Legume Root Response to Symbiotic Infection. Enzymes of the Peribacteroid Space

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Three of the major soluble partitioned pools in the symbiotic cell, namely the host cell cytoplasm, the cytoplasm of the bacterial symbiont and the space between the two partners (the peribacteroid space) were assayed for selected enzymes. Whilst alanine dehydrogenase was found almost totally in the bacterial cell cytoplasm, other enzymes were more widely spread. Amongst these α-mannosidase, previously described as a vacuolar marker enzyme in eukaryotes, was found largely in the peribacteroid space.

These results are discussed in relation to models of peribacteroid membrane biogenesis.

Introduction

During the initial invasion of plant root cells by symbiotic *Rhizobium* the membrane which surrounds the prokaryote is initially donated by the plant plasma membrane [1] but it is unclear as to whether the plasma membrane continues to contribute material during the subsequent massive proliferation of these membranes. In many ineffective (non-N_2-fixing) symbioses the peribacteroid membrane has a much shorter life time than in effective (N_2-fixing) symbioses. It may be therefore that the formation and maintenance of these organelle-like structures is important in the establishment and maintenance of a properly regulated symbiosis.

Results and Discussion

Antiserum was raised against pure α-mannosidase from jack bean which gave quantity dependent precipitate heights when challenged with antigen in a Laurell-type "rocket" immunoelectrophoresis system (Fig. 1 a). Antigenic cross-reactivity with authentic *Glycine may* nodule α-mannosidase was established simply by putting nodule $50000 \times g$ supernatent into a well in the agar punched closer than normal to one containing the original antigen. The enzymes were allowed to diffuse overnight before electrophoresis as normal the next morning. The fusion of the two rockets indicates a common antigenic identity (Fig. 1 c).

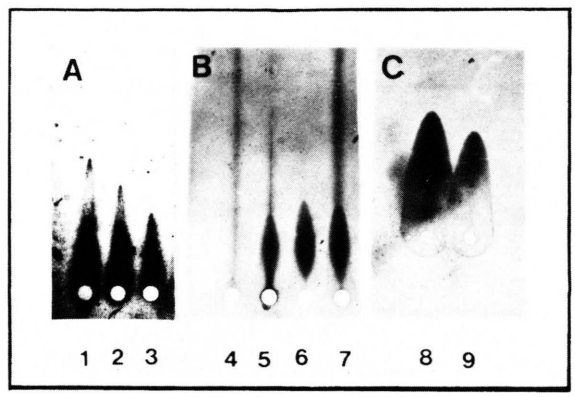

Fig. 1. Immunoelectrophoresis into agarose gels containing 200 µl IgG per plate. Lanes 1–3 and 8 contain authentic jack bean α-mannosidase and lane 9 contains authentic soybean α-mannosidase (Plate C is magnified ×13). Lane 4: 20 µg peribacteroid membranes. 5: 100 µg bacteroids with peribacteroid membranes intact. 6: 5 µg peribacteroid space. 7: 100 µg host cell cytoplasm.

Bacteroids both retaining and devoid of surrounding peribacteroid membranes were prepared as before [2]. From this fraction an osmotic shock fraction, containing the molecules present within the peribacteroid space, the peribacteroid membranes and also the released bacteroids were purified [2]. It can be seen from the activities of various enzymes (Table I) that some enzymes were asymmetrically distributed amongst these fractions. In particular alanine dehydrogenase, a bacterial cytoplasmic marker, was found overwhelmingly in the $50000 \times g$ supernatent from French-pressed bacteroids with little appearing in then nodule cytoplasm or peribacteroid space fractions, indicating minor bacteroid

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breakage upon homogenization. Conversely the nodule cytoplasmic marker enzyme, asparagine synthetase, was confined to this fraction alone, demonstrating that little, if any, cytoplasm adhered to the outer surface of the peribacteroid membranes. This view is reenforced by electron micrographs (Fig. 2) of the bacteroid and peribacteroid membrane fraction, showing no discernable cytoplasmic contamination. It thus appears likely that the peribacteroid space fraction used was largely free of plant or bacteroid-derived soluble components.

Table I. % distribution of soluble enzymes in symbiotic cell fractions.

	Host cytoplasm	Peri-bacteroid space	Bacterial cytoplasm
alanine dehydrogenase	11.40	0	88.60
asparagine synthetase	100	0	0
α-galactosidase	100	0	0
α-glucosidase	74.73	9.27	16.0
α-mannosidase[a]	56.5	43.48	0
protease[b]	35.00	17.50	47.60
protein	96.52	1.42	2.12

[a] 100% Activity here corresponds to 23% of the total cellular activity, 77% being recovered as particulate enzyme.
[b] Measured as PNP release from PNP phenylalanine.

By a comparison of the heights of the rockets formed from standard antigen with those of authentic samples (Fig. 1 b) calculations could be made concerning the concentration of α-mannosidase in each sample. As is shown in Fig. 1, preparations of whole bacteroids with surrounding membranes, when preincubated with 0.5% Triton X-100, showed a discernable rocket upon immunoelectrophoresis, whereas similarity treated purified peribacteroid membranes did not. It is very unlikely that any of this α-mannosidase came from the prokaryote, as previous studies [3] and our own unpublished immunoreactive studies show a total absence of this enzyme in bacteroids or free-living cells of *Rhizobium japonicum* strain 61-A-101. Furthermore almost all of that activity could be accounted for within the peribacteroid space fraction which gave a prominent rocket precipitate upon immunoelectrophoresis. Since, on a protein

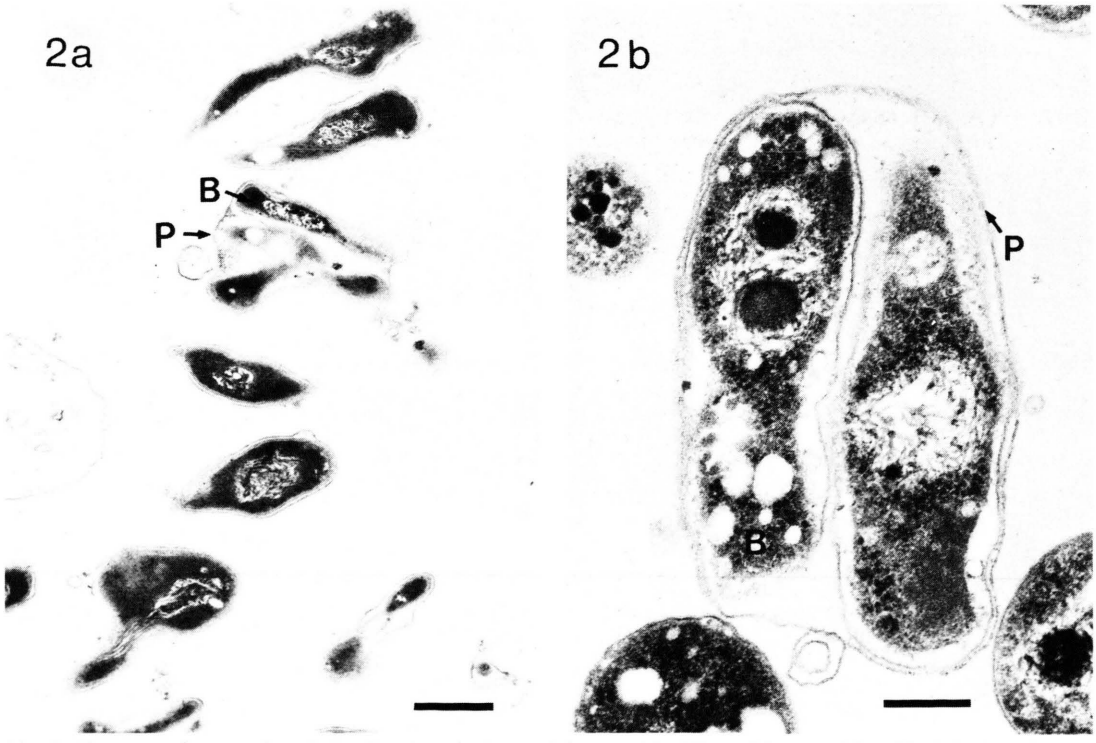

Fig. 2. Electron micrographs of the fraction designated bacteroids (B) and bacteroids with intact peribacteroid membranes (P). a: magnification × 21 000 (Bar = 0.5 μm); b: magnification × 57 000 (Bar = 0.2 μm).

basis, the concentration of α-mannosidase was some 15 times higher in the peribacteroid space fraction than in the nodule cytoplasm it appears clear that the space between the bacteroids contains α-mannosidase which cannot be accounted for by contamination.

If we assume that components of organelles and their matrices are provided by the ER, as is currently thought [4] then we would expect α-mannosidase, a vacuolar marker enzyme in yeast and higher plants [5, 6] to be made by rough microsomes. In infected cells of soybean nodules vacuoles never fully develop. Mature symbiotic cells show no central vacuole as in normal root cells, and only fragments of tonoplasts are sometimes faintly observable [1, 7] but α-mannosidase enjoys an activity cycle independent of central vacuole development [3]. It is known that α-mannosidase exhibits a relatively wide particulate distribution on this tissue [3] and we have previously speculated that although vacuole development and maintenance is shut down in infected tissue, the enzyme α-mannosidase is still made but is rerouted elsewhere. How then does this molecule get into peribacteroid spaces? If the peribacteroid membrane is formed from plasmamembrane, microbodies would be expected to be formed from either ER or Golgi, but first fuse with the plasma membrane, releasing their contents outside the cell. Such microbodies are rare [1] and furthermore a membrane recycling model now demands the selective reuptake of α-mannosidase into nascent peribacteroidal structures. We know of no evidence to support these dictates. Conversely a membrane recycling model would work if α-mannosidase was made on cytoplasmic polyribosomes and later specifically transported across the peribacteroid membrane and into the peribacteroid space by a hitherto unelucidated mechanism.

We must conclude therefore from the data presented that peribacteroid membrane proliferation is supported directly by the biosynthetic parts of the endomembrane system in continuity with the vacuome in a fashion independent of the plasma membrane.

Acknowledgement

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Experimental

Glycine max var. mandarin was supplied by K. Behm GmbH, Hamburg and *Rhizobium japonicum* strain 61-A-101 was from the American Type Culture Collection. Tissue was grown and prepared as previously [3].

Cell fractionation was accomplished by following the scheme detailed in [2].

Samples for electron microscopy were prepared as in [2] and examined as in [7].

Antiserum was raised as before [8] in male Deutsche Großsilber rabbits using α-mannosidase isolated commercially from jackbean (Sigma, München) as antigen. Immunoelectrophoresis was performed using an LKB Multiphor kit and methods recommended by the manufacturer (LKB, Uppsala, Sweden).

Estimations of lytic enzyme activity was as in [9], alanine dehydrogenase was assayed as by Müller and Werner [10] and asparagine synthetase as β-aspartylhydroxamate formation following the method of Ravel [11].

Protein determination [12] was carried out on 8% TCA precipitated material.

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