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How is leghemoglobin involved in peribacteroid membrane degradation during nodule senescence?

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An increase in the rate of succinate and glutamate uptake by isolated symbiosomes from French bean nodules was observed in the presence of iron plus H_2O_2. The lipid bilayer, and not proteins involved in transport, seems to be the major target of radical attack. Leghemoglobin in the presence of a 6-fold excess of H_2O_2 (where heme breakdown and iron release occurred) provoked also an increase in peribacteroid membrane permeability. In contrast, this hemoprotein in the presence of a 2-fold excess of H_2O_2 (where a protein radical was generated) was without effect. We suggest that in vivo the release of heme iron may constitute the major process concerning the involvement of leghemoglobin in the degradation of the peribacteroid membrane during nodule senescence.

Peribacteroid membrane; Senescence; Lipid peroxidation; Leghemoglobin; Dicarboxylate transport; French-bean nodule; Symbiosome

1. INTRODUCTION

In legume root nodules, symbiotic *Rhizobium* are separated from the host cell cytosol by the peribacteroid membrane (PBM), forming a structure called the symbiosome [1]. The PBM appears to play an essential role in the interaction between the two partners, as indicated by its selective permeability [2,3]. For example, the PBM has been shown to contain a dicarboxylate carrier [2,4] which may correspond to the symbiotic protein nodulin 26 [5], but the membrane is poorly permeable to some amino acids and sugars [2,3].

During the early stages of senescence, or when nodulated plants are submitted to nitrate stress, the PBM is one of the first structures to be degraded and its disappearence parallels the loss of nitrogen-fixing capacity [6]; indeed, loss of the PBM could well participate in the senescence process and even provoke it. In this context, it is interesting to note that symbiosomes are bathed in a leghemoglobin (Lb)-rich cytosol. This myoglobin-like hemoprotein is susceptible to autoxidation [7] which leads to the formation of superoxide anions ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2); these reactive species can also be formed in nodules by several other reactions [8]. In the presence of molar ratios for H_2O_2:Lb of 1 or 2, this hemoprotein can generate a tyrosine phenoxyl radical together with the ferryl species [9]. On the other hand, in the presence of excess H_2O_2, heme loss from Lb is observed, leading to the existence of free iron [10]. All of these reactions are favoured during senescence when Lb autoxidation is accelerated by the acidification of the nodule cytosol [11] and the activity of O_2-derived species scavenging enzymes decreases [12]. As a consequence, an increase in non-protein-bound iron concentration parallels the decline of Lb content in senescing nodules [13]. The involvement of iron in membrane lipid peroxidation is well known: its role has been shown both in the decomposition of lipid peroxides and in the initiation step itself [14,15]. It also has been recently proposed that a tyrosine peroxyl radical, derived from the tyrosine phenoxyl radical, is an important species in myoglobin-induced lipid peroxidation [16].

Clearly, PBM degradation will interfere with the regulated exchange of signals and metabolites between the partners, and thus may stimulate nodule senescence. In the present study, the ability of iron released from Lb, and activated Lb itself, to perturb the permeability of the peribacteroid membrane of French bean nodules was investigated. The involvement of Lb in damage to the symbiosomes during senescence is discussed within the framework of the interaction and metabolic exchange between symbionts.

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Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; Lb, leghemoglobin; PBM, peribacteroid membrane; PMSF, phenylmethylsulfonyl fluoride.

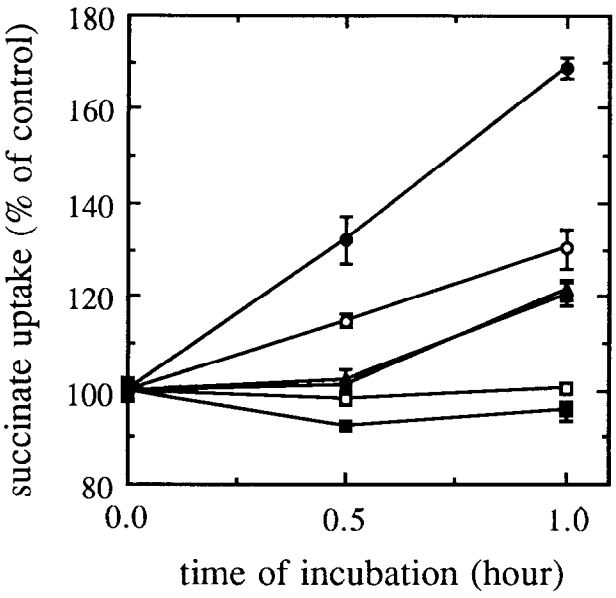

Fig. 1. Effect of iron and H_2O_2 on the rate of succinate uptake by isolated symbiosomes. Equal aliquots (1 ml) of a symbiosome suspension were incubated either with standard wash medium or wash medium supplemented with 100 μM $FeCl_3$, 100 μM $FeSO_4$ and 400 μM H_2O_2, at 25°C. At the indicated times, 100 μl of the incubation mixture was sampled for a 5 min succinate uptake assay as described in section 2. The control (100%) rate of succinate uptake was 1.2 nmol/min/mg protein. Three separate experiments were performed for each reaction medium and the values shown are means ± S.E.M. Closed symbols indicate incubations with, and open symbols those without, the radical generating system. (■, □) Sucrose medium; (▲, △) mannitol medium; (●, ○) NaCl medium.

2. MATERIALS AND METHODS

2.1. *Materials*

French beans (*Phaseolus vulgaris* L. cv. Contender) were grown in a glasshouse (temperature range 20–27°C) and supplied with a nitrogen-free solution as previously described [2]. Plants were inoculated with *Rhizobium leguminosarum* biovar *phaseoli* strain 9–6. Nodules were harvested when they were 3–4 weeks old. All chemicals were purchased from Sigma except [U-^{14}C]succinic acid (5.55 GBq/mmol) from Dositek (Orsay, France), and oxonol V from Molecular Probes Inc., Eugene, OR (USA). Lb was purified from French bean nodules as described in [13]. Lb *a*, used in our experiments, was separated from Lb *b* by chromatography on DEAE-cellulose and isoelectric focusing.

2.2. *Preparation of symbiosome and bacteroid fractions*

The preparation of symbiosome and bacteroid fractions in mannitol medium was as described elsewhere [17]. The operations were exactly the same when sucrose was used in place of mannitol. With NaCl medium, the first steps were performed in mannitol medium because of the instability of symbiosomes in highly ionic solutions: around 40 g of freshly harvested nodules were crushed in a glass mortar at 4°C, in 100 ml of homogenization medium containing 25 mM MES-Tris buffer, pH 7.0, 8% (w/v) mannitol, 2.5% (w/v) Ficoll 400, 5% (w/v) Dextran T40, 2 mM $MgSO_4$, 5 mM EDTA, 1% BSA, 5 mM DTT, 1 mM PMSF, and adjusted to 600 mosmol/l. The homogenate was filtered through a 200 μM nylon mesh. A first centrifugation (150 × *g*, 10 min, 4°C) eliminated cell debris. During a second centrifugation (300 × *g*, 10 min, 4°C) most of the symbiosomes were sedimented. The pellet was then resuspended in the NaCl medium containing KH_2PO_4/K_2HPO_4 buffer, pH 7.0, 1.6% NaCl and 2 mM $MgSO_4$, and centrifuged again (300 × *g*, 3 min, 4°C). This last fraction was layered onto Percoll gradients (each Percoll solution has the same composition as NaCl medium) performed as previously described [17]. Symbiosomes were diluted in NaCl medium and pelleted (300 × *g*, 2 min, 4°C). The pellet was then resuspended in NaCl medium and equilibrated at 25°C, 30 min before kinetic experiments. A bacteroid fraction was obtained by vortexing symbiosomes vigorously for 2 min, to rupture PBMs.

2.3. *Peroxidation assays*

Membrane peroxidation was initiated by the addition of different volumes of $FeSO_4$, $FeCl_3$, H_2O_2 or Lb to 0.7 ml or 1 ml of symbiosome suspension and incubated at 25°C. All iron-containing solutions were prepared in ultra-pure water, bubbled with argon and conserved on ice. Lb was pre-mixed with H_2O_2 before addition to prepared fraction. After the desired time of incubation, 100 μl samples were taken up for kinetic assays.

2.4. *Transport studies*

Symbiosomes (around 0.1 mg of protein) were incubated with 10 kBq of [U-^{14}C]succinate or glutamate at a final concentration of 100 μM in a total volume of 300 μl, under aerobic conditions at 25°C. Two samples of 100 μl from individual assays were taken at 0.5 and 5 min after the addition of the purified fraction and each diluted into 5 ml of wash medium [17]. They were then filtered through GF/F Whatman filters, and washed again with 5 ml. Filters were counted into 4 ml of Aqualuma Plus, using a Beckman liquid scintillation counter.

2.5. *Protein estimation*

Protein concentrations were estimated according to [18] after solubilizing in 0.01% (w/v) Triton X-100 with BSA as a standard.

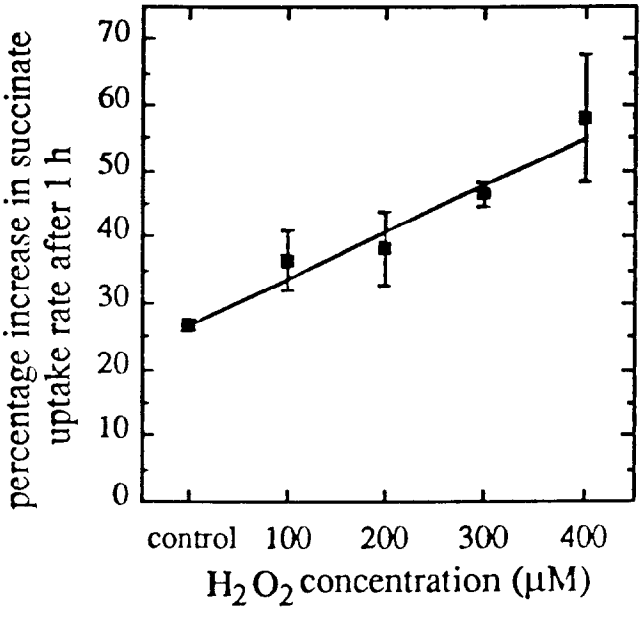

Fig. 2. Effect of increasing concentrations of H_2O_2 on the enhancement of succinate uptake by symbiosomes. Symbiosomes (0.7 ml) were incubated with 100 μM $FeCl_3$, 100 μM $FeSO_4$ and the indicated concentration of H_2O_2, at 25°C for 1 h. At time 0 and 1 h, 0.1 ml of the incubation mixture was sampled and used in a 5 min succinate uptake assay, as described in section 2. Each point shown is the difference in the rate of uptake between the two sampling times. The control contained neither H_2O_2 nor iron and represents the usual change in rate over 1 h at this temperature. 100% corresponds to a rate of 1.2 nmol succinate/min/mg protein. Values shown are the means ± S.E.M., $n = 3$.

2.6. *Membrane energisation*

Formation of an electrical potential difference ($\Delta\psi$) across the PBM of intact symbiosomes (interior positive) was measured by following the quenching of oxonol fluorescence (excitation wavelength 580 nm, emission 650 nm) in a Perkin-Elmer (Norwalk CT, USA) model LS-5 luminescence spectrophotometer. Approximately 500 μg of symbiosome protein and 4 μM oxonol were added to 3 ml of mannitol wash buffer (25 mM MES-Tris buffer, pH 7.0, 8% (w/v) mannitol, 2 mM $MgSO_4$); quenching was initiated by adding 1 mM Na-ATP; after several minutes, 4 μM CCCP was added.

3. RESULTS AND DISCUSSION

3.1. *Succinate transport*

Anny effect of radicals on the structure of the PBM should be reflected in changes of the permeability of the membrane. These changes could include activation or inhibition of specific transport proteins, or non-specific permeabilisation. A dicarboxylate carrier capable of rapid succinate transport across the PBM has been characterized in symbiosomes from various legumes [2,4]. In French beans, succinate uptake by bacteroids is substantially faster than that by intact symbiosomes at about 100 μM succinate [2]. We have taken advantage of this difference to test the effect of radical-generating systems on PBM permeability, since disruption of the PBM can be monitored as an increase in the rate of uptake. At this concentration, which is close to the apparent K_m of the PBM system, activation of the carrier will also be observed as an increase in uptake rate.

Isolated symbiosomes incubated at 25°C usually exhibited a slight increase in the rate of succinate transport with time, regardless of incubation conditions, when osmotica other than sucrose were used (Fig. 1). The structure of French bean symbiosomes (numerous bacteroids enclosed by a fragile membrane) probably contributed to the release of free bacteroids under these conditions. In the same way, the high viscosity of sucrose could explain the higher stability of the symbiosomes in this osmoticum.

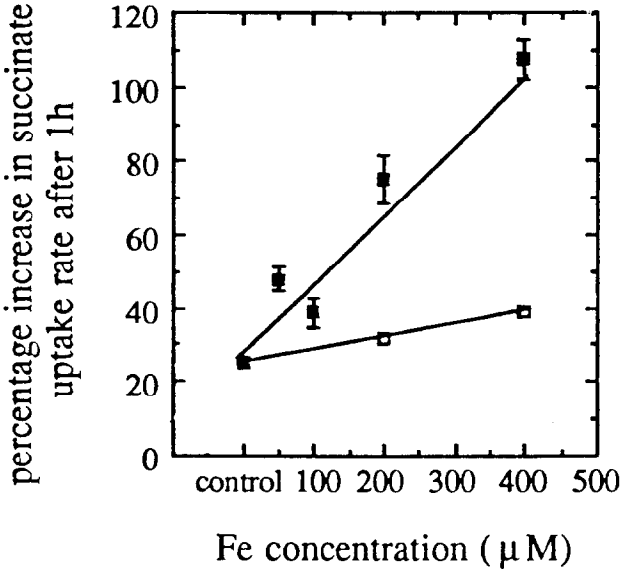

Fig. 3. Effect of increasing concentration of iron on the enhancement of succinate uptake by symbiosomes. Symbiosomes (0.7 ml) were incubated with $FeCl_3$ and $FeSO_4$ at the indicated concentrations, with (■) or without (□) 100 μM H_2O_2. Experimental details as in Fig. 2.

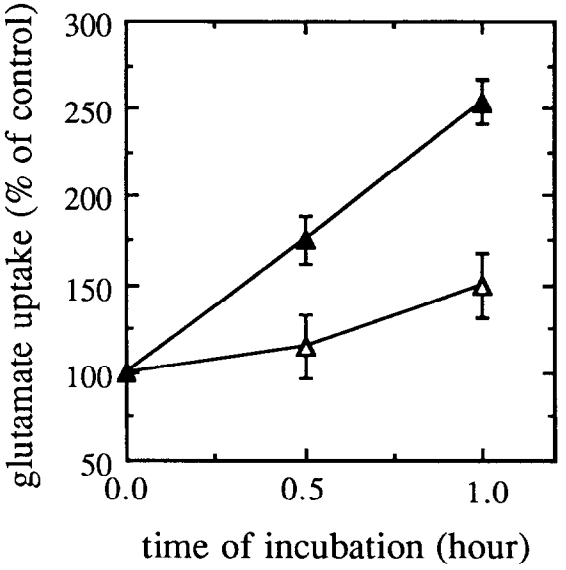

Fig. 4. Effect of iron and H_2O_2 on the rate of glutamate uptake by isolated symbiosomes. Glutamate uptake was measured as described in section 2. The control (100%) rate of glutamate uptake was 0.26 nmol/min/mg protein. Three separate experiments were performed with (▲) or without (△) 100 μM $FeCl_3$, 100 μM $FeSO_4$ and 400 μM H_2O_2 and the values shown are means ± S.E.M.

The radical-generating system was constituted using H_2O_2 and equimolar amounts of ferrous and ferric iron ions, which have been previously shown to cause peroxidation in various membranes [19,20], including the PBM [13]. This mixture is likely to exist in vivo since ferric ions can be-reduced by O_2^- [14]. In order to observe effects within a reasonable period of time, symbiosomes were purified in phosphate buffer using NaCl as osmolyte. Although preparation in a medium with such a high ionic strength led to a fall in symbiosome yield, it avoided rapid quenching of free radicals seen with the sugars. When symbiosomes were submitted to the radical-generating system an acceleration of this phenomenon was clearly observed (Fig. 1), indicating peroxidation-induced changes in membrane permeability. When carbohydrates (mannitol or sucrose) and MES-Tris buffer were used in preparation media, as in previous work [2,4], we failed to observe a significant difference between the control and the fraction incubated with iron and H_2O_2 over 1 h (Fig. 1). The iron-chelating and hydroxyl radical-quenching activity of these carbohydrates can easily explain this result.

Changes in symbiosome permeability to succinate were studied as a function of both iron and H_2O_2 concentrations. In these experiments, the increase in succinate uptake rate observed in the controls was subtracted

from that which occurred under peroxidation conditions. Fig. 2 shows clearly that the degree of PBM permeabilisation increased with H_2O_2 concentration. When no iron ions were added to the incubation medium, H_2O_2 appeared to be without effect up to a concentration of 400 μM, indicating that the observed effect was really caused by radical generation. This was further confirmed by the dramatic effect of increasing iron ion concentration on the rate of succinate uptake by the treated symbiosomes (Fig. 3). When iron was added without H_2O_2, membrane permeabilisation was observed at a lower but reproducible extent. This may have been caused by traces of performed lipid peroxides in our preparations, which, in the presence or iron, would decompose to chain-propagating peroxyl and alkoxyl radicals [22].

3.2. *Glutamate uptake and permeability of the PBM to protons*

To assess whether the above effects on succinate uptake were due to a specific enhancement of the PBM dicarboxylate carrier or a general effect on membrane permeability, two additional transport functions of the PBM were examined.

Glutamate uptake by free bacteroids is rapid and is catalysed by a specific carrier; the PBM, on the other hand, is virtually impermeable to this amino acid [2,3]. Thus the rate of glutamate uptake by symbiosome fractions can also be used to monitor changes in the permeability of the PBM. Fig. 4 shows that exposure of symbiosomes to the radical-generating system led to a marked increase in the rate of glutamate uptake, although an even greater effect could be expected on the basis of the high capacity of glutamate uptake by bacteroids [2]. The initial rate of glutamate uptake was indeed the result of contamination by free bacteroids, which are often present in symbiosome suspensions [2,3]. Incubation of symbiosomes with the radical-generating system presumably increased the access of glutamate to the bacteroids by damaging the PBM.

The PBM from different legumes has been shown to possess a P-type ATPase which pumps protons into the symbiosome and energises the membrane [23–25]. French bean PBM possesses a similar ATPase, and in Fig. 5A–C we show that it can also catalyse the formation of a $\Delta\psi$ (positive inside) in intact symbiosomes (monitored by quenching of oxonol fluorescence). Free bacteroids do not contribute to this energisation and have little effect on oxonol fluorescence quenching [23]; consequently, damage to the PBM should result in decreased quenching. Incubation of isolated symbiosomes at 25°C for 1 h led to a slight decrease in their ability to form a $\Delta\psi$ (Fig. 5A–C), but exposure to a radical-generating system markedly stimulated this decline

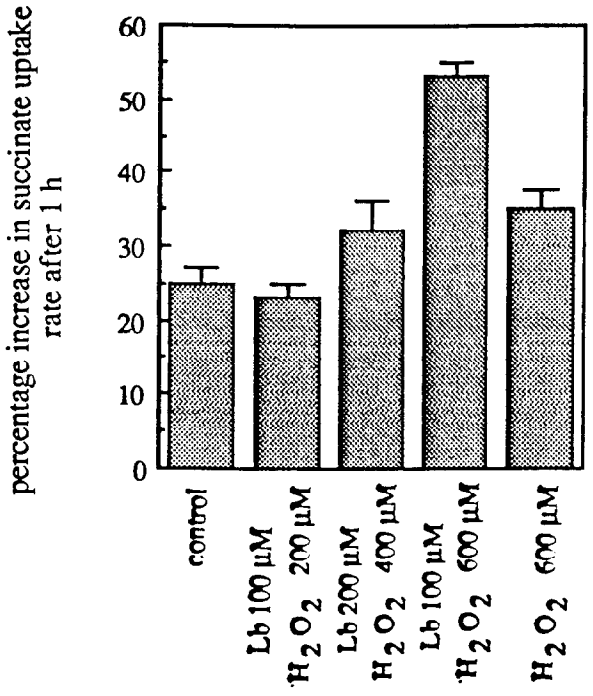

Fig. 6. Effect of Lb on the rate of succinate uptake by symbiosomes. Symbiosomes (0.7 ml) were incubated with the indicated concentration of Lb and/or H_2O_2, at 25°C for 1 h. When the two compounds were added together, they were pre-mixed before incubation commenced. Other experimental conditions were as described in Fig. 2.

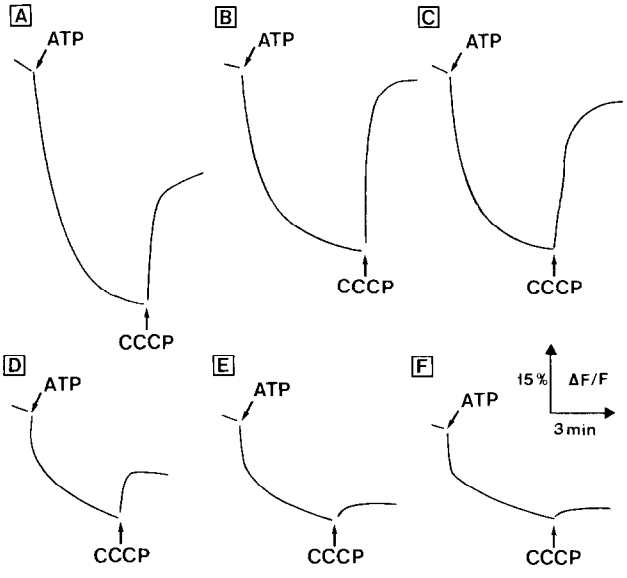

Fig. 5. Effect of iron and H_2O_2 on the formation of a membrane potential across the PBM of isolated French bean symbiosomes. Symbiosomes (0.7 ml) were incubated with or without 100 μM $FeCl_3$, 100 μM $FeSO_4$ and 400 μM H_2O_2, at 25°C for 1 h. At the times indicated, 0.1 ml of the incubation mixture was sampled and used to measure membrane potential (monitored as the quenching of oxonol fluorescence upon addition of 1 mM ATP, as described in section 2. Quenching is indicated by a downward deflection of the fluorescence trace). Where indicated, 5 μM of the protonophore, CCCP, was added, resulting in dissipation of the membrane potential and an increase in oxonol fluorescence. (A) Control t = 0; (B) control t = 30 min; (C) control t = 60 min; (D) iron + H_2O_2 treated t = 15 min; (E) treated t = 30 min; (F) treated t = 60 min.

(Fig. 5D–F). After only 15 min exposure, $\Delta\psi$ formation was severely hampered and it declined further over the subsequent 45 min (Fig. 5). In parallel experiments with the same symbiosomes, no significant effect on PBM ATPase activity per se was noted (results not shown).

Taken together, these results clearly show that general PBM permeability, rather than specific transport activity, was enhanced by the radical treatment.

3.3. *Significance for in vivo senescence*

The effect of H_2O_2 and iron ions seen here with symbiosomes is similar to that noted in studies on liposomal membranes where lipid peroxidation caused an increase in non-specific permeability of membranes [21]. Thus peroxidized symbiosomes can be considered as bacteroids surrounded by a deteriorating PBM, allowing more direct contact between the microsymbionts and the external medium: the permeability of symbiosomes gradually came to resemble that of the bacteroid. Although the treatment we have used is more drastic than that likely to occur in vivo, it nonetheless shows the effect of lipid peroxidation on the PBM and may mimic those changes which occur during nodule senescence, albeit over a longer time.

When isolated symbiosomes were incubated in the presence of 100–400 μM Lb previously mixed with a 2-fold excess of H_2O_2, which is known to generate a protein radical [9], the observed increase of succinate uptake was not significantly different from that produced by H_2O_2 alone (Fig. 6). Obviously, under our conditions the protein radical derived from the reaction of Lb with H_2O_2 had little effect on the permeability of the symbiosomes. These results differ from those obtained with purified PBM where a lipid peroxidation was observed with Lb in the presence of a 2-fold excess of H_2O_2 [13]. It should be noted, however, that under certain conditions lipid peroxidation can occur without inducing significant membrane leakage in the short term [21]. Furthermore, as radical reactions are site-specific (when formed, radicals must be very close to their target to cause lipid peroxidation), the access of activated Lb to membrane material may limit the reaction in intact symbiosomes. Purified PBM, obtained by rapid vortexing of the symbiosomes, may lose its original organization. Consequently, the affinity of both lipid peroxidation-generating species could be very different for purified PBM and the PBM of intact symbiosomes in which the integrity of the internal compartment is preserved. An accumulation on the external face of symbiosomes is conceivable for positive iron ions since this side may be negatively charged [23], whereas this may not occur with Lb. It is also possible that extensin-like hydroxyproline-rich glycoproteins on the PBM may confer some protection [26]. Even if these proteins are not eliminated by the vortexing procedure, the lipid bilayer could become accessible in at least some of the released membranes because of the heterogenous orientation of purified PBM vesicles (2/3 right side out, based on ATPase latency measurements).

In contrast, when isolated symbiosomes were incubated in the presence of 100 μM Lb previously mixed with a 6-fold excess of H_2O_2, an important change in the permeability of the symbiosomes was observed; this effect is significantly higher than that observed with 600 μM H_2O_2 (Fig. 6). It was previously shown that molar ratios for H_2O_2:Lb of 5 or greater cause breakdown of the heme and release of iron ions [10]. Thus, this iron release appears responsible for the increase in symbiosome permeability described above.

On the basis of the results obtained here, we suggest that in vivo Lb can contribute to PBM degradation via iron release during the early stages of senescence, rather than by the attack of protein radicals. The observation that cytosolic free iron accumulates before PBM degradation occurs supports this hypothesis. Iron released from Lb could become associated with the PBM and catalyse lipid peroxidation.

Our assays cannot rule out the existence of a specific interaction between activated Lb and certain PBM constituents. Radicals are able to produce chemical changes in protein structures, and histidine and proline are known potential sites of attack [27]. Direct inactivation of protein activity in other systems has been seen with comparable radical-generating mixtures [28]. Nevertheless, under our experimental conditions, damage to the ATPase did not occur and there was no evidence for a direct effect on the dicarboxylate carrier.

The most important implication of the current work is that lipid peroxidation could modify one of the major properties of the PBM – its very selective permeability. This membrane, which limits metabolic exchange between the two partners, seems to be essential for the stability of the symbiosis, and controls the capacity of the bacteroid to take up cytosolic components. A non-specific increase in permeability caused by lipid peroxidation would result in a general availability of metabolites to the bacteroids. This phenomenon could create a dramatic imbalance in host cell metabolism and contribute to the death of the tissue.

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