Short Communication

Preparation and Fractionation of *Rhizobium* Bacteroids by Zone Sedimentation through Sucrose Gradients

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ABSTRACT

Zone sedimentation through sucrose gradients was used for preparing *Rhizobium* bacteroids from lupin nodules and for separating them into slowly and rapidly sedimenting fractions.

The purity of the bacteroids was established by electron microscopy and by enzyme assays, and they were shown to contain a CO-insensitive cytochrome c oxidase.

Bacteroids sedimented more rapidly than broth-cultured *Rhizobium* bacteria, and bacteroids from old nodules sedimented more rapidly than bacteroids from young nodules.

There appear to be at least two forms of bacteroid in old nodules: slowly sedimenting bacteroids with moderate colony-forming ability resembling the bacteroids found in young nodules, and rapidly sedimenting bacteroids with much lower colony-forming ability.

*Rhizobium* bacteroids have usually been prepared from homogenized nodule tissue by differential centrifugation (5, 7, 8, 10). Although such procedures give good separations of soluble plant proteins from bacteroids, the complete removal of plant membrane fragments and particulate enzymes such as Cyt c oxidase from the bacteroid fraction is difficult, and has led in some cases to the use of techniques such as detergent treatment (7) that would be expected to have detrimental effects on bacteroid viability.

In this paper we report the use of sucrose gradient sedimentation to obtain clean bacteroid fractions and to demonstrate changes in the sedimentation rate of bacteroids during lupin nodule development.

MATERIALS AND METHODS

Blue lupins (*Lupinus angustifolius* L. cv. Bitter Blue, inoculated with *Rhizobium* strain NZP 2257) were grown under controlled environment conditions (9). Growth of *Rhizobium* NZP 2257 in broth cultures without mannitol, preparation of sterile and nonsterile nodule homogenates, bacteroid counts, and bacteroid colony formation assays on media containing 0.055 M mannitol were as previously described (10).

Sucrose gradient centrifugation was carried out using linear 20-ml gradients of 12 to 30% (w/v) sucrose in 50 mM tris-HCl, pH 7.4, equilibrated at 0 to 5 C before use. All centrifugations were at about 2,000g for 15 min at 0 to 5 C, using the HB4 swinging bucket rotor of a Sorvall RC-2 centrifuge. For preparing total bacteroids, up to 5 ml of filtered nodule homogenate (in 0.5 M mannitol, 50 mM tris-HCl, pH 7.4) was applied to each gradient, and after centrifugation the bacteroid zone was detected by eye and removed from the top with a Pasteur pipette. The bacteroids were then rinsed twice by centrifuging for 5 min at 6,000g and resuspending in mannitol-tris buffer. For resolving different classes of bacteroids, 1 ml of bacteroid suspension or filtered nodule homogenate was applied to each 20-ml gradient, and after centrifugation the tubes were punctured with a hot needle and 1-ml fractions collected from the bottom. The bacteroids were located by absorbance measurements at 600 nm. Comparisons of sedimentation rate were made only between gradients matched volume prepared in the same batch and centrifuged simultaneously.

For sterile gradient work all solutions were autoclaved, the gradient-making device and centrifuge tubes were sterilized with 70% (v/v) ethanol in water for 10 to 20 min followed by three rinses with sterile distilled H2O, all manipulations were carried out in a laminar flow hood, and each gradient was covered with a 3-mL layer of sterile paraffin oil.

Electron microscopy of bacteroids was carried out by the method of Craig and Williamson (4). Phosphoenolpyruvate carboxylase, L-malate dehydrogenase, and Cyt c oxidase were assayed as described by Christeller et al. (2), Ochoa (6), and Cooperstein and Lazaro (3), respectively. A unit of Cyt c oxidase was defined as the amount required to produce a change of 0.10/min in the log10 A550 of a reduced Cyt c solution at 25 C. For CO inhibition studies, CO produced by the reaction of formic acid with concentrated H2SO4 was pumped as a fine stream of bubbles through a proton of the reduced Cyt c solution for 1 min at 4 C, prior to the assay.

RESULTS AND DISCUSSION

Preparative Centrifugation. Preliminary experiments showed that zone sedimentation of filtered nodule homogenates gave good separations of an opaque zone containing 35 to 40% of the bacteroids from a red supernatant layer containing leghemoglobin and other plant proteins. The remaining bacteroids were found in a pellet at the bottom of the gradient, together with plant cell wall fragments, starch-filled organelles, fragmented nuclei, and aggregated plant membranes (Fig. 1). The distance traveled by the opaque bacteroid zone was proportional to the time of centrifugation, and our routine conditions were chosen to leave it in the middle of the gradients.

When fractions collected from gradients were assayed for the plant enzyme P-enolpyruvate carboxylase, more than 99% of the recovered activity was found in the supernatant layer. No activity was detected co-sedimenting with the bacteroid zone.
although the sensitivity of the assays was such that 0.05% of the recovered activity should have been detectable (2). Assays for l-malate dehydrogenase, used by Cutting and Schulman (5) to distinguish between soybean bacteroids and mitochondria, showed that only 1.1% of the activity in nodules co-sedimented with the bacteroid zone.

Reijnders et al. (7) found that bacteroids prepared by differential centrifugation from pea root nodules contained Cyt c oxidase activity. They attributed this to mitochondrial contamination, and they found that they could selectively remove the activity by a 30-min incubation in 1 M KCl/1% sodium deoxycholate at 4 C. We found with lupins that 2% of the Cyt c oxidase activity of day 11 nodules and up to 20% of the activity of older nodules co-sedimented with the bacteroid zone in sucrose gradients. Since only 35 to 40% of the bacteroids sedimented in the middle zone, these results imply that 5 to 6% of the Cyt c oxidase activity of day 11 nodules, which contain relatively few bacteroids (10), was bacteroid-associated, while in older nodules up to 60% of the activity was bacteroid-associated.

Another 30 to 50% of the Cyt c oxidase activity of nodule homogenates was recovered from a separate zone within and just below the supernatant. This zone contained all of the activity recovered from sucrose gradient centrifugation of lupin root homogenates, and was presumed to contain the mitochondria.

Attempts to remove selectively the Cyt c oxidase activity of bacteroids by the technique of Reijnders et al. (7) were unsuccessful; although 1 M KCl/1% sodium deoxycholate at 4 C destroyed the Cyt c oxidase activity, it also lysed the bacteroids giving a clear viscous solution from which nothing could be pelleted at 6,000g. Similar results were obtained with mannitol-tris buffer supplemented with either 1% of 0.3% sodium deoxycholate; and while 0.1% sodium deoxycholate resulted in only partial lysis of the bacteroids after 30 min at 4 C, the fraction recovered by pelleting at 6,000g retained 49% of the initial bacteroid Cyt c oxidase activity.

It seemed unlikely that these results could be attributed to mitochondrial contamination, and more probable that our assays were detecting the terminal Cyt c oxidase of the bacteroid respiratory chain. Appleby (1) has reported that the succinate-oxidase pathway of aerobically prepared soybean bacteroids, unlike that of mitochondria and broth-cultured Rhizobium bacteria, is CO-insensitive. We therefore tested the effects of CO on the Cyt c oxidase activity of our fractions. As shown in Table I, bubbling CO through the substrate inhibited the Cyt c oxidase activity of lupin root homogenates and of the supernatant fraction from nodule homogenates, but did not inhibit the activity of purified bacteroids. Bubbling N2 through the substrate had no effect on either activity. We concluded that there are two Cyt c oxidases present in lupin nodules, and that the activity that co-sediments with the bacteroids represents a bacteroid enzyme rather than a mitochondrial one.

This conclusion was supported by electron microscopy of bacteroids from the middle zone, which showed that they had similar morphology to that seen in thin sections of nodule tissue (4) and were contaminated by fewer than 50 fragments of plant membrane and less than one mitochondrion per 1,000 bacteroid cross-sections (Fig. 1). Bacteroids prepared from similar nodule tissue by differential centrifugation followed by several washes in a buffer of low osmolarity contained more than 130 fragments of plant membrane per 1,000 bacteroid cross-sections.

Table I. Effect of CO on the Cytochrome C Oxidase Activity of Root and Nodule Fractions from Lupin

<table>
<thead>
<tr>
<th>Days</th>
<th>Fraction</th>
<th>Cytchrome C oxidase activity</th>
<th>% -CO</th>
</tr>
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<tbody>
<tr>
<td>11</td>
<td>root homogenate</td>
<td>510</td>
<td>54</td>
</tr>
<tr>
<td>11</td>
<td>nodule homogenate</td>
<td>3060</td>
<td>51</td>
</tr>
<tr>
<td>11</td>
<td>nodule supernatant</td>
<td>3550</td>
<td>26</td>
</tr>
<tr>
<td>11</td>
<td>bacteroids</td>
<td>50</td>
<td>105</td>
</tr>
<tr>
<td>14</td>
<td>root homogenate</td>
<td>400</td>
<td>28</td>
</tr>
<tr>
<td>14</td>
<td>nodule homogenate</td>
<td>4730</td>
<td>29</td>
</tr>
<tr>
<td>14</td>
<td>nodule supernatant</td>
<td>3560</td>
<td>26</td>
</tr>
<tr>
<td>14</td>
<td>bacteroids</td>
<td>400</td>
<td>106</td>
</tr>
</tbody>
</table>

Fig. 1. Electron micrographs of lupin bacteroids prepared by sucrose gradient sedimentation (× 8,100). a: Day 17 bacteroids from the middle zone of a gradient; b: bacteroids and plant cell debris from the pellet at the bottom of the same gradient.
Bacteroid Sedimentation Rates. No matter how thin the layer of bacteroid suspension was initially, the opaque band after centrifugation was observed to be quite broad (Fig. 2), suggesting that bacteroids might have a wide range of sedimentation coefficients. This was confirmed by the observation that slowly and rapidly sedimenting fractions recovered from such gradients sedimented at different rates when recentrifuged (Fig. 2). Bacteroids from old nodules were usually found to sediment more rapidly and give a broader distribution than bacteroids from younger nodules (Fig. 3). Bacteroids from all ages of nodule sedimented more rapidly than *Rhizobium* bacteria from broth cultures (Fig. 3).

These different sedimentation rates were used to test a hypothesis concerning the bacteroids from mature nodules able to form colonies on bacteroid culture media of low osmolarity (10). Two alternatives were envisaged: either all of the bacteroids from mature nodules might have a uniformly low ability to form colonies, or else there might be more than one form of bacteroid. In the experiments shown in Figure 4, bacteroid fractions obtained by sucrose gradient centrifugation of nodule homogenates were tested for colony-forming ability as well as for absorbance. With bacteroids from day 11 nodules, which have a colony-forming efficiency of about 10% on low osmolarity medium (10), the colony-forming fraction co-sedimented with the rest of the bacteroids. With bacteroids from day 18 nodules, which have a colony-forming efficiency of only 0.3% on low osmolarity medium (10), the fraction able to form colonies had a much lower sedimentation rate than the majority of the bacteroid population.

The simplest interpretation of this result is that bacteroids from old nodules are a mixture of two forms: slowly sedimenting bacteroids with moderate colony-forming ability, and rapidly sedimenting bacteroids with much lower colony-forming ability. Changes in bacteroid viability (10) and sedimentation rate (Figs. 3 and 4) during nodule development can be interpreted as the conversion of most but not all bacteroids into the rapidly sedimenting form.

**Fig. 2.** Resolving power of zone sedimentation. a: First cycle sedimentation of day 17 bacteroids. Arrow shows direction of sedimentation. Fractions from the two shaded areas were pooled. b: Second cycle sedimentation of day 17 bacteroids; O: slowly sedimenting fraction from a; *: rapidly sedimenting fraction from a.

**Fig. 3.** Zone sedimentation of bacteria and bacteroids of different ages. a: Broth-cultured bacteria; O: day 13 bacteroids; *: day 20 bacteroids; Δ: day 27 bacteroids.

**Fig. 4.** Zone sedimentation of bacteroids in sterile gradients. a: Day 11 bacteroids; b: day 18 bacteroids. O: Bacteroid absorbance; Δ: absorbance of broth-cultured bacteria in a parallel gradient; *: bacteroid colony-forming units.

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**LITERATURE CITED**