# LEGHEMOGLOBIN: DIFFERENT ROLES FOR DIFFERENT COMPONENTS?

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SUMMARY. The ratio of leghemoglobin c content to leghemoglobin a content in soybean (Glycine max) root nodules drops in the early stages of plant growth and becomes constant during flowering and fruiting. These data bring into question the assumption that leghemoglobin components have a single biochemical role in nitrogen fixation.

INTRODUCTION. Leghemoglobins are dioxygen-binding hemeproteins which surround nitrogen-fixing bacteroids in the root nodules of various legumes. Although not involved directly in reduction of dinitrogen to ammonia (1), leghemoglobins are physiologically necessary for leguminous symbiotic nitrogen fixation in vivo (2,3). Recent studies on the biochemical and physiological role of leghemoglobins emphasize reversible binding of dioxygen to leghemoglobins and the involvement of leghemoglobins in facilitated dioxygen diffusion (4-7). Leghemoglobin is almost invariably heterogeneous (8); in soybean root nodules there are two major and two minor components (9, 10). Nevertheless, implicit in leghemoglobin function studies is the assumption that all leghemoglobin components function similarly. By following the relative concentrations of the two major soybean leghemoglobin components as a function of the age of the soybean plants from which the leghemoglobin was isolated, we have tested that assumption and found it questionable.

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METHODS. Soybean plants (Glycine max var. Amsoy) were grown in a field well innoculated with Rhizobium japonicum. As a precaution commercial innoculum was used during one growing season (summer, 1974, Experiment 1) but not during the other (summer, 1975, Experiment 3). The number of nodes along the main stem of each plant was the measure of physiological age; physiological age varied among plants of the same chronological age.

Acrylamide polymerization and gel electrophoresis were carried out in quartz tubes (0.5 cm diameter, 10.0 cm long). The polymerization solution was 0.05 M Tris acetate or Tris chloride, pH 8.0, with 7.5% acrylamide and 0.2% bisacrylamide. Ammonium persulfate (0.75 ml of a 10% solution per 30 ml acrylamide solution) and N,N,N',N'-tetramethylethylenediamine (5 µl per 30 ml acrylamide solution) initiated polymerization.

Experiment 1. Fresh nodules (5-6 g) from plants of the same physiological age were blended in 0.05 M Tris acetate (75 ml), pH 8.0, and centrifuged. The same buffer was used throughout all subsequent steps. The supernatant was brought to 65% saturation with ammonium sulfate and again centrifuged. To the supernatant at 65% saturation were added excess oxidizing agent (10 drops 0.017 M potassium ferricyanide solution per 6 g nodules) and then ammonium sulfate to reach 80% saturation in ammonium sulfate. After centrifugation the reddish pellet was redissolved in buffer and dialyzed against three changes of buffer (600 ml per dialysis of 3-12 hours). Sucrose was added to dialyzed, oxidized leghemoglobin (metleghemoglobin) to a final 10% sucrose concentration. Samples of metleghemoglobin in 10% sucrose and 0.05 M Tris acetate, pH 8.0, were applied to preelectrophoresed 7.5% polyacrylamide gels (50 µl per gel) and were electrophoresed at ca. 23° C with constant current of 3 ma per gel. After electrophoresis the gels were scanned at 400 nm, without removal from their tubes, on a Gilford 240 spectrophotometer equipped with a linear transport assembly and specially machined tube holders. Each peak was integrated manually with a Gelman planimeter. Relative peak areas were calculated. Because the results of Experiment 1 exhibited some dependence upon the amount of metleghemoglobin in the electrophoresis sample, the samples were subjected to a further experiment.

Experiment 2. After five months storage in sucrose, Tris acetate solutions at 4° C the samples from Experiment 1 were centrifuged, diluted to constant concentration (judged by 620 nm absorbance measured on a Cary 17 spectrophotometer), and electrophoresed as described above.

Experiment 3. Procedures were the same as in Experiment 1 except for the following. To avoid drying of nodules, plants were removed from sunlight when harvested and nodules were immediately detached and stored in liquid dinitrogen until blended. Tris chloride buffer, pH 8.0, was used. The pellet from 80% ammonium sulfate saturation was redissolved in 0.05 M Tris chloride, pH 9.2, and dialyzed against the same buffer twice (600 ml per 3 hour dialysis) and against 0.05 M Tris chloride, pH 8.0, once (600 ml, 3-12 hours). Samples were diluted to constant concentration (judged by Soret band maximum absorbance at ca. 406 nm measured on a Cary 17 spectrophotometer) and electrophoresed at 0° C.

RESULTS. At all stages of plant development leghemoglobin c (the electrophoretically fast component) was present in excess over leghemoglobin a (the electrophoretically slow compo-

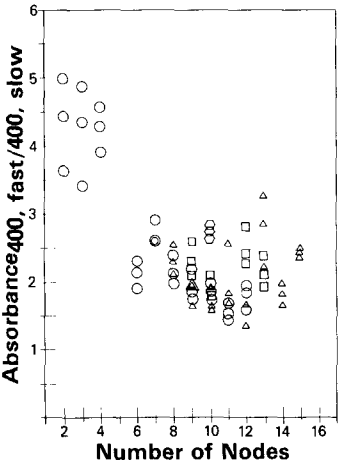


Figure 1. Ratios of integrated absorbance peaks (from gel scans at 400 nm after polyacrylamide gel electrophoresis in 0.05 M Tris acetate, pH 8.0) as a function of plant age (number of nodes). The ratio is metleghemoglobin c (fast component) to metleghemoglobin a (slow component). Each geometric figure represents the average of three calculations on data from the same gel. Different geometric figures for the same age represent different nodule samples. Repeated geometric figures for the same age represent replicates. Data are from Experiment 2 described in text.

nent), assuming similar extinction coefficients for both components at 400 nm in the gels. However, the ratio of leghemoglobin c to leghemoglobin a declined during the early stages of plant growth (2-4 nodes) to a ratio which remained constant during plant flowering and fruiting (Figure 1).

Electrophoresis gel scans in Experiments 1 and 3 exhibited two major bands and two minor bands. After storage the minor bands were no longer evident, but the changes in leghemoglobin c to leghemoglobin a ratios in Experiment 2 were nearly identical to those in Experiments 1 and 3. Evidence that the bands detected at 400 nm were metleghemoglobins was given by wavelength scans of the band centers (Figure 2). The presence or absence of innoculum during germination and the presence or absence of acetate or chloride in the buffer had no significant effects on the results. A leghemo-

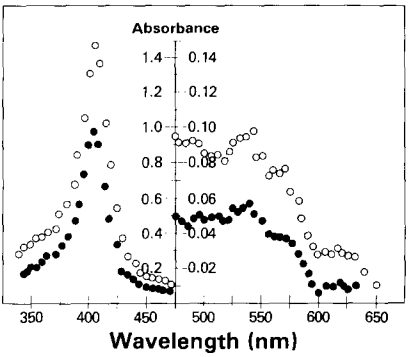


Figure 2. Optical spectra of metleghemoglobin bands in the gel after electrophoresis. Open circles: metleghemoglobin c. Filled circles: metleghemoglobin a. Data are from Experiment 2 described in text.

globin concentration effect on the ratios (Experiment 1) was almost completely eliminated by dialysis against pH 9.2 buffer (Experiment 3), a procedure which is reported (8) to remove bound nicotinic acid.

DISCUSSION. Whether the observed changes in leghemoglobin ratios with increasing plant age resulted from increases in the absolute amounts of leghemoglobin a per nodule and/or decreases in the absolute amounts of leghemoglobin c per nodule or changes in the relative rates of synthesis or degradation of the two leghemoglobins could not be determined from these experiments. Nevertheless, the behavior over time of the leghemoglobin c to leghemoglobin a ratio can be interpreted in terms of biochemical and physiological role(s) for leghemoglobins. A constant concentration ratio might have meant 1) similar or identical roles for the two components but heterogeneity in the apoprotein synthesized by the plant or 2) a functional interaction between the components like the relationships among cytochromes in electron transport systems. The variable concentration ratio which was observed could mean 1) simi-

lar or identical roles but different locations and/or different physiological conditions for optimum functioning of the two components, akin to the similarity in the roles of fetal and adult human hemoglobins, 2) similar or identical roles but different rates of synthesis or degradation of the two components, or 3) dissimilar roles. The data reported here do not disprove the assumption that all leghemoglobin components have the same biochemical and physiological role, but they raise the possibility that the two major components of soybean leghemoglobin may have dissimilar roles. More comparative data on the properties of leghemoglobins a and c are needed. One pertinent observation is that leghemoglobin a has a higher affinity for dioxygen than has leghemoglobin c (11). Soybean root nodules are reported to adapt to abnormal dioxygen pressures (12). Changes in the ratio of leghemoglobin components may play an important role in such adaptation.

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