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Absence of Synproportionation Between Oxy and Ferryl Leghemoglobin

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The synproportionation reaction between ferryl leghemoglobin and oxyleghemoglobin does not occur, at least under conditions where this process could be clearly demonstrated with myoglobin and hemoglobin. In contrast, a cross synproportionation can occur between oxyleghemoglobin and ferryl myoglobin or between ferryl leghemoglobin and oxymyoglobin. The non-exposure, at the surface of the leghemoglobin molecule, of the nearest tyrosine residue to the heme group could explain this behaviour. Thus leghemoglobin *per se* does not appear to be able to act as an antioxidant in removing H_2O_2 by synproportionation. However, in the presence of ascorbate and/or glutathione which can reduce ferryl leghemoglobin, this hemoprotein could act as an H_2O_2-removing antioxidant, in a process similar to that described for myoglobin. This could also explain why, despite the absence of synproportionation, ferryl leghemoglobin is not detected in nodule extracts.

Keywords: Leghemoglobin, nitrogen fixation, senescence, hydrogen peroxide, myoglobin, hemoglobin

INTRODUCTION

Leghemoglobin (Lb), a monomeric heme protein of Mr approx. 16 000, has been found to be necessary, amongst other nodule-specific processes, for efficient nitrogen fixation in legume root nodules.[1] The major function of this hemoprotein *in vivo* appears to be to transport oxygen to the bacteroids at a concentration that is compatible with both bacteroidal nitrogenase activity and respiration.[1] In functioning nodules, Lb exists mainly in the reduced form[2] but, because of the low O_2 partial pressure present in the central tissue of root nodules, only about 20% is oxygenated to give oxyleghemoglobin (oxyLb).[3] OxyLb has been shown to undergo slow autoxidation to the ferric form with the concomitant production of superoxide radicals, which disproportionate to

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form H_2O_2.[4] The formation of potentially damaging oxygen-derived species, such as H_2O_2, in root nodules, may additionally occur as a result of the strong reducing conditions required for nitrogen fixation, and the action of several proteins including ferredoxin, uricase and hydrogenase.[5] As with hemoglobin (Hb) and myoglobin (Mb), ferrous Lb has been shown to react with H_2O_2 to form ferryl Lb, Lb(IV).[6] The ferric form of Lb also reacts with H_2O_2 and in this case at least one globin-derived radical is formed during the course of the reaction;[7] the quenching of this radical appears to involve either intra- or intermolecular processes.[8] The ferryl forms of Hb and Mb have been shown to synproportionate with their respective oxy forms, to produce the respective ferric species,[9,10] *i.e.* Fe(IV) + Fe(II)O_2 → 2 Fe(III). This has been shown by two different methods: by mixing the ferryl and the oxy forms or by submitting the oxy form to a continuous flux of H_2O_2, the ferryl form resulting from reaction of some of the oxy form with H_2O_2 subsequently reacting with the remaining oxy.[10] The possibility that oxyMb could play a role in the elimination of H_2O_2 has therefore been suggested[9] and a similar novel antioxidant role for oxyHb has been proposed.[10]

In the present paper, we show that ferryl Lb and oxyLb do not appear to undergo synproportionation. Despite the non-occurrence of this reaction which would have been expected to remove ferryl Lb, this species is not observed in senescing nodules; other modified forms are however observed. The possible reasons for this are discussed.

MATERIALS AND METHODS

Preparation of the Different Lb and Mb Forms

Soybeans (*Glycine max*) were grown in a glasshouse, and the Lb*a* purified from the root nodules as described previously.[11] Ferric Lb was generated by oxidation with ferricyanide, followed by chromatography on a Sephadex G-15 column; ferrous Lb was obtained as described by Aviram *et al.*[6] Ferryl Lb was obtained either from the ferrous form[6] or the ferric one in the presence of glutathione.[12] In the latter case, excess glutathione was immediately removed by filtration through a membrane filter disc with a Mr cut-off of 10 kDa (Filtron, Coignières, France). The Lb concentration was determined by using a millimolar extinction coefficient of 14.7 at 574 nm for oxyLb and of 10.4 at 543 nm for ferryl Lb.[6]

Myoglobin (Mb) from horse heart was obtained from Sigma (Sigma-Aldrich, Saint Quentin Fallavier, France). The oxyMb was prepared as indicated above for oxyLb. Conversion to the ferryl form was performed immediately prior to use by addition of a 4:1 excess of H_2O_2: heme to a ferric solution;[9] the excess H_2O_2 was removed by addition of catalase. The Mb concentrations were determined according to Whitburn *et al.*[13]

Spectrophotometric Assays

Reaction of Lb with the H_2O_2 generating system was monitored by recording repetitive scans over the range 450–750 nm. Reaction mixtures contained 50 μM Lb, 5 mM glucose and glucose oxidase (10 μg/ml) in 25 mM KH_2PO_4/KOH buffer, pH 7.4. Glucose oxidase, which catalyzes the reaction glucose + O_2 → gluconic acid + H_2O_2, was added to start the reaction. The absorption maxima for the Lb forms studied were as follows: 543 nm for ferryl Lb, 630 nm for ferric Lb, 574 nm and 541 nm for oxyLb.

The synproportionation reaction was followed at 630 nm (absorption maximum for both ferric Lb and ferric Mb); reactions, in 10 mM KH_2PO_4/ KOH buffer (pH 7.4), were started by mixing oxyLb*a* with ferryl Lb*a* or ferryl Mb, or ferryl Lb*a* with oxyMb, prepared separately as described above.

Obtaining the Nodule Extracts

To avoid oxidation processes, nodules (4 and 11 weeks old) were crushed in chilled, chelexed and

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degassed 25 mM KH_2PO_4/KOH buffer (pH 7.4) containing 10% insoluble PVP (w/v). After centrifugation at 8000 g to eliminate cell debris and bacteroids, extracts were directly applied on to narrow range (4.6—5.7) immobiline isoelectric focusing gels which have been shown to be able to separate the different redox forms of Lb*a* without interference from other Lb components (Lb*b*, *c* and *d*).[11]

Immunoblotting

Following isoelectric focusing, proteins were transferred from the gel to an Immobilon-P (0.45 μm) polyvinylidene difluoride (PVDF) microporous membrane (Millipore, Saint Quentin en Yvelines, France) for 1 h, at 4°C. The membrane was blocked overnight with a solution of 3% BSA (w/v) and incubated, for 1.5 h, with rabbit polyclonal antibodies (10 mg/ml) raised against purified soybean ferric Lb*a*. Proteins of interest were visualized by the goat anti-rabbit horseradish peroxidase development method.[14]

RESULTS

Assays with a Continuous Flux of H_2O_2

To our knowledge, until now the generation of ferryl Lb has only been studied in conditions where Lb and H_2O_2 (at ratios ranging from 1:2 to 1:4) are mixed to start the reaction.[6,7] In the first part of this work, H_2O_2 was produced in a continuous flow—generated during the glucose oxidase-catalyzed oxidation of glucose (see Materials and Methods)—to better mimic physiological conditions. Under similar conditions, ferryl Hb has been shown to synproportionate with oxyHb to give the ferric form.[10] When this H_2O_2-generating system was mixed with oxyLb, the disappearance of the 574 nm and 541 nm peaks without the appearance of a new peak indicated that some heme degradation was occurring (Figure 1). Even on modifying the ratio of the concentrations of the

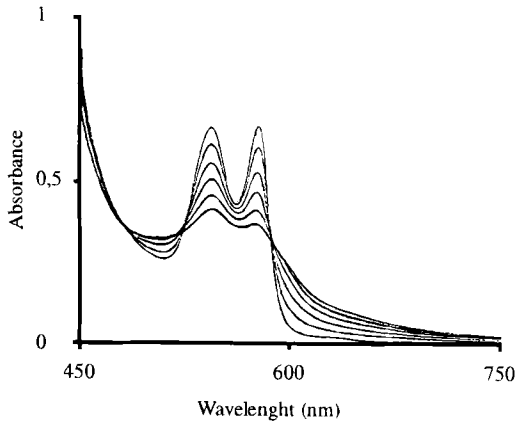

FIGURE 1 Reaction of oxyLb*a* with the H_2O_2 generating system. Reaction mixtures containing oxyLb*a* (51 μM) in air-saturated 25 mM KH_2PO_4/KOH buffer (pH 7.4) and glucose (5 mM) were supplemented with glucose oxidase (10 μg/ml) to initiate a continuous flux of H_2O_2. Repetitive scans were recorded immediately after glucose oxidase addition and then at 20-min intervals.

H_2O_2-generating system to Lb, we were unable to detect either the ferryl or the ferric forms of Lb. Similar results have been previously obtained on direct mixing of oxyLb with H_2O_2,[6,15] where Lb degradation was also observed (the ferryl form was obtained only with the deoxygenated ferrous Lb[6]). When ferric Lb was exposed to the glucose/glucose oxidase system, a compound exhibiting absorption maxima at 628 nm and 575 nm was obtained, which appears identical to that detected in experiments where ferric Lb and H_2O_2 were mixed directly.[12] This spectrum differs markedly from that of Lb(IV) and has recently been ascribed to a species arising from an intramolecular heme-protein cross-link, probably involving a tyrosine radical.[7] Since aerobic conditions are necessary for the functioning of the glucose oxidase, analogous experiments with ferrous Lb could not be performed.

Assays with Ferryl Lb and OxyLb

In a second set of experiments, the possible interaction of stoichiometric amounts of previously prepared (see Materials and Methods) ferryl Lb

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and oxyLb was studied. The ability of ferryl Lb*a* to autoreduce was first examined: as shown by Figure 2 (curve A), a slow spontaneous formation of ferric Lb from Lb(IV) was observed. In experiments with oxyLb*a*, a slow autoxidation of this form of the protein to the ferric form was observed (Figure 2, curve B), in agreement with previous studies.[4]

On mixing ferryl Lb*a* and oxyLb*a*, an increase in absorbance at 630 nm due to ferric Lb was observed (Figure 2, curve C). However, the rate of formation of this species was not significantly different from the sum of the rates observed with the corresponding concentrations of ferryl Lb*a* and oxyLb*a* alone (Figure 2, curves A and B), clearly indicating that a synproportionation reaction does not occur.

The lack of such a process with Lb could be due to the stability of the ferryl or oxy oxidation states of Lb compared to the corresponding Mb and Hb species (cf. previous studies which have shown that the ferryl state of Lb is particularly stable[6]), or due to steric/electrostatic interactions between Lb molecules slowing down possible electron transfer processes. In order to examine these possibilities, evidence for possible cross-synproportionation between oxyLb*a* and ferryl Mb was examined. These species were found to be capable of undergoing synproportionation (Figure 2, curve D) at a rate that appeared similar to that obtained on mixing oxyMb and ferryl Mb (Figure 2, curve E). Synproportionation occurred also between ferryl Lb*a* and oxyMb, although at a slightly slower rate than that observed with oxyLb*a* and ferryl Mb (data not shown).

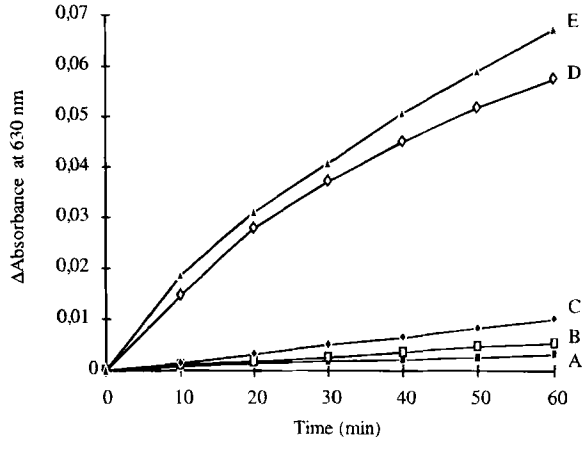

FIGURE 2 Ferric Lb formation. Reaction mixtures contained, in 10 mM KH_2PO_4/KOH buffer (pH 7.4), ferryl Lb*a* (52 μM) alone (curve A), oxyLb*a* (50 μM) alone (curve B), oxyLb*a* (50 μM) plus ferryl Lb*a* (52 μM) (curve C) or ferryl Mb (51 μM) (curve D), oxyMb (47 μM) and ferryl Mb (51 μM) (curve E). 630 nm is the wavelength of a typical absorption maximum of both ferric Lb and ferric Mb.

Is Ferryl Lb Present *in vivo*?

The existence of the synproportionation reaction of Hb has been suggested as a possible explanation for the difficulties encountered in the identification and quantification of ferryl Hb *in vivo*.[10] Based on the results described above, it appeared interesting to look for the presence of ferryl Lb in nodules, especially in senescing ones, where the nodules antioxidant defences are decreased.[16] To limit possible redox processes during the preparation of the extracts, a minimal purification procedure was used (see Materials and Methods). In order to examine the Lb forms present in these crude extracts immunological detection was employed. In such experiments, the ferryl form of Lb*a* was shown to cross-react with polyclonal antibodies raised against ferric Lb*a*. On narrow range immobiline gels, we were unable to detect ferryl Lb*a*, which has a pI of 5.1.[8] In contrast, two other bands, corresponding to pI of 5.38 and 5.45 were detected, together with ferric Lb*a* (Figure 3, lane A). The species giving rise to the band with pI 5.45 was green in colour and had an identical absorption spectrum and pI to that reported previously for the the so-called green compound, formed during reaction of ferric Lb with H_2O_2.[8] The species giving rise to the other band did not exhibit a «normal» Lb type absorption spectrum and was most probably another Lb degradation product. None of these bands appeared in extracts from 4 weeks old nodules. A positive control was performed in

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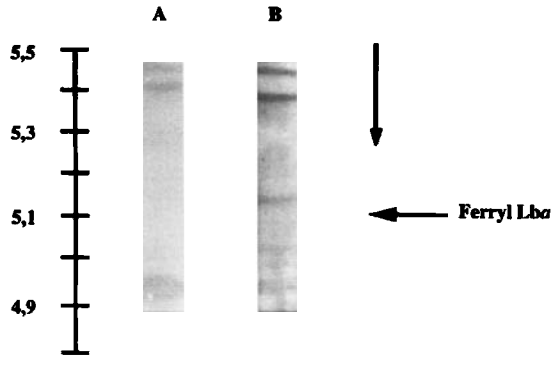

FIGURE 3 Detection of modified Lbs in extracts of 11 weeks old soybean nodules. The different forms of Lb*a* were revealed by immunoblotting with a polyclonal antibody raised against ferric Lb*a*. The vertical arrow represents the migration direction and the scale indicates the pH gradient. Lane A: 11 weeks old soybean nodules extract. Lane B: positive control: 11 weeks old soybean nodules extract plus exogenous ferryl Lb*a* prepared as described in Materials and Methods.

adding exogenous ferryl Lb*a* to the nodule breis: in this case a band corresponding to this ferryl Lb*a* was detected (Figure 3, lane B), indicating that the «minimal purification-electrophoresis» procedure could indeed detect the ferryl form.

DISCUSSION

The results presented here indicate that a synproportionation reaction between ferryl Lb and oxyLb does not occur, at least under conditions where this process could be clearly demonstrated with Mb and Hb. In the synproportionation process between oxyMb and ferryl Mb, an electron transfer mediated by an amino acid in the vicinity of the heme moiety has been suggested as the key process,[9] as no contact between the edges of the heme moieties is possible. The involvement of tyrosine residues in electron transfer between oxy- and ferryl Hbs has been proposed, since acetylation of these residues with N-acetylimidazole produced a decreased rate of synproportionation.[10] A similar inhibition of the synproportionation process has been observed with myoglobin on treatment with this agent.[10] Examination of the three-dimensional structure of the ferric nicotinate complex of soybean Lb*a* obtained from X-ray crystallographic studies, shows that electron transfer involving the nearest Tyr residue to the heme group (Tyr 133), where radical generation is believed to occur,[17] is unlikely, as this residue is not significantly exposed on the outer protein surface (Figure 4). The second Tyr residue in this form of the protein (Tyr 30) is also only slightly exposed and is probably not accessible enough to interact with another protein molecule. The third one (Tyr 25) is very exposed on the surface, but is a very long distance from the heme centre, and hence electron transfer via this residue might be expected to be very slow, if it occurs at all. The partially buried position of Tyr 133 may explain the absence of synproportionation in the case of Lb: the distance between two partially buried Tyr 133 residues of two Lb molecules is probably too long to allow electron transfer. This hypothesis is further reinforced by the results obtained on mixing the oxy form of Lb with ferryl Mb or the oxy form of Mb with ferryl Lb,

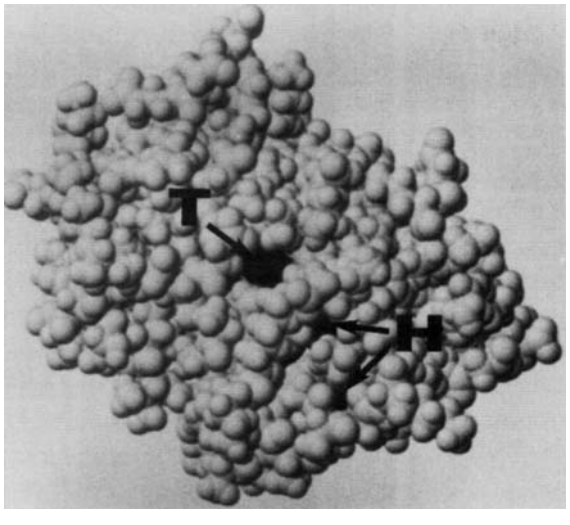

FIGURE 4 Molecular model of soybean Lb*a* showing that tyrosine 133 is partially buried. The heme group is noted «H» and Tyr 133 «T».

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where the favourably exposed Tyr 103 residue at the surface of the Mb protein could allow this exchange with Lb Tyr 133, possibly via other Lb aminoacid residues (as the distance between the two Tyr residues is significantly lower than in the previous case).

In contrast with Hb and Mb, Lb does not appear to be able to act as an antioxidant by removing H_2O_2 via synproportionation. Thus the formation of ferryl Lb leads to an inactivation of this hemoprotein as this form cannot act as a oxygen carrier.[6] Ferryl Lb appears to slowly autoreduce; a possible explanation could be that an amino acid residue in the globin protein is oxidized in the process. Here again, the role of Tyr 133, which appears to be near the heme moiety, can be suggested. This process, if it occurs, should lead to the formation of a protein radical which could be quenched by intra-molecular cross-links.[8] This mechanism could explain the observed degradation of the structural integrity of Lb during multiple cycling of Lb III → Lb(IV) → Lb III.

In vivo such nodules probably possess efficient ferryl Lb reduction processes: ascorbate and glutathione, which are present at high concentrations in the nodule cytosol,[16] are good candidates for this role.[12,18] In this framework, the combination of Lb plus ascorbate/glutathione could be regarded as an H_2O_2-removing antioxidant, in a process similar to that described for Mb.[19] This could also explain why, despite the absence of synproportionation, ferryl Lb was not detected in nodule extracts, although we cannot completely exclude the presence of traces of this form (Figure 3). One of the modified Lbs evidenced in extracts of senescing nodules appears to be similar, or identical, to the so-called green compound.[8] This species has previously been proposed to arise from reaction between ferric Lb and H_2O_2, resulting in formation of a phenoxyl radical which subsequently forms an intramolecular cross-link between the heme ring and the globin.[8] This suggestion is strengthened by the detection of ferric Lb in the same extracts; this form of Lb may arise from the more rapid autoxidation of the ferrous forms of Lb as a result of the drop in cytoplasmic pH during senescence.[20] Thus, the simultaneous presence of ferric Lb and H_2O_2 can lead to the formation of this green compound, which is reduced neither by glutathione nor by ascorbate.[8] This result is in agreement with the extraction, from soybean nodules, of modified Lbs, the concentration of which increases during ageing.[21] On the other hand, the possible formation of Lb radicals in senescing nodules may be of great biological importance. These radicals have indeed been shown, *in vitro*, to transfer damage to the peribacteroid membrane[22] which is, *in vivo*, one of the first structures to be degraded during senescence.[23]

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