THE ABSENCE OF OXIDIZED LEGHEMOGLOBIN IN SOYBEAN ROOT NODULES DURING NODULE DEVELOPMENT*

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SUMMARY: To prevent autoxidation leghemoglobin was extracted from soybean root nodules of different ages in an atmosphere of carbon monoxide. Spectrophotometric examination of the leghemoglobins revealed that, during the functional life of the nodules, leghemoglobin exists only in its reduced form. Therefore, the decline of nitrogenase activity in older root nodules cannot be attributed to the accumulation of oxidized leghemoglobin.

INTRODUCTION

Leghemoglobin is the myoglobin-like hemeprotein found in the nodules which form on the roots of leguminous plants when they are invaded by bacteria of the genus Rhizobium. These nodules are the site of symbiotic dinitrogen fixation. An important role for Lb in dinitrogen fixation is suggested by the correlation between nodule Lb content and dinitrogen fixation activity. There is considerable evidence suggesting that Lb functions in facilitating oxygen diffusion within root nodules (1), supplying oxygen for bacterial oxidative phosphorylation without disturbing the anaerobic conditions necessary for the maintenance of nitrogenase, the rhizobial enzyme responsible for dinitrogen fixation. Thus, Lb in its reduced state as Lb^{2+} is the functional form of the molecule.

Spectrophotometric examinations of nodules from 21-28 day old soybean plants demonstrated that in vivo Lb exists only in the reduced state as either Lb^{2+} or $Lb^{2+}O_2$, and it was estimated that only about 20% of the Lb was oxygenated (2).

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Abbreviations: Lb, leghemoglobin; Lb^{2+}, ferroleghemoglobin; $Lb^{2+}O_2$, oxyleghemoglobin; $Lb^{2+}CO$, carbon monoxyleghemoglobin; Lb^{3+}, ferrileghemoglobin.

Early attempts to isolate and purify Lb invariably resulted in preparations which contained Lb^{3+} (3-5). Although numerous methods have since been developed to prevent autoxidation some preparations still contained traces of Lb^{3+} (2).

In spite of the indications that in nodules from 21-28 day old plants Lb exists only as Lb^{2+} and $Lb^{2+}O_2$ there was still a need for clarification of the status of Lb during the life of the nodule. We had observed that the rate of dinitrogen fixation per unit Lb decreased with increasing age and size of nodules (6) and wished to see whether this observation correlated with a progressive oxidation of Lb^{2+}, thereby rendering it ineffective in facilitated oxygen diffusion.

This report describes the results of experiments designed to determine the oxidation state of Lb in nodules throughout their growth and development.

METHODS

Soybean plants (Glycine max var. Kanrich) were infected with Rhizobium japonicum (strain 61A76), cultured, and their root nodules harvested as des-

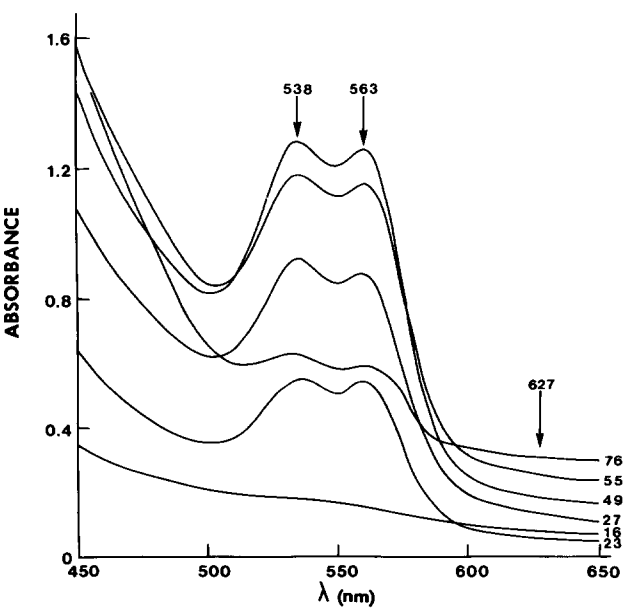

Fig. 1. Spectra of nodule extracts prepared under CO from plants of different ages. Plant age in days as indicated at the right of each spectrum.

cribed previously (7). Samples of nodules were taken on 13 separate days between the 12th day after sowing when nodules first appear and the 83rd day when nodules are senescent as indicated by their decreased ability to reduce acetylene.

The nodules were extracted in four volumes (v/w) of phosphate buffer (0.01 M, pH 6.8) and Polyclar AT (0.25 g per g nodules) in a Sorval Omnimixer with a chamber modified for gas flushing. In the extraction procedure advantage was taken of the high affinity of Lb^{2+} for CO (8,9) and the complete unreactivity of Lb^{3+} with CO. Before homogenization commenced the chamber was flushed with argon and CO was introduced to a partial pressure of 0.25 atmospheres. A low speed supernatant of the homogenate was clarified by centrifugation at 190,000 x g for 40 minutes in a Beckman Type 50Ti rotor. All steps in the procedure were carried out at 4°C. The spectra (650nm-450nm) were recorded immediately with a Beckman Acta V double beam spectrophotometer.

RESULTS AND DISCUSSION

The spectra obtained with extracts of nodules of various ages are shown in Figure 1. Their absorption maxima at 538nm and 563nm are typical of $Lb^{2+}CO$ (2). The complete lack of absorption peaks at 627nm indicates that Lb^{3+} is absent at all stages of the growth cycle. No evidence of an absorption peak at 627nm was seen even when four-fold more concentrated nodule extracts were

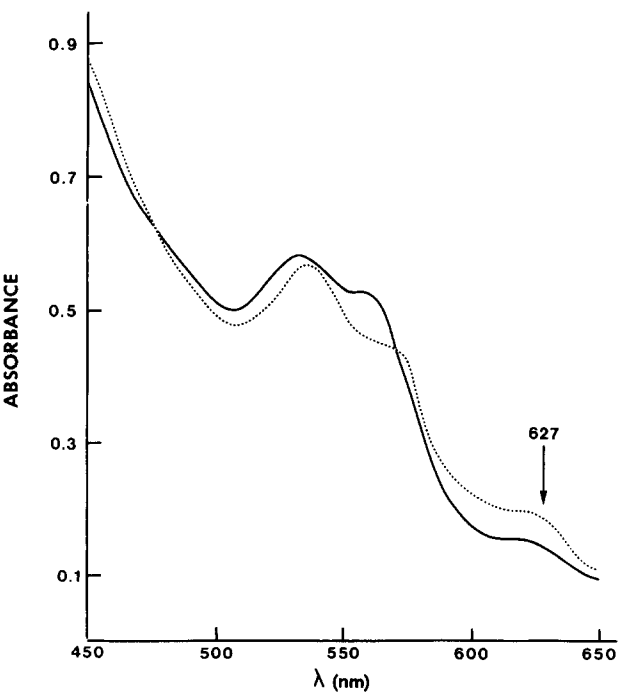

Fig. 2. Spectra of nodule extracts prepared under argon or air in the absence of CO. Nodules from 27 day old plants extracted in argon, solid line. Nodules from 32 day old plants extracted in air, broken line.

examined. On the other hand, samples which were extracted in argon without CO showed an absorption peak at 627nm which was more pronounced if the extraction was done in air or if Polyclar AT was omitted (Figure 2).

Some previous reports indicated that Lb preparations contained ferrihemochrome (4,5), which led to the suggestion that a ferrohemochrome might be the native form of the pigment (10). Since ferrihemochrome has α and β absorption peaks at 560nm and 527nm and ferrohemochrome at 554nm and 526nm and neither have absorption peaks in the 627nm region (11) they would not be detected in the presence of a large excess of $Lb^{2+}CO$. If present in large amounts the ferrihemochrome would be expected to cause some shift in the β absorption peak while the ferrohemochrome would displace both the α and β absorption peaks. More recently it has been shown that the ferrihemochrome described earlier results from the combination of Lb^{3+} with a low molecular weight ligand at low pH (11). The ligand has been shown to dissociate from Lb at neutral pH (12) and has been identified as nicotinic acid (13). Its low affinity for Lb at pH 6.8 and the reported absence of ferrihemochromes in Lb preparations made at this pH (2) would indicate that it is not a complicating factor in our interpretation of the spectra in Figure 1.

The results demonstrate that if Lb^{3+} is present in vivo it exists in very small amounts even in older root nodules. This was surprising both from the reports that $Lb^{2+}O_2$ has a relatively high autoxidation rate compared with vertebrate hemoglobins (9,14), and by analogy with hemoglobin and myoglobin which, when partially deoxygenated, are readily autoxidized (15). The reported autoxidation rates for $Lb^{2+}O_2$ may reflect the method by which the Lb was prepared. Recently, a new purification procedure has been described which produces stable preparations of $Lb^{2+}O_2$ (16) and it would be interesting to know the autoxidation rates of these preparations.

Our inability to detect Lb^{3+} does not exclude the possibility that it forms, since a system could operate in vivo to remove it rapidly. Lb^{3+} might be degraded immediately after its formation or alternatively, a system analogous to

the ferrihemoglobin reductase of red blood cells might restore Lb^{3+} to its functional Lb^{2+} form.

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