obtained from plants grown in the absence of nitrate were reported as being less than 10%, suggesting that reduction (either enzymatic or direct) of nitrate was a major, if not the only, source of NO (*69*). However, very recent reports have suggested that this may not be the case. Thus, it has been reported that root nodules contain an NO synthase activity that generates NO from arginine in a manner akin to mammalian NO synthases (*73*), and a recent EPR study has reported the detection of signals from LbNO in frozen intact root nodules from plants grown in the complete absence of nitrate (*74*). The levels of this complex were found to be highest in young nodules, decreased in mature nodules, and almost completely absent in old and senescent species. These reports, if they are confirmed, together with earlier studies that have demonstrated that NO can play a role in regulating gene transcription via a sensory system involving proteins of the "fix" family (*75–77*), suggest that the formation and levels of NO may play a vital role in determining the nitrogen-fixing activity of root nodules.

The determination of the crystal structures of these different forms of Lb have also allowed previous suggestions that the heme pocket of Lb is larger and more flexible than those of other heme proteins (*15*, *23*, *37*, *78*), to be examined in detail. Superposition of the backbone carbon trace of the E and F helices of the Lb structure with that obtained from HbCO and MbCO provides direct confirmation of the larger size of the heme pocket and the altered orientation of the distal histidine residue for both lupin (*30*) and soybean proteins (*15*, *23*). This greater size and flexibility also explains a further unusual property of Lb: at slightly acidic pH values the distal His of Fe(III) (met) Lb can move close enough to the iron atom to become a ligand (*15*), whereas at higher (neutral) pH values it can swing right out of the heme pocket to allow entrance of large ligands such as fatty acids, nicotinic acid, and isoquinoline to occur (*30*). In contrast to this larger pocket, the Lb structure shows a somewhat narrower "entrance" with the α-carbon of the E7 (distal) His nearer to the plane of the heme ring by *ca.* 1.5 Å compared to hemoglobin and myoglobin, but with the imidazole ring at approximately 90° to the angle found in these other proteins (*30*).

Early suggestions [e.g., (*79*)] that Lb might act to facilitate nitrogen fixation by acting as an ancillary nitrogen carrier have been shown to be unlikely. Thus, although it has been reported that N_2 complexes of both Fe(III) and Fe(II) Lb can form, these only exist to any significant extent when nitrogen gas is employed at high pressure. Thus, 1.2 at-

mospheres of N_2 were required to give half formation of the Fe(III) adduct and 1.7 atmospheres for half formation of the Fe(II) form (*80*). These species are unlikely to be of biological significance (*37*).

B. Other Molecules

It has been established in a number of studies that Lb(II) behaves in a somewhat similar manner to myoglobin and hemoglobin with respect to its binding of a number of small inorganic and organic ligands (*37*). Thus, it is known that Lb can form stable complexes with toxic groups such as azide, cyanide, alkylisocyanides, and fluoride in a manner similar to other heme proteins (*81*). The kinetics of many of these binding reactions have been examined using fast laser pulses to monitor reactions after photodissociation of the complexes, and these values have been compared with those for other heme proteins (*55*). In many cases the pattern of high on and slow off rate constants seen with diatomic molecules is mirrored with these organic ligands [see, for example, (*81*)]. The formation of the fluoro species has been suggested (*37*) to be of use in the detection of Fe(III) Lb in nodule slices or extracts. When used in large excess [concentrations up to 0.1 M have been employed (*2*, *82*)], the fluoride will displace other ligands and give rise to a characteristic (and diagnostic) UV-vis absorption band at 608–610 nm (*83*). This complex is in a pure high-spin state, which has proved useful in the detection of Lb by use of EPR spectroscopy (*37*). The EPR parameters of a number of other Lb–ligand complexes have been studied in some detail (*68*).

However, unlike most mammalian heme proteins, Lb(III) is also known to readily form complexes with a number of small carboxylic acids including acetate, propionate, butyrate, and valerate (*84*). The reaction with acetate has been shown to occur in a proton-dependent reaction, pK_a 4.8 (*84*), to form a high-spin complex (*85*). This complex is only slowly reduced by powerful reductants such as sodium dithionite (*83*). The pK_a coincides with that of acetic acid, and it was suggested (*84*) that undissociated acetic acid binds to the heme iron. However, it has also been suggested that this the pK_a may arise from another ionizable group on the protein and that this group may need to be protonated before the acetate ion can ligate to the iron ion (*37*). These observations and those with other ligands (discussed later), form the basis of an "electrostatic gate" model of binding of ligands to Lb at high pH values (*86*, *87*), with electronic interactions between anionic ligands and the ionized residue(s) on the protein restricting access (*23*). The exact nature of the residues that give rise to this gate

and its significance *in vivo* requires further study. The early observation (*84*) that larger carboxylates bind to Lb(III) almost as readily as acetate led to the suggestion that the heme pocket of Lb must be larger and more open than that of myoglobin, in which little or no binding is observed. This suggestion was subsequently borne out by X-ray crystallographic studies (*23*, *29*).

The pocket is large enough to accommodate ligands such as imidazoles (though these appear to be somewhat different complexes from those seen with myoglobin and hemoglobin), nicotinic acid (pyridine-3-carboxylic acid) and various derivatives (e.g., the 5-bromo and 5-fluoro species), pyridine and some substituted species, a wide variety of amines, including some long-alkyl-chain species, and isoquinoline (*37*, *88–90*). The binding of nicotinic acid to Lb has been the subject of several studies as a result of the observation that this ligand is often bound to Lb extracted from root nodules (*37*, *88*, *89*, *91*). It is known that a number of nicotinic acid derivatives will not react with Fe(III) Lb, suggesting that the ligand makes a number of close contacts with the surrounding globin protein. Thus, amide and *N*-methyl derivatives of nicotinic acid do not readily bind. The binding of nicotinic acid, like that of acetate, is pH dependent, pK_a 4.9, and the dissociation constants for the Fe(II) and Fe(III) complexes are 33 μM and 1.3 μM respectively (*89*, *90*). The pH dependence and lack of reaction with the *N*-methyl derivative have been interpreted in terms of reaction of the un-ionized ring nitrogen with the heme iron and interaction of the ionized carboxyl group with a protonated residue on the apoprotein. These predictions have been borne out by the crystal structure of the soybean Lb–nicotinate complex, with the species interacting with the carboxyl substituent being either the imidazole nitrogen of the distal histidine or possibly the phenolic —OH group of Tyr30 (*23*).

V. Oxidation of Fe(II) Leghemoglobin

A number of species that are known to be or may be present in root nodules can oxidize Fe(II) Lb. These species include nitrite (*92*), superoxide (*93*), and peroxides (*94*). The process would be expected to be relatively facile, as the reduction potential for the Fe(III) Lb/Fe(II) Lb couple has been reported to be +0.22 V at pH 7 and +0.27 V at pH 6 (*95*). Even in cases in which considerable care has been taken during the extraction process, most extracts from root nodules contain at least traces of the Fe(III) form of the protein. Whether this arises

during the extraction process or is present in the intact root nodule has been the subject of some debate [e.g., (*12*, *37*)]. Detection of the Fe(III) form of the protein by optical spectroscopy in intact root nodules is fraught with difficulty, due to the light scattering produced by the nodules and an incomplete knowledge of the different ligand species that might be present and their respective contributions. Early studies suggested that Fe(III) Lb could not be detected in soybean root nodules (*96*). However, in a review article (*97*) Klucas and Becana have recently reported the results of experiments carried out on old intact nodules attached to roots and maintained under an atmosphere of either air or 100% oxygen, which have suggested that low concentrations of Fe(III) Lb can be present. These nodules were exposed to fluoride (presumably at high concentrations), and the absorbance at the 625-nm parent peak and at 610 nm for the fluoride complex monitored. The low initial absorbance at the former wavelength is rapidly lost, and an increase in absorbance at 610 nm is observed. These changes have been ascribed to the formation of the Fe(III) fluoride complex (*97*). A similar treatment with nicotinate also resulted in a decrease in absorbance at 625 nm and an increase in absorbance at 557 and 526 nm, which correspond to the absorption maxima of the Fe(III) nicotinate complex. Both experiments have been interpreted in terms of the prior presence of low levels of Fe(III) Lb in the intact nodules (*97*). However, it is impossible to rule out the possibility that the high concentrations of these two ions, which have a high affinity for Fe(III) Lb, are enhancing formation of this species through alteration of the redox potential. In contrast, recent EPR studies on intact nodules from soybean plants of different ages that were rapidly frozen immediately after harvesting provided no evidence for high-spin complexes of Fe(III) Lb, which have very distinct g values of *ca.* 6 and 2 (*74*). Whether low-spin forms of the Fe(III) protein were present was more difficult to discern due to the relatively weak nature of these features and the presence of strong absorption bands from the NO complex of Lb(II) (also discussed later). Thus, it is still not unequivocally established whether Fe(III) species of Lb are present at any significant levels in young and mature nodules. The situation with senescent nodules is somewhat different, and it is likely that intact Fe(III) Lb and/or partially degraded Fe(III) Lb are present (*74*, *97*).

Reaction of Fe(II) Lb with superoxide radicals has been suggested as an important route to the formation of Fe(III) Lb via Eq. (1) (*93*). The precise nature of the reaction remains to be fully established; it has been reported that the process is inhibited *in vitro* by the presence of the enzyme superoxide dismutase (SOD), which removes the super-

oxide radical [Eq. (2)] (*93*). It should be noted, however, that $O_2\cdot^-$ also appears to be able to act as a reductant for the Fe(III) Lb, reducing it to the Fe(II) form [Eq. (3)], and that the process is ameliorated by the presence of SOD (*93*). The latter reaction [Fe(III) to Fe(II)] appears to be considerably slower than the former. The significance of these $O_2\cdot^-$-dependent processes will depend on the form of the protein present initially. Current evidence is that the major form in functional nodules is the Fe(II) form, and it is likely that the major effect of $O_2\cdot^-$ generation *in vivo* will be oxidative [generating inactive Fe(III) Lb] rather than reductive (which might be protective).

$$\text{Fe(II)} + O_2\cdot \rightarrow \text{Fe(III)} + O_2^{2-} \tag{1}$$

$$2O_2\cdot^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \tag{2}$$

$$\text{Fe(III)} + O_2\cdot^- \rightarrow \text{Fe(II)} + O_2. \tag{3}$$

The LbO_2 adduct is known to undergo a slow autoxidation to the Fe(III) form with release of $O_2\cdot^-$ (*93*). This process is usually slow, but the rate may be enhanced under certain conditions (e.g., pH, temperature, concentration of Lb, and the presence of certain metal ions, chelators, and anions) (*93*, *97*). Thus, the high levels of LbO_2 present in intact nodules and the drop in pH observed in old and senescent nodules [pH values of *ca.* 6.4 have been reported for young nodules, and *ca.* 5.5 in old or stressed species (*98*)] would be expected to enhance formation of the Fe(III) form, with release of $O_2\cdot^-$.

Incubation of soybean bacteroid preparations with nitrite (0.4 mM) has been shown to markedly inhibit ethylene reduction and concomitantly be capable of inducing the oxidation of LbO_2 to Fe(III) Lb (*92*). The inhibition of nitrogen fixation in these bacteroids has been ascribed to both a direct inactivation of the nitrogenase and the effect of nitrite in converting LbO_2 into an inactive form (*92*). In contrast, direct evidence for (uncatalyzed) autoxidation processes *in vivo* is lacking because, despite numerous efforts, significant amounts of the Fe(III) Lb have not been detected in intact root nodules. This is probably due to efficient reduction of the Fe(III) form to active Fe(II) Lb. In contrast to the autoxidation of LbO_2, LbNO is resistant to both autoxidation and oxidation by added oxidants such as $[Fe(CN)_6]^{3-}$ (*68–70*); it cannot therefore be readily converted back to the oxygen-binding form of Lb even by oxidation/reduction cycles.

$$2O_2\cdot^- + 2H^+ \rightarrow H_2O_2 + O_2. \tag{4}$$

Reaction of deoxy Fe(II) Lb with small excesses of H_2O_2 results in rapid changes in the UV-vis spectrum of the heme center, with the appearance of new absorption bands at 416 nm (ε 97.2 $mM^{-1} \cdot cm^{-1}$) and 543 nm (ε 10.4 $mM^{-1} \cdot cm^{-1}$), with a shoulder at 575 nm (*94*). The second-order rate constant for this reaction is 2.24×10^4 $M^{-1} \cdot s^{-1}$ (*94*). These changes have been ascribed to the oxidation of the Fe(II) center by the peroxide to give a water molecule and a species that has optical properties similar to Compound II, the Fe(IV)=O species in peroxidases:

$$\text{Fe(II)} + H_2O_2 \rightarrow \text{Fe(IV)=O} + H_2O. \quad (5)$$

Approximately 1.5 equivalents of hydrogen peroxide are required per mole of heme to obtain maximum yields of this species (*94*). This species is remarkably stable, with little decay over 12 h at 25°C in the absence of reducing agents (*94*). The corresponding species formed in the case of myoglobin and hemoglobin decay much more rapidly.

The number of oxidizing equivalents present in this species has been examined by titration with reducing agents such as sodium dithionite. Two equivalents of the latter are required per mole of oxidized Lb, whereas only one equivalent was required to reduce Fe(III) Lb (*94*). When similar reactions were carried out with larger excesses of H_2O_2, a more rapid conversion of Fe(II) to Fe(IV) was observed. From the absorbance changes at 543 nm due to the Fe(IV) species, the yield is somewhat reduced with excess hydrogen peroxide above 1:4 (Lb:H_2O_2). Under such conditions the Fe(IV) absorbance is gradually lost due to slow reactions resulting in destruction of the heme chromophore (*94*). Such an interpretation is in line with studies carried out using chelating ligands with a high affinity for iron. In these studies no significant release of iron was observed until a greater than 4:1 excess of hydrogen peroxide was employed (*99*). It should be noted that the release of iron by this method may not accurately reflect the release of iron in other situations, as such high-affinity chelating ligands may remove iron from the protein, where it would otherwise have remained bound. Thus, the assessment of heme degradation by UV-vis absorbance measurements, which reflect early stages of heme degradation, do not necessarily correlate with the release of iron as measured by other methods.

When similar reactions were carried out with LbO_2, the absorbance changes were much slower. Early studies with mammalian heme proteins suggested that such reactions might give rise to free hydroxyl radicals via one-electron reduction of the hydrogen peroxide (*100*), but

it is now believed that this reaction proceeds via the formation of a high-oxidation-state species similar to that described earlier, at least when low excess peroxide levels are employed. No evidence for "free" hydroxyl radical formation has been obtained under these conditions by product analysis (*99*) or EPR spin trapping (*101*). When higher concentrations of hydrogen peroxide were employed, which can result in heme degradation with release of iron [typically at peroxide : heme ratios $> 6:1$; LbO_2 appears to be less sensitive than the Fe(II) form], some hydroxyl radicals may then be generated by the iron released (*99*). With low concentrations of peroxide and intact LbO_2, the reaction presumably involves displacement of the dioxygen by the hydrogen peroxide and reaction as described earlier. Such changes are readily monitored using spectrophotometry by the absorption decrease at 574 nm or the increase at 625 nm. As with the species formed from the deoxy form of the protein, this species is very stable in the absence of reducing agents. Oxidation of ligated forms of Lb can also occur; thus, it has been shown that the Lb(II) nicotinate can be oxidized to the Lb(III) form by hydrogen peroxide. Further oxidation to higher oxidation states does not occur, however (*94*). The nature of this oxidation, and in particular the fate of the second oxidizing equivalent from the hydrogen peroxide, remains to be established.

VI. Oxidation of Fe(III) Leghemoglobin

Reaction of H_2O_2 with Fe(III) Lb gives lower yields of Fe(IV)=O Lb than those seen with Fe(II) (*94*, *102*). The absorbance changes observed during reaction with low excesses of the peroxide were ascribed to the formation of both the Fe(IV)=O species and an additional (green) compound (*102*). The latter is characterized by UV-vis absorbance bands at 410 and 630 nm (Fig. 3). This species has since been shown to be very stable and has been further isolated and characterized (see Section IX,A) (*103*). The nicotinate complex of Fe(III) Lb does not undergo these reactions (*94*).

The formation of Fe(IV)=O Lb suggests that the Fe(III) center is oxidized by only one of the two oxidizing equivalents from the peroxide. The fate of the second equivalent has been the subject of much study. In early work, in which the ability of Fe(III) Lb/H_2O_2 mixtures to initiate lipid peroxidation and oxidation of organic molecules was examined, it was postulated that the second oxidizing equivalent was released as a hydroxyl radical:

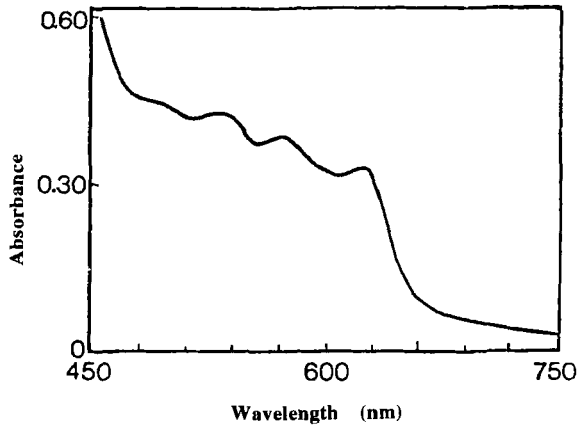

FIG. 3. Formation of the additional (green) compound on reaction of soybean Fe(III) Lb (50 μM) with H_2O_2 (100 μM) in 25 mM KH_2PO_4/KOH buffer, pH 7.4, containing 0.1 mM DTPA. Visible range spectrum recorded 120 s after addition of H_2O_2 at 25°C.

$$\text{Fe(III)} + H_2O_2 \rightarrow \text{Fe(IV)=O} + \text{HO}\cdot. \quad (6)$$

Further studies on this system and related work with the analogous mammalian heme proteins myoglobin and hemoglobin have shown that free hydroxyl radicals are not released, at least with low excesses of hydrogen peroxide, when oxidative damage to the heme and release of iron is minimal (*101*). These reactions therefore appear to occur in a similar manner to other peroxidase enzymes in which the initial oxidation by the peroxide involves a two-electron transfer from the heme iron to the peroxide. However, unlike peroxidases, the second oxidizing equivalent does not appear to be retained by the heme center for any significant length of time, and the second equivalent is rapidly dissipated into the surrounding protein (globin) matrix [Eq. (7); (*101*)]. It appears therefore that the initial reaction gives an Fe(IV)=O species and a heme (porphyrin) radical cation. The radical cation, unlike those present in classical peroxidases, reacts with the surrounding protein by what appears to be an electron-transfer process to oxidize one or more globin residues with concomitant reformation of the intact heme ring (*101*). Thus, within a very short period the reaction gives an Fe(IV)=O center identical to that formed with Fe(II) Lb, and one or more globin radicals. This situation is similar to what occurs with myoglobin (*104–110*):

$$\text{Fe(IV)=O (heme}^{+}\cdot\text{)(globin)} \rightarrow \text{Fe(IV)=O (heme)(globin}^{+}\cdot\text{)}. \quad (7)$$

Though there is now some considerable evidence that globin radicals are formed in these reactions, it is still not absolutely clear where the oxidizing equivalent resides and why. In initial (direct, room temperature) EPR studies a multiplet signal was detected on reaction of the Fe(III) form of the protein with low excesses of hydrogen peroxide (Fig. 4) (*101*). This signal was only detected shortly after initiation of the reaction. Monitoring of the intensity of the EPR signal by carrying out time-course experiments in which the magnetic field was set to that corresponding to the position of one of the absorption lines, allowed the half-life of the species to be determined as approximately 40 seconds (Fig. 5). This globin-derived species was observed with all three types of Fe(III) Lbs tested (*a*, *c1*, and *c3*) and was also detected with a number of other hydroperoxides and two-electron oxidants. The former observation suggests that the minor changes in sequence and structure between the isoforms are unimportant in determining the course of the reaction; the latter observation suggests that a common mechanism occurs, with similar globin-derived radicals generated in all cases. The latter strongly suggests that the globin radical does not contain any part of the original oxidant (e.g., is not an adduct formed by addition of the hydroperoxide to an amino acid). In further experiments in which the Lb was iodinated before treatment with hydrogen peroxide (*101*), the EPR signal was not observed. The condi-

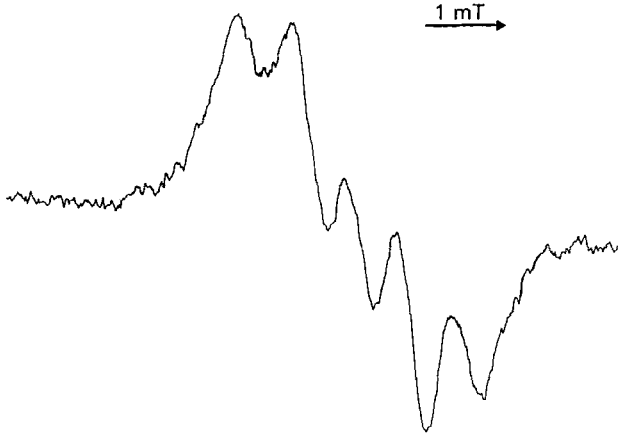

FIG. 4. EPR spectrum observed immediately after mixing soybean Fe(III) Lb (250 μM) with H_2O_2 (250 μM) at pH 7.4. Reaction studied using a two-way stopped-flow mixing system inserted into the cavity of the EPR spectrometer. The signal is assigned to a sterically constrained tyrosine phenoxyl radical formed at position 133 (reproduced with permission from Davies, M. J.; Puppo, A. *Biochem. J.* **1992,** *281*, 197–201).

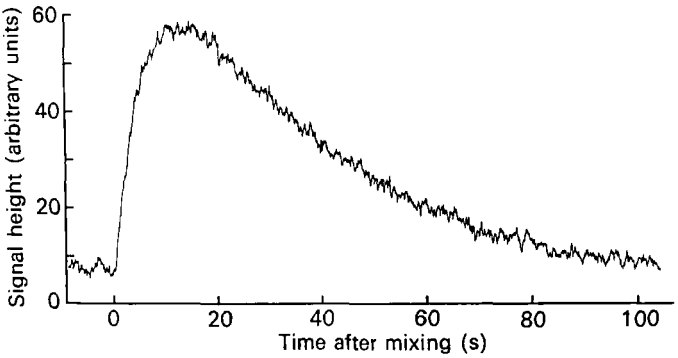

FIG. 5. Build-up and decay with time of the EPR signal (see Fig. 4) from the tyrosine phenoxyl radical formed at position 133 of the soybean protein on reaction of Fe(III) Lb (250 μM) with H_2O_2 (250 μM) at pH 7.4 (reproduced with permission from Davies, M. J.; Puppo, A. *Biochem. J.* **1992,** *281*, 197–201).

tions under which this iodination was carried out were such that only the Tyr residues in the protein ought to be affected, suggesting that the Tyr residues present in the globin are either the site of the radical species or involved in the generation of the radical.

Determination of the *g* value of the observed signal (2.0044) and analysis of the hyperfine coupling constants suggest that the signal is a Tyr-derived species, with both the *g* value and the coupling pattern consistent with the presence of a Tyr phenoxyl radical (*101*). This species does not, however, have coupling constants identical to those of a free Tyr phenoxyl radical [see, for example, (*111*)], particularly with respect to the coupling of the hydrogens of the methylene ($-CH_2-$) group, which attaches the aromatic ring to the backbone. The variation between these values is consistent with the surrounding globin structure forcing the radical to adopt a fixed conformation with little free rotation round the methylene-to-ring bond (*112*). This species also has somewhat different coupling constants with regard to the methylene hydrogens than those reported for analogous Tyr phenoxyl radicals in other proteins (*113–124*), suggesting that the conformation of such radicals is not universal in proteins.

In the soybean form of the protein, which was used in these experiments, there are three Tyr residues, with one of these, that at position 133, near the heme ring (Fig. 6). This was suggested to be the site of the radical on account of its proximity to the heme center at which the initial oxidation must be occurring (*101*). Further studies with a variety of scavenging agents demonstrated that the radical can undergo a number of reactions. Thus, its signal was lost when high per-

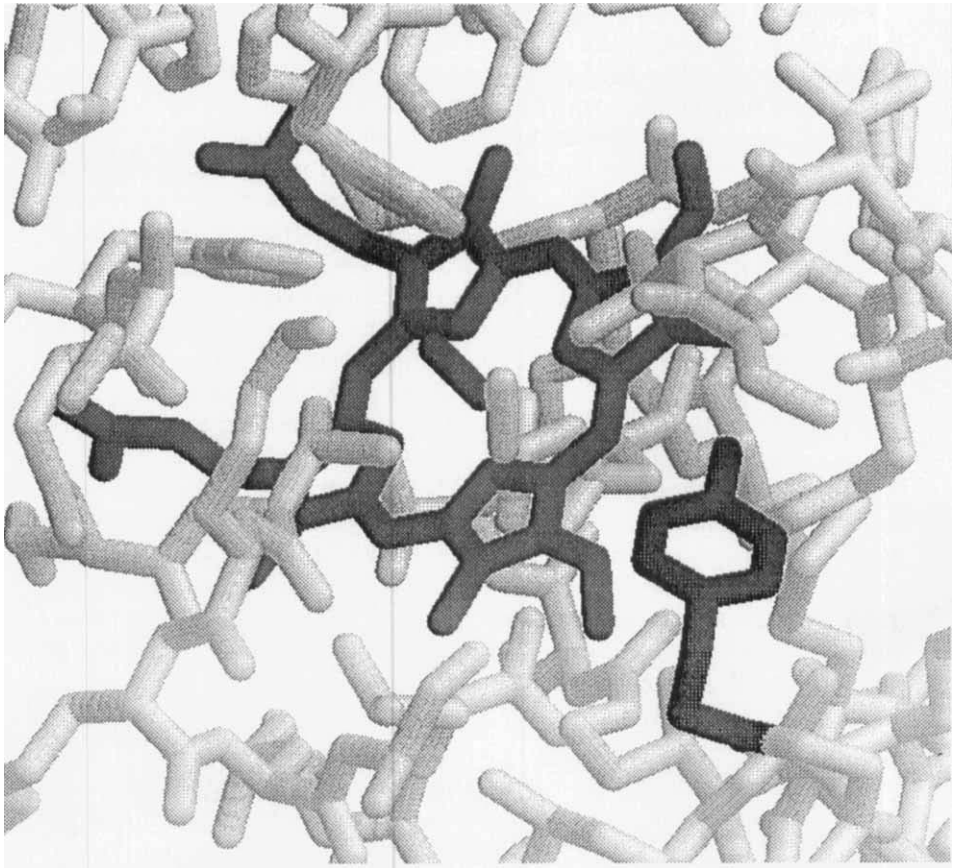

FIG. 6. Position of the Tyr133 residue in soybean metLb in relation to the heme ring. Computer-generated molecular model using crystal coordinates (*23*) (Ellis and Freeman, unpublished data).

oxide concentrations were employed, a process assigned to reaction between the radical and excess hydrogen peroxide. The signal was also diminished in intensity, or lost completely, when various heme-binding agents (cyanide, azide, nicotinic acid) were used or reducing agents were added (see Section IX,B). The latter was attributed to one-electron reduction of the radical (*101*). The observation that the globin-derived radical reacts with agents expected to be present solely in the aqueous phase, and not expected to penetrate far into the protein structure, suggests that the radical must be present on the protein surface, or not far removed from it.

Further studies with one of the lupin forms of Lb have helped elucidate the site of the radical (*112*). This protein contains only two Tyr residues, with only one of them conserved when compared to the soybean structure. Thus, it was hypothesized that if the radical had identical parameters and similar kinetics for formation and decay, then it could be assigned to the single conserved residue (Tyr133 in soybean and Tyr138 in lupin). This proved to be the case, with a very similar EPR signal observed with the Fe(III) form of the lupin protein on treatment with small excesses of hydrogen peroxide (*112*). The signals obtained in experiments with the lupin protein were better resolved than those with the soybean protein, and this allowed a detailed analysis of the splitting pattern and coupling constants to be carried out. The spectra were computer simulated using this data, adding further support to the theory that the globin radical is indeed a Tyr-derived phenoxyl radical (*112*).

The mechanism of formation has been postulated to involve rapid one-electron oxidation of the aromatic ring by the heme radical cation to give a Tyr radical cation [Eq. (8); (*101*, *112*)]. Using model phenols such species undergo rapid deprotonation to give a phenoxyl radical with rate constants *ca.* 10^{10} $dm^3 \cdot mol^{-1} \cdot s^{-1}$ (*125*, *126*). Thus, these model studies are consistent with the formation of a radical within the globin polypeptide:

$$\begin{aligned} \text{Fe(IV)=O (heme}^{+\cdot}\text{)(globin-Tyr-OH)} &\rightarrow \text{Fe(IV)=O (heme)(globin-Tyr-OH}^{+\cdot}\text{)} \\ &\rightarrow \text{Fe(IV)=O (heme)(globin-Tyr-O}\cdot\text{)} + \text{H}^{+}. \end{aligned} \tag{8}$$

In the experiments reported in these studies, little attempt was made to quantify the extent of formation of the radical. Such studies are technically challenging, especially when the kinetics of formation of the radical are poorly defined. However, from the intensity of the signals the steady-state concentration of the radical is considerably less than that expected from the concentrations of protein and oxidant employed. Thus, it appears that the radical is not formed in a very efficient manner. This suggests that there are other routes to the loss of the second oxidizing equivalent from the heme center or there are other pathways that result in the loss of the Tyr radical.

In an effort to determine whether other radical species are generated on the globin, EPR spin-trapping experiments were carried out using the spin trap DMPO (5,5-dimethyl-1-pyrroline-*N*-oxide). In these experiments, unlike those carried out with myoglobin, no significant signals were observed (*101*). At the time the interpretation

was in terms of nonformation of additional radicals, but later work has shown this to be incorrect. The incorrect conclusion probably arose from the fact that the spin trap chosen was not ideal, with the trapping rate constants rather slow (i.e., other species formed were not trapped and hence not observed). These negative experiments did, however, allow one possible mechanism of formation of the observed radical to be ruled out. The trap reacts rapidly with HO· (k *ca.* 2×10^9 $M^{-1} \cdot s^{-1}$) to give a spin adduct with distinctive EPR parameters and a long half-life (*127*, *128*). The absence of this well-characterized product suggests that free HO· are not formed (*101*). Similarly, the absence of alkoxyl radicals (and related species), which also give long-lived and well-characterized spin adducts, in experiments in which hydroperoxides (and similar compounds) were used to generate the radicals, rules out the involvement of these radicals (*101*).

Subsequent studies using alternative spin traps have demonstrated that other radical species are generated on the globin when Fe(III) Lb is incubated with low excesses of hydrogen peroxide (*129*). Thus, incubation in the presence of the spin trap MNP (2-methyl-2-nitrosopropane) has resulted in the detection of broad, partially anisotropic EPR signals. The partial anisotropy of these signals is believed to be due to the trapping of relatively large, slowly tumbling radical adducts. These are believed to be trapped globin-derived radicals (*129*). Similar experiments carried out with other spin traps, such as the nitrones PBN (α-phenyl-*N*-*t*-butylnitrone) and POBN [α-(pyridine-1-oxide)-*N*-*t*-butylnitrone], also give EPR signals that have been assigned to globin-derived radicals (*129*). The formation of these species does not appear to be affected by the presence of dioxygen in the reaction mixture. This suggests that the radicals are not formed from an oxygen-derived species (such as a peroxyl radical) and that they do not react rapidly with oxygen. There are, however, two important caveats to these suggestions. First, it is very difficult to make such reactions completely anoxic, as oxygen may be generated from the decomposition of hydrogen peroxide. Second, the high concentrations of spin traps used in these experiments (to obtain reasonable signal-to-noise ratios) may result in reaction with dioxygen being uncompetitive. In only one case, with the nitroso spin trap DBNBS (3,5-dibromo-4-nitrosobenzene sulfonic acid), were signals observed in the absence of hydrogen peroxide. In the latter case the signals have been assigned to a novel compound arising from a chemical reaction of the heme moiety with the trap and not spin trapping. Subsequent oxidation of the initial product gives the observed signal (*129*).

In the case of POBN at least two adducts were detected, and the EPR signals were almost completely isotropic in nature, in contrast with those detected with MNP and PBN. This suggests that the radicals present have considerable mobility (*129*). The trapping with POBN, which is a highly hydrophilic trap and partitions very poorly into hydrophobic solvents, suggests that the radicals detected are present on the outer surface of the globin. The situation with the other traps is less clear, as PBN and MNP partition readily into hydrophobic media. Unfortunately, the spectra obtained do not give extensive information as to the nature of the radicals formed. Thus, the spectra observed with PBN and MNP give little information due to their anisotropic nature, whereas the sharper lined spectra observed with POBN do not allow much information to be obtained because the adducts formed do not show a dramatic change in hyperfine coupling constants (*128*). However, the signals observed with MNP suggest that the added radical is carbon-centered in nature.

To obtain further information about the species trapped in these experiments, enzymatic digestion experiments were performed on the spin adducts (*129*). This technique, which has been successfully employed to obtain information on the nature of radicals trapped from other proteins, DNA, RNA, and complex carbohydrates (*130*, *131*), relies on the fact that many of the trapped species are stable for considerable periods of time. The macromolecular radical adduct can therefore be treated with either nonspecific or specific proteases that will release smaller fragments, at least some of which should still have the spin trap attached. This process may be aided by the fact that many proteolytic enzymes degrade damaged proteins more readily than the corresponding undamaged parent macromolecule (*126*, *132*). Such fragments would be expected to tumble rapidly in solution and hence give isotropic spectra, from which further information may be obtained. When such experiments were carried out with the Lb adducts detected on treatment of Fe(III) Lb with hydrogen peroxide in the presence of MNP, a conversion of the anisotropic signal to more isotropic features was observed. The spectra observed at the end of this process consisted of a single triplet, which is consistent with the trapping of at least one tertiary carbon-centered radical (*129*). Though these experiments do not allow complete identification of the protein radical, they suggest that the radicals are formed at stabilized sites (i.e., tertiary rather than secondary or primary).

Recent studies on the analogous myoglobin reactions have suggested that a highly resonance-stabilized radical that is trapped via the C-3 position on the indole ring (*108*, *110*), can be formed on the

side chain of Trp residues. This is a tertiary site and would be expected to be formed readily due to the ease of oxidation of such Trp side chains. Thus, it is possible that a similar species may form and become trapped in the Lb experiments described earlier, and that such a radical may be the source of the tertiary nitroxide signals seen with MNP (and possibly the signal observed with PBN and one of those detected with POBN). The detection of two distinct species with POBN as the trap suggests that, even if this speculation is correct, other types of radical must also be present. Recent studies have shown that phenoxyl species can be trapped under certain circumstances, and in particular when other decay routes (such as dimerization) are inhibited by, for example, steric factors (*133*). Thus, some, but not all, of these spin-adduct signals may arise from the trapping of Tyr-derived species. The additional radical species may arise via two (or more) competing damage-transfer pathways within the protein or may be species formed along a single pathway. Evidence from the analogous myoglobin experiments suggests that more than one pathway exists, with both tyrosine and tryptophan-derived species observed (*104–110*). Recent studies have also implicated histidine-derived radicals (*109*), though it should be noted that some of this evidence has been obtained from the study of mutant proteins (from site-directed mutagenesis) from which key amino acids (e.g., the Tyr residues) have been removed. Such data might be misleading, for removal of a key residue may merely switch damage transfer to another pathway and hence other final sites that are not normally damaged to any great extent. Considerable work therefore still needs to be carried out in order to obtain a complete picture of the radical species that are formed in these reactions.

VII. Reduction of Fe(IV)=O Leghemoglobin

As mentioned earlier, Fe(IV)=O Lb is particularly stable, as least when compared to the analogous species detected with other heme protein systems (*94*). This species remains observable 12 h after its formation under favorable conditions and can be isolated from other forms of the protein on isoelectric focusing gels. However, in the presence of reducing agents or readily oxidizable compounds it undergoes further reaction, resulting in its conversion to a lower oxidation state. Some of these reactions are biologically significant, as they repair the oxidized species.

A. Reaction with H_2O_2

Reaction with excess hydrogen peroxide results in the oxidation of the heme ring and release of iron, though the exact mechanism of this type of reaction is unknown. It may involve oxidation of a further molecule of hydrogen peroxide (*101*) with consequent formation of the HOO· radical, which may then react with the heme ring, possibly at the methylene bridge sites. The latter is consistent with known degradation reactions of the heme ring with loss of the iron atom (*99*).

B. Reaction with Ascorbate

It has been reported recently that addition of ascorbate to Fe(III) Lb immediately prior to the addition of small excesses of hydrogen peroxide results in transient formation of Fe(IV)=O Lb as monitored by UV-vis spectrophotometry (*134*). The situation when ascorbate is added after formation of Fe(IV)=O Lb is more complex due to the formation of additional species during the reaction of Fe(III) Lb with the hydrogen peroxide. In the former case, with 1 mM ascorbate and 50 μM Lb, the conversion back to Fe(III) is complete in about 2.5 minutes (Fig. 7) (*134*). Under these conditions a further slow reduction of the Fe(III) species is also observed (discussed later). The observation of isobestic points is consistent with the presence of only the Fe(IV)=O and Fe(III) species in the reaction system. As expected, the rate of the Fe(IV)=O to Fe(III) conversion was found to depend on the ascorbate concentration and is associated with oxidation of the ascorbate. The stoichiometry of the ascorbate oxidized to hydrogen peroxide added is 1:1. This ratio implies that both oxidizing equivalents in the peroxide are responsible for ascorbate oxidation and hence that the protein-derived radicals are also removed. The reduction of the Fe(IV)=O form by ascorbate has been shown to be a one-electron transfer reaction by the use of EPR spectroscopy. In these experiments intense signals due to the ascorbyl radical were observed (*134*). This process may occur via reaction of ascorbate at the heme edge, which is known to be exposed to agents present in bulk solution (Fig. 8).

Similar results were obtained when Fe(IV)=O Lb was generated from Fe(II) Lb, though in this case both oxidizing equivalents from the hydrogen peroxide are believed to remain at the heme center before being removed by reaction with ascorbate.

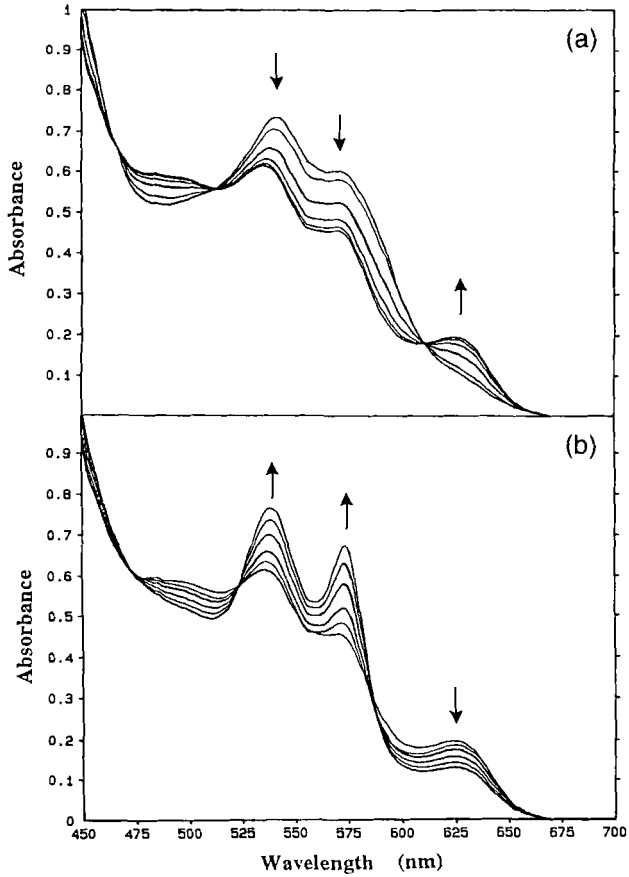

FIG. 7. Spectrophotometric changes during the reduction of soybean Fe(IV)=O Lb by ascorbate. *Fe(III)* Lb (50 μM) was mixed with H_2O_2 (100 μM) immediately after addition of 1 mM ascorbate at pH 7.4 (25 mM KH_2PO_4/KOH buffer containing 0.1 mM DTPA). Reactions were run at 25°C, and repetitive scans were recorded at 30-s intervals (a) and then 30-min intervals (b). Data are representative of experiments carried out in triplicate [reprinted from *Phytochemistry, 39,* Moreau, S.; Puppo, A.; Davies, M. J.; The reactivity of ascorbate with different redox states of leghaemoglobin, pp. 1281–1286, copyright (1995), with kind permission of Elsevier Science Ltd, The Boulevard, Langford Lane, Kidlington, OX5 1GB, UK].

C. Reaction with Glutathione, Other Thiols, and Related Species

As in the case of ascorbate reactions, addition of glutathione (GSH; 1 mM) to Fe(III) Lb (50 μM) before reaction with hydrogen peroxide results in a decreased lifetime of the Fe(IV)=O species (*102*). The loss

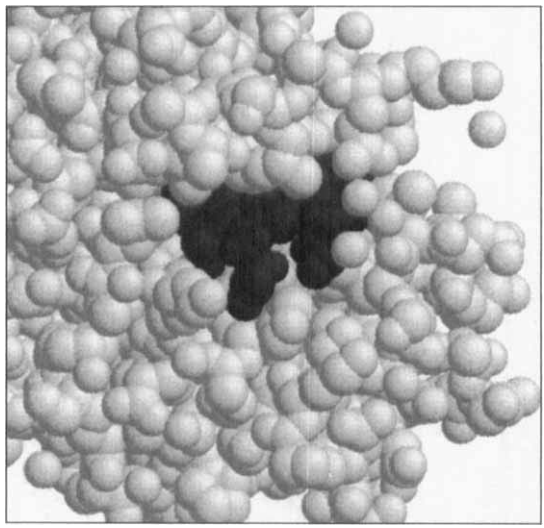

FIG. 8. The heme edge (dark shaded residues) in soybean Fe(III) Lb is partially exposed on the surface of the protein. Computer generated molecular model using crystal coordinates (*23*) (Ellis and Freeman, unpublished data).

of the UV-vis absorption bands of Fe(IV)=O Lb is accompanied by the reappearance of those for Fe(III) Lb, with good isobestic points confirming that no other species is involved (Fig. 9). The reduction to Fe(III) Lb is accompanied by formation of oxidized glutathione [glutathione disulfide (GSSG), assayed using NADPH in the presence of GSSG reductase] in approximately stoichiometric amounts (H_2O_2 added:GSH formed), again suggesting that both oxidizing equivalents from the hydrogen peroxide are utilized. The conversion of GSH into GSSG is also believed to occur via a one-electron (radical) process, because glutathione thiyl radicals have been detected by EPR spectroscopy. Similar behavior is observed with Fe(II) Lb treated with small excesses of hydrogen peroxide (*102*).

As might be predicted on the basis of these data, a number of other thiol compounds have been shown to reduce Fe(IV)=O Lb to the Fe(III) state with concomitant formation of thiyl radicals (detected by use of EPR spin trapping using 5,5-dimethyl-1-pyrroline *N*-oxide) (*135*). In some cases, however, other species are formed, and these have been identified from their UV-vis absorption spectra as novel sulfur species formed by nucleophilic attack on the tetrapyrrole ring by the thiol group (*135*). The ability of a thiol to undergo two such competing reactions is dictated by steric and electronic characteristics

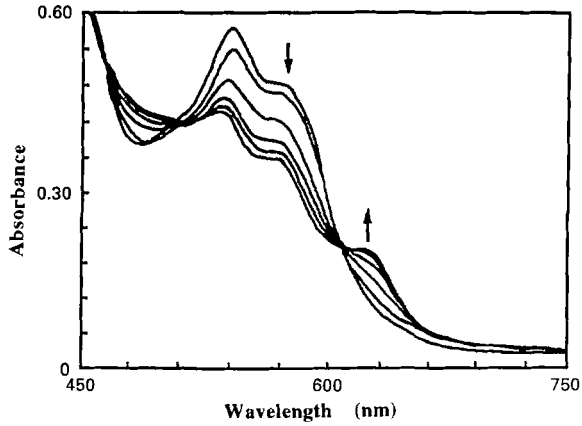

FIG. 9. Visible range spectral changes during the reduction of soybean Fe(IV)=O Lb by GSH. Fe(III) Lb (50 μM) reacted with H_2O_2 (100 μM) at pH 7.4 (25 mM KH_2PO_4/KOH buffer containing 0.1 mM DTPA). Repetitive scans were recorded 1 min after addition of H_2O_2 and then at 3-min intervals (reproduced with permission from Puppo, A.; Monny, C.; Davies, M. J. *Biochem. J.* **1993,** *289*, 435–438).

of the thiol. Addition of cysteine results in reduction to the Fe(III) [and subsequently to Fe(II) Lb] and formation of the Fe(III) and Fe(II) sulfur derivatives via nucleophilic reactions. The Fe(II) sulfur derivative appears in this system to be formed from the corresponding Fe(III) sulfur compound, suggesting that nucleophilic addition can be followed by heme reduction. The difference in behavior between cysteine and GSH may arise from the greater steric bulk of the latter, which may inhibit reaction at the heme edge. This hypothesis is supported by the observation that the behavior of *N*-acetylcysteine is intermediate between the two.

Cysteamine (2-mercaptoethylamine) gives extremely rapid heme reduction with formation of Fe(II) Lb and little formation of the sulfur derivatives of Lb. 3-Mercaptopropionic acid on the other hand gives only slow reduction of Fe(IV)=O Lb, even slower reduction to Fe(II) Lb, and some sulfur-Lb formation. This suggests that the overall charge on the reducing agent can have a major influence on the kinetics and the extent of competition between the two pathways (*135*). These differences have been rationalized in terms of the negatively charged gate in the globin, which gives access to the heme. Electronic interactions between the negative charge on the protein and the reductant may slow down the reduction with 3-mercaptopropionic acid and give an enhanced rate with positively charged cysteamine. In contrast the formation of sulfur–Lb derivatives always appears to be rel-

atively slow and is only therefore a significant competing reaction when reduction at the heme center is slow. The exposed nature of the heme edge in Lb (*23*) (Fig. 8) may also explain the much greater prevalence of nucleophilic processes as compared to myoglobin (*136*), where such reactions appear to occur much less readily.

Reduction of Fe(IV)=O Lb by disulfides does not occur to any significant extent, except in the case of oxidized lipoic acid, which brings about slow reduction of Fe(IV)=O Lb to Fe(III) Lb (*135*). No radical species were detected by EPR spectroscopy during the reaction, though such species are very likely generated by one-electron oxidation of the disulfide. No reaction between Fe(IV)=O Lb and hydrogen sulfide has been observed, unlike the situation with myoglobin (*137*).

D. Reaction with Other Reductants

Other chemical reducing agents can bring about loss of Fe(IV)=O Lb. Thus, ferrocyanide can reduce Fe(IV)=O Lb to Fe(III) Lb, though this reaction is relatively slow and complex [as judged by changes in the absorption spectra (*94*)]. In contrast, reaction with dithionite appears to be quantitative and has been used to estimate the concentration of Fe(IV)=O Lb (*94*). The Fe(IV)=O Lb does not undergo reaction with dioxygen, carbon monoxide, cyanide, or nicotinic acid (*94*). Similarly, it does not appear to undergo reaction with Fe(II) Lb (i.e., synproportionation to give two molecules of Fe(III) Lb does not occur) (*138*). The potential reaction of Fe(IV)=O Lb with lipids and membranes is discussed in Section IX,D.

VIII. Reduction of Fe(III) Leghemoglobin

A. Enzymatic

There is considerable evidence that functional root nodules possess efficient means of reducing Fe(III) Lb back to Fe(II) Lb. It has been shown that this occurs via a number of different mechanisms, including both enzymatic and nonenzymatic pathways [reviewed in (*97*, *139*)].

Early studies by Appleby (*140*) showed that bacteroids slowly reduce Fe(III) Lb to Fe(II) Lb under anaerobic conditions. It was suggested that an enzymatic process was involved. Later reports [e.g., (*141*, *142*)] confirmed that a specific enzymatic Fe(III) Lb reduction system is present in root nodules, and the nature of this enzymatic

action has been characterized in some detail (*143*). The protein responsible for the activity was initially characterized in lupin nodules but has since been shown to be present in other nodules (e.g., soybean). It is a FAD-containing species that also contains active thiol groups, but no catalytic metals (*139*, *143–147*). The enzyme has been purified in a number of cases. There are significant differences in size and other properties of the enzyme from different species (e.g., the molecular mass varies from 60 kDa for the lupin form to 100 kDa for the soybean). In lupin nodules there are positive correlations between activity of the enzyme and symbiotic performance as measured by the rate of nitrogen fixation and the Lb content (*148*). The protein from soybean root nodules is present primarily in the plant fraction of the nodules (91% of the enzymatic activity), with only low levels present in the bacteroids (*143*). The isolated protein appears to reduce all eight forms of soybean Lb equally effectively. Several different forms of this species have been identified in soybean nodules, suggesting that there are a number of modified or different forms of the protein present (*149*). The activity of this enzyme requires a reductant (NADH or NADPH) and dioxygen but does not require an intermediate electron carrier. Activity is decreased if the Fe(III) Lb is complexed with a number of small ligands such as acetate, nicotinate, and nitrate, and is also inhibited by the presence of exogenous catalase, but not by superoxide dismutase. The latter observation suggests that a peroxide-type intermediate may be formed during the catalytic cycle (*97*, *139*). The rate of reduction does not appear to depend on the species that finally ligates Fe(II) Lb; thus, identical rates of reduction are observed with O_2 or CO ligated in the axial position. As with nonenzymatic reduction (discussed later) the enzymatic process is pH dependent. Rate constants are constant over the pH range 7.6 to 6.5, but increase at lower pH values, with a threefold increase at pH 5.2 (*143*). Further studies and partial sequencing of the enzyme have shown that this protein is similar to myoglobin reductase, and the NADH : cytochrome b_5 reductase from erythrocytes (*147*).

B. Nonenzymatic

There are a number of low-molecular-weight species present in root nodules that might act as reductants for Fe(III) Lb. In mature root nodules there are significant levels of NADH and NADPH (150–200 μM), free cysteine (200 μM), reduced glutathione (40–150 μM) and reduced ascorbate (1–2 mM). The exact concentrations depend on the age of the nodules and the method by which the extraction and quan-

tification is carried out (*97*, *139*, *150*). The reduction catalyzed by a number of these species is pH dependent. Rate constants for the reduction of Fe(III) Lb by high concentrations (1 mM) of NADH or NADPH were found to increase with change in pH from pH 7.0 to 5.2, with maximum rates observed at the lowest value. The reduction was O_2 dependent and was inhibited by superoxide dismutase and catalase (*93*, *151*). These results implicate superoxide radicals and/or hydrogen peroxide as the reductants. However, at higher pH and physiological levels of reductant (400 μM), the rate constants are very small (*151*). Furthermore, only ascorbate and cysteine of these reductants appear to be able to reduce Fe(III) Lb at significant rates (*139*). A number of nonphysiological thiols also appear to be able to bring about reduction. In a number of cases, including cysteine, competing processes generating sulfur–Lb species have been shown to occur (*135*). If such sulfur–Lb species could be detected in intact root nodules, it might give an indication of the occurrence and importance of cysteine-mediated reduction of Fe(III) [and Fe(IV)=O] Lb *in vivo*.

Disulfides do not appear to be capable of reducing Fe(III) Lb to Fe(II) Lb (*135*). Reaction with sulfide itself appears to be different and gives rise to a Fe(III) Lb–sulfide complex rather than reduction or nucleophilic attack (*135*). The complex is, however, susceptible to reduction by strong reducing agents such as dithionite and ascorbic acid.

It has also been reported (*152*) that incubation of Fe(III) Lb with a large excess of ascorbic acid (10 mM) can result in the formation of LbO_2 together with heme degradation products due to the generation of radicals (from autoxidation of excess ascorbate). More recent work has cast doubt on the significance of this type of reaction (*134*).

Rate constants for many of these reduction processes have been shown to depend on a number of factors including pH, the presence of metal ions, and cofactors. Thus, it has been reported that the reduction of Fe(III) Lb by NADH can be stimulated by addition of Mn(II) ions (*139*). This is thought to be due to the formation of superoxide radicals, which then reduce the Fe(III) center. Flavins have been suggested as stimulators of Fe(III) Lb reduction (*139*). Thus, in the presence of NADH or NADPH, free flavins can act as efficient reducing agents in a process that appears to be independent of mediators such as superoxide radicals (no effect of SOD). This direct reaction may be aided by the exposed nature of the heme edge of Lb (Fig. 8) (*23*, *29*). This type of process does not require dioxygen, and may be a significant pathway *in vivo* due to the high flavin content of root nodules (*139*).

IX. Reactions of Globin-Derived Radicals

A number of reactions of the globin radicals have been elucidated, and these can be readily grouped into various categories.

A. Reactions with the Heme Ring

During the reaction of Fe(III) Lb with hydrogen peroxide, the yield of Fe(IV)=O Lb is relatively low compared to reaction with the Fe(II) state. However a further species is formed, which can be detected by the UV-vis absorption bands at 410 and 630 nm (*94*, *102*, *103*). The observation that this species is produced only under conditions in which globin radicals are formed suggested that it results from subsequent reactions of the globin radical (*153*). The stability of this additional (green) species has allowed it to be isolated from reaction mixtures by isoelectric focusing using immobiline gels with a 5.7 to 4.7 pH gradient. The green compound has an isoelectric point of 5.45. Electroelution has allowed this species to be further characterized, and UV-vis and fluorescence spectra have been determined (*153*). The visible spectrum is completely different from Fe(III) Lb in that the Soret band (410 nm) is much less intense and broader, and the charge-transfer bands are dramatically altered, with a broad absorption at 630 nm. The product reacts with strong reducing agents such as dithionite or ascorbate (Fig. 10), which results in the formation of a product with UV-vis bands similar to those of LbO_2. This reduction is slow and occurs over approximately 18 h (*153*).

The pyridine hemochromogen spectrum of the product provides strong evidence for alterations to the heme ring but with no loss of iron (*153*). The UV-vis spectrum is very different from other heme degradation products such as biliverdin. SDS-PAGE analysis gives a single band with similar R_f to Fe(III) Lb, suggesting that the protein is not a fragment or dimer. The suggestion that it contains a modified heme ring (and iron), has been confirmed by EPR spectra, which demonstrate the presence of high-spin Fe(III) in a distorted environment. The usual iron absorption at $g = 6$ was not observed, but a sharp peak at g *ca.* 4.3 was detected together with a broad background signal. This may be due to an envelope of signals from completely free or partially bound Fe(III) (*153*).

The possibility that addition of a globin radical to the heme ring gives an altered heme moiety has been confirmed by heme extraction experiments (using acidic 2-butanone) following treatment of soybean Fe(III) Lb with a small excess of hydrogen peroxide. Measure-

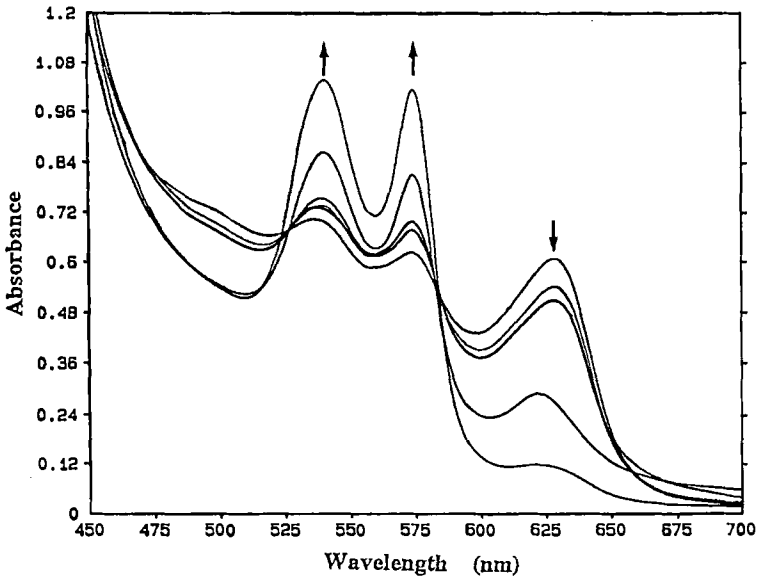

FIG. 10. Visible range spectral changes during the reduction of the additional (green) species formed on reaction of Fe(III) Lb (50 μM) with H_2O_2 (100 μM) at pH 7.4 (25 mM KH_2PO_4/KOH buffer containing 0.1 mM DTPA) at 25°C, with subsequent addition of 1 mM ascorbate. Scans were recorded 0, 1, 2, 6, and 18 h after the addition of ascorbate; spectral changes are indicated by arrows. Data are representative of experiments carried out in triplicate [reprinted from *Phytochemistry, 39,* Moreau, S.; Puppo, A.; Davies, M. J.; The reactivity of ascorbate with different redox states of leghaemoglobin, pp. 1281–1286, copyright (1995), with kind permission of Elsevier Science Ltd, The Boulevard, Langford Lane, Kidlington, OX5 1GB, UK].

ment of the ratio of the absorption of Soret band to that at 280 nm was used to monitor heme removal from the holoprotein. With untreated Fe(III) Lb, heme retention was of the order of 4.4% (i.e., near-quantitative removal), whereas with the green compound heme retention was calculated as *ca.* 63%. The green compound is therefore believed to be a cross-linked species arising from reaction of a globin radical with the heme (*153*). Similar species have been demonstrated on reaction of myoglobin with hydrogen peroxide (*154–156*). Though the exact nature remains to be confirmed, it has been suggested that in the case of soybean the species arises via a mechanism involving reaction of the Tyr133 phenoxyl radical with either one of the vinyl groups of the heme moiety or a methylene bridge site; further reaction of the initial adduct gives the stable green compound (*153*). Examination of molecular models of both the lupin and soybean forms of Lb from the crystal coordinates [(*23, 29*); (Ellis and Freeman, personal

communication)] show that the Tyr residue (138 and 133 respectively) is aligned in such a way that the phenolate oxygen atom is in close proximity to a vinyl group and methylene bridge (Fig. 6). This suggestion therefore seems very reasonable. Proteolytic digestion of the green compound and sequence analysis of the fragments, in a manner similar to that for the corresponding myoglobin heme-protein cross-linked species (*154*, *155*), should allow the nature of this link to be confirmed.

The heme-to-globin cross-linking process seems to be an inherent feature of proteins in which a radical center is generated in the vicinity of the heme. The formation of such species has been suggested as a marker of exposure to reaction with hydrogen peroxide *in vivo* (*155*, *156*). It is interesting to note that it has long been known that exposure of functional root nodules to stress (e.g., absence of light) results in the formation of "green" nodules (*157*). The green coloration may be a result of the formation of hydrogen peroxide and its subsequent reaction with Lb. It has been reported that at least two green pigments are formed on oxidative damage to Lb both *in vitro* and *in vivo* (*12*, *67*, *157*, *158*). One of these, called cholegobin, originates from oxidative attack on the ring without loss of iron (*158*), whereas biliverdins arise from heme degradation with concomitant loss of both the iron and carbon monoxide (*152*, *159*). Pigments that have these characteristics have been partially purified from senescent pea nodules (*12*), but no further characterization has been reported. It has also been reported that nodules from aging gram plants (*Vicer arietinum*) retain their pink color longer, and do not develop a green coloration until they are much older, when they are kept in a nutrient medium containing ascorbic acid (*37*, *160*). This result is in accord with the protective effects of ascorbic acid against oxidative damage, and with the suggestion that oxidative radical-mediated damage is of importance in the aging of nodules *in vivo*.

Further *in vitro* studies have been carried out, and it has been shown that Fe(III) Lb obtained from common bean (but not soybean) can be reduced by glycine at alkaline pH with the formation of glyoxylate and hydrogen peroxide (*159*). The LbO_2 produced during this reaction is rapidly degraded to a green product that still contains iron and has an absorption maximum at 697 nm. Although the significance of this process *in vivo* remains to be established, it has obvious parallels to the radical processes already described. It is also interesting to note that the formation of the green pigment can be stimulated by pretreatment of the Lb with a proteolytic enzyme (carboxypeptidase) and is inhibited by superoxide dismutase and catalase (*159*).

The latter observations are in accord with the formation of the pigment from a hydrogen peroxide–mediated radical process. A similar formation of green products has been observed during the reaction of Fe(III) Lb with ascorbate (10 mM). This process again results in the transient formation of LbO_2 and rapid breakdown to biliverdin-like materials (*152*). Analysis of the products has shown that in the case of the pea and vetch Lbs, only a single major biliverdin isomer (the b form) was detected, whereas with soybean and the common bean protein, mixtures of products were obtained [*ca.* 30% a isomer, 50% b, and 20% d; (*152*)]. The difference between these Lb forms is of interest. The lack of specificity is indicative of random damage (as might be envisaged from a "free" radical type of reaction, i.e., a low-molecular-weight rapidly diffusing molecule), whereas the highly specific damage seen with pea and vetch is more suggestive of site-specific damage induced by a bound oxidant (i.e., a radical centered on a particular residue). These suggestions relating to the type of radical (either "free" or protein bound) are in accord with the observation that the formation of green products is inhibited by superoxide dismutase and catalase (*152*).

B. Reactions with Low-Molecular-Weight Species

Spectroscopic evidence has been obtained for the reaction of the Tyr phenoxyl radical derived from soybean Lb with a number of low-molecular-weight species. Thus, it has been demonstrated by EPR that the globin radicals can react with ascorbic acid, cysteine, glutathione, Trolox C (a water-soluble analog of vitamin E), thiourea, salicylate, and desferal (desferrioxamine) (*101*, *102*, *134*). Other compounds such as 2-deoxyribose and mannitol either do not react or react slowly. In a number of cases radicals from these reactions could be detected, for example the ascorbate radical, the cysteine and glutathione thiyl radicals, the Trolox C phenoxyl radical, and the desferal nitroxide species (*101*, *102*, *134*). In the case of glutathione, complete loss of the phenoxyl radical was not observed, whereas with an equivalent concentration of cysteine it was. This suggests that access to the radical by species present in bulk solution might be limited (*102*). The accessibility problem is strongly supported by molecular modeling (from the crystal coordinates) of the soybean protein, which shows that the Tyr133 phenoxyl radical is only partially exposed on the protein surface (Fig. 11) (*112*).

It should be noted that with a number of these compounds, reaction occurs with both the Fe(IV)=O heme center and the Tyr radical. Rel-

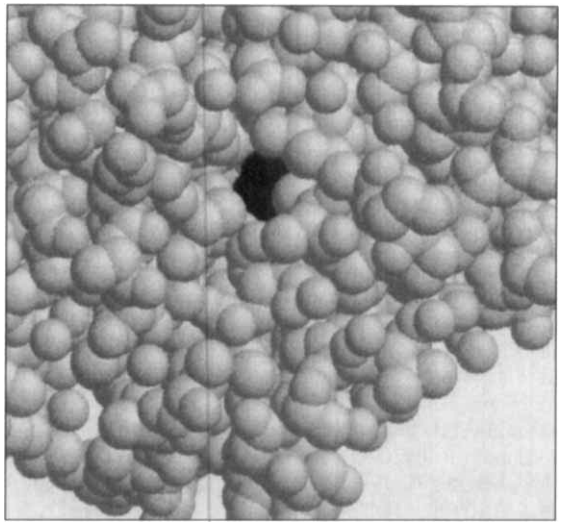

FIG. 11. The Tyr133 residue (dark-shaded residues) in soybean Fe(III) Lb is only partially exposed on the protein surface. Computer-generated molecular model using the crystal coordinates obtained (*23*) (Ellis and Freeman, unpublished data).

atively little is known about the reactions of the other globin-derived radicals (*129*). However, the stoichiometric conversion of ascorbate to dehydroascorbate (*134*) and reduced glutathione to GSSG (*102*) with respect to the amount of hydrogen peroxide added suggests either that the other globin-derived radicals are present at low concentrations or, more probably, also react rapidly.

C. Reactions with Other Leghemoglobin Molecules

Examination by SDS-PAGE of reaction mixtures in which Fe(III) Lb was incubated with a two-fold excess of hydrogen peroxide has provided evidence for the formation of dimeric species (Fig. 12) (*103*). It has been shown that the native protein migrates abnormally fast and appears at the bottom of the gel (*103*, *159*). However a distinct band at *ca.* 32 kDa is observed, with little evidence for other species. No dimerization was observed with apoLb, confirming the role of the heme group in this process. Confirmation of the dimerization process was obtained using size-exclusion HPLC on columns previously calibrated with horse myoglobin (M_r *ca.* 16 kDa) and carbonic anhydrase (M_r 31.5 kDa). In contrast to untreated samples, in which a single band with a retention time very similar to that observed with horse

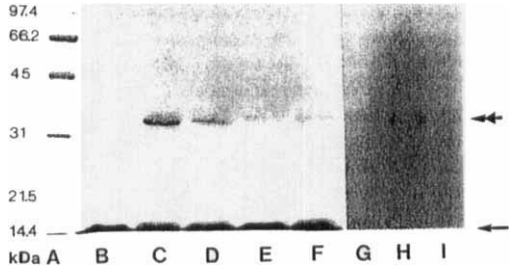

FIG. 12. Dimerization of soybean Lb and its inhibition by ascorbate, glutathione, and tyrosine. Aliquots (30 μg of protein each) of incubations containing untreated 1 mM soybean Lb (lane B), or Lb incubated for 2 h in the presence of 2 mM H_2O_2 without (lane C) or with 5 mM ascorbate (lane E), 5 mM glutathione (lane D) or 5 mM *p*-tyrosine (lane F) were analyzed by SDS-PAGE. Molecular weight markers were in lane A. The abnormally fast migration of monomeric Lb was evidenced by Western blotting with polyclonal antibodies raised against purified soybean Lb_c in the absence (lane G) or in the presence (lane H) of H_2O_2, and a blank was performed with egg lysozyme (MW 14.4 kDa) (lane I). The position of the monomeric Lb is indicated by the arrow and that of the dimeric Lb by the double arrow [reprinted from *Biochim. Biophys. Acta, 1251,* Moreau, S.; Davies, M. J.; Puppo, A.; Reaction of ferric leghemoglobin with H_2O_2: formation of heme-protein cross-links and dimeric species, pp. 17–22, copyright (1995), with kind permission from Elsevier Science—NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands].

myoglobin was observed, the treated samples (two-fold excess of hydrogen peroxide over Fe(III) Lb) gave two bands, a major band corresponding to the parent material and a weaker band that eluted with approximately the same retention time as carbonic anhydrase, consistent with the presence of a dimer (Fig. 13) (*103*). Integration of the peak areas suggests that the dimer represents approximately 6% of the initial Lb. Sufficient material could be obtained from such chromatographic separations to allow further studies to be carried out on nature of the cross-linking. Unlike the situation with sperm whale myoglobin, in which dimer formation is also observed (*109, 161*), no evidence was obtained for the presence of dityrosine after acid hydrolysis of the dimeric material (*103*). Thus, unlike myoglobin the cross-linking does not appear to occur via the dimerization of two tyrosine phenoxyl radicals. This is not unexpected because the phenoxyl radical site is only slightly exposed on the surface of the protein (Fig. 11) (*112*), unlike the case of sperm whale myoglobin in which the Tyr151 residue is very accessible (*161*).

The mechanism of formation of this dimer was further investigated by examining the effect of various putative radical scavengers on the intensity of the dimer band on SDS-PAGE gels (Fig. 12). These stud-

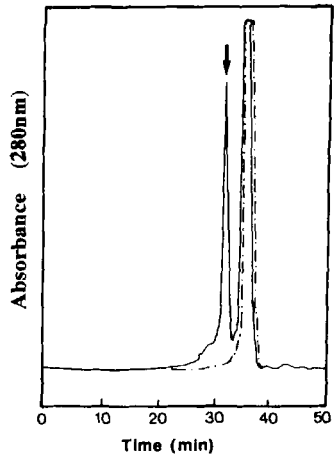

FIG. 13. High-pressure liquid chromatography of H_2O_2-treated Lb. Aliquots (100 μL) of reaction mixtures containing 1 mM soybean Fe(III) Lb incubated for 2 h in the presence of 2 mM H_2O_2 were chromatographed. The trace marked (——) shows the aromatic amino acid absorbance at 280 nm; the peak marked with an arrow is that of the dimer. Control experiments were performed with Lb alone (-·-·-) [reprinted from *Biochim. Biophys. Acta, 1251,* Moreau, S.; Davies, M. J.; Puppo, A.; Reaction of Fe(III) leghemoglobin with H_2O_2: formation of heme–protein cross-links and dimeric species, pp. 17–22, copyright (1995), with kind permission from Elsevier Science—NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands].

ies have demonstrated that ascorbate completely inhibits dimer formation, and glutathione and *para*-tyrosine also significantly inhibit its generation. In contrast, *ortho*- and *meta*-tyrosine have little effect. The reason is not immediately apparent, but may be due to steric factors. The results are in accord with dimerization being a radical process, but not via dimerization of two tyrosine-derived phenoxyl radicals (*103*). The cross-linking may involve dimerization of the reactive radicals detected in spin-trapping experiments (*129*).

D. Reactions with Membranes

A number of studies have examined the possible role of both Fe(IV)=O and globin radicals in the initiation of membrane peroxidation. Thus, it has been reported that although reaction of Fe(III) Lb with hydrogen peroxide (two-fold excess) initiates peroxidation of peribacteroid membranes from French beans (as measured by the yield of malondialdehyde), similar reactions with Fe(II) Lb and hydrogen peroxide did not give a similar effect (*162*). Little oxidation was ob-

served in the absence of hydrogen peroxide, whereas much higher levels of oxidative breakdown products are observed with a 5- or 10-fold excess. In the latter, however, it is known that significant release of iron occurs, and it is possible that the lipid oxidation is due to the reaction of the iron with excess peroxide (*99*). The observation that with Fe(III) Lb and a low excess of peroxide, damage is initiated, whereas none is observed with Fe(II) Lb [which is a more efficient method of generating Fe(IV)=O], suggests that Fe(IV)=O is not able to initiate damage to membrane fractions (*162*). This is not entirely unexpected because Fe(IV)=O would not be normally expected to have ready access to membrane lipids (or other membrane components) due to the surrounding protein. Recent studies with symbiosomes from French beans have supported the suggestion that Fe(IV)=O alone cannot initiate lipid oxidation (*163*). Isolated symbiosomes exposed to Fe(III) Lb that had been preincubated with a two-fold excess of hydrogen peroxide did not appear to suffer damage as monitored by the uptake of succinate (a useful marker of symbiosome membrane integrity). Enhanced uptake of succinate was observed, however, with six-fold excesses of hydrogen peroxide when iron release is likely to have occurred (*163*).

A number of groups have suggested that metal ions derived from Lb and/or trace transition-metal ions can play a role in initiating or propagating damage (*162–164*), but the mechanism by which this occurs is poorly understood. It is possible that the release of iron from the heme protein (possibly as a result of oxidative damage) or from a build-up of trace-metal ions in old nodules by other routes, may be responsible for the catalysis of radical generation, though the details remain uncertain. Recent reports have demonstrated that low-molecular-weight chelates of transition-metal ions are present in old nodules (*162*, *164*), and it has been suggested that hydroxyl radicals may be a key species (*164*). It should be noted, however, that specific products of hydroxyl radical attack were not identified, and it is possible that the oxidation of dimethyl sulfoxide observed may be due to the formation of other radicals. Other studies have also reported that hydroxyl radicals are not involved (*162*). Previous studies using fluorescence quenching (*165*) have suggested that Lb can interact with peribacteroid membranes, possibly via specific binding sites. The presence of such "receptors" might be expected to facilitate damage induced by either protein radicals or high-oxidation-state species.

In the light of the evidence reviewed here that at least some of the globin radicals formed on reaction of Fe(III) Lb with hydrogen peroxide are present on the surface of the protein and that these species

can react both with low-molecular-weight species and other protein molecules, it was of interest to determine whether these species could transfer damage to a membrane (i.e., initiate lipid oxidation). Of particular relevance is whether the Lb-derived radicals could initiate damage to the peribacteroid membrane that surrounds the microsymbiont *in vivo* (Fig. 2) and whether such reactions play a role in the loss of nitrogen-fixing ability of nodules and nodule senescence. The latter membrane appears to be more sensitive to oxidation than others (e.g., microsomes prepared from identical nodules) (*162*), possibly as a result of the different compositions of these structures (e.g., the high lipid : protein ratio of the peribacteroid membrane).

Incubation of purified peribacteroid membranes (PBMs) with soybean Fe(III) Lb/hydrogen peroxide and analysis of the extent of Lb dimerization by SDS-PAGE have demonstrated that the presence of the PBMs decreases (in a dose-dependent manner) the extent of protein dimerization (*129*) (Fig. 14). This has been interpreted as evidence for interaction of the radicals involved in the dimerization reaction with the PBMs. Further evidence for such a process has been obtained from examining the intensity of the signal (which is directly proportional to the radical concentration) of the Tyr-derived phenoxyl radical generated from soybean Fe(III) Lb and H_2O_2 in the presence of added PBMs by stopped-flow EPR spectroscopy. Inclusion of the PBMs (>0.3 mg protein/mL) resulted in a statistically significant decrease in concentration of the phenoxyl radical, consistent with scavenging by membrane components (*129*). Such a process would be expected to result in the formation of one or more radical species in the membrane fractions.

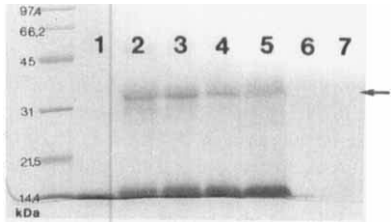

FIG. 14. Quenching by peribacteroid membranes (PBMs) of the dimerization process of Fe(III) Lb in the presence of H_2O_2. Fe(III) Lb (210 μM) was incubated with H_2O_2 (420 μM) for 1 h in the absence (lane 2) or in the presence of PBMs at the following protein concentrations: 0.23 mg/mL (lane 3), 1.16 mg/mL (lane 4) and 2.33 mg/mL (lane 5). Lane 1 represents Fe(III) Lb alone and lane 6 and 7 PBMs plus H_2O_2 and PBMs alone, respectively (reprinted with permission from: Moreau, S.; Davies, M. J.; Mathieu, C.; Herouart, D.; Puppo, A. *J. Biol. Chem.*, **1996,** *271*, 32557–32562).

Inclusion of PBM fractions in Fe(III) Lb/hydrogen peroxide spin-trapping experiments has also provided evidence for such interactions. Thus when PBMs (*ca.* 3 mg protein/mL) were added to an equimolar amount of soybean Fe(III) Lb and hydrogen peroxide in the presence of the spin trap PBN, significant changes were observed in the EPR spectra as compared to control spectra in the absence of the membrane fractions (Fig. 15) (*129*). The additional features present are consistent with the presence of large, slowly tumbling radical adduct species and have been assigned to membrane-derived radicals.

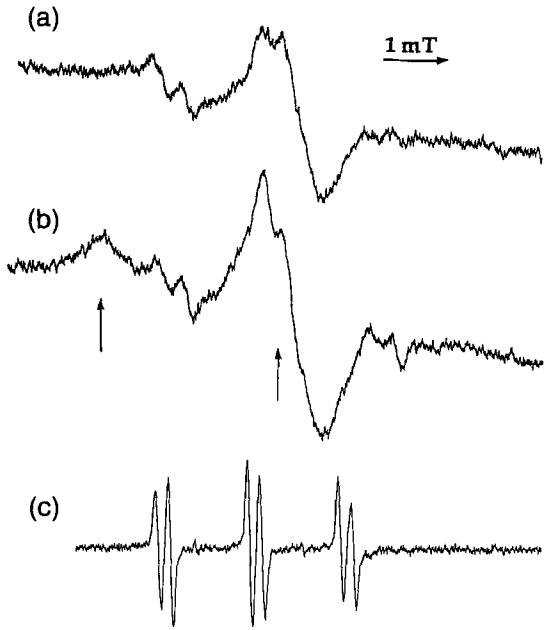

FIG. 15. EPR spectra observed on reaction of soybean Fe(III) Lb (500 μM) with H_2O_2 (1 mM) and the spin trap PBN (46 mM) in the absence or presence of peribacteroid membranes (PBMs). (a) Incubation carried out for 2 h at 29°C in the absence of added membrane fractions; signal assigned to one (or more) large protein-derived radical adducts to the spin trap. (b) Incubation carried out for 2 h at 29°C in the presence of added membrane fractions (2.95 mg protein/mL); signal assigned to one (or more) large protein-derived radical adducts to the spin trap together with a further species (arrowed features) believed to arise from a radical generated from the membrane fraction. (c) As (b) but spectrum of the organic layer obtained after extraction of the membrane fractions with an equal volume of N_2-degassed toluene; signal assigned to at least one lipid-soluble radical adduct arising from radical induced damage to the membrane fraction (reprinted with permission from: Moreau, S.; Davies, M. J.; Mathieu, C.; Herouart, D.; Puppo, A. *J. Biol. Chem.*, **1996,** *271*, 32557–32562).

To obtain further information on the nature of these intermediates, solvent extraction experiments were carried out. When toluene was added, the radical adducts were observed only in the organic phase. This is consistent with the presence of lipid-derived radicals (or other hydrophobic species). The signals observed from the toluene extracts are considerably more mobile than those seen in the aqueous system, suggesting that the slow tumbling is due to the formation of a lipid radical in the membrane, which is prevented from rotating rapidly by the membrane. The sharp (isotropic) nature of the signals in these organic extracts has allowed further fine structure of the EPR signals to be observed. The hyperfine coupling constants are consistent with a lipid-derived species (*129*).

There is now considerable evidence for an interaction of the globin-derived radicals with the peribacteroid membrane resulting in a transfer of the radical into the membrane (*129*). The membrane-derived radicals would be expected to participate in chain reactions in the presence of oxygen, and this would result in peroxidative damage to the membrane. Lipid peroxidation reactions are known to result in widespread alterations to membrane structure and can lead to irreversible loss of function. Such reactions may play a role in the disruption of the peribacteroid membrane during nodule senescence, when conditions for peroxide formation and subsequent radical reactions would be expected to be favorable. An obvious corollary is that species that protect against the formation of Fe(III), Fe(IV)=O and globin radicals might inhibit the reactions and hence nodule degradation. These may include nonenzymic reactions (e.g., with ascorbate, cysteine, and glutathione), all of which are present in nodules at high concentrations, and enzymatic reactions brought about by superoxide dismutase, catalase, Fe(III) Lb reductase, and possibly other enzymes. Manipulation of the levels of activity of these species is an obvious potential strategy for increasing the lifetime and activity of the nitrogen-fixing processes. Some evidence suggests that this hypothesis is indeed correct—for example, the observation that the nodules from aging gram plants remain pink for longer periods in the presence of added ascorbate in the growth medium (*160*). Although significant protection by addition of exogenous materials such as ascorbate and thiols is unlikely to be of any great practical utility, the manipulation by genetic means of the enzymes that regulate the production of these low-molecular-weight materials and the concentration and activity of the protective enzymes within the host plant and bacteroid may be realistic and achieveable goals. Work toward these targets is already under way.

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