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Immunofluorescent and Immunogold Localization of Glutamate Dehydrogenase in the Nodules of Soybean

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Abstract

Soybean nodules have been examined for localization of glutamate dehydrogenase, using a monospecific antiserum to the plant enzyme prepared from lupin nodules. By immunofluorescence techniques, glutamate dehydrogenase was located in the cytoplasm at the periphery of infected cells adjacent to air spaces. A more precise localization within soybean-nodule mitochondria was shown by immuno-electron microscopy using protein A-gold labelling of thin sections.

Such a localization of glutamate dehydrogenase is consistent with a possible role either in assimilation of any overflow of ammonia from the operation of the glutamine synthetase/glutamate synthase system or, alternatively, in the reprocessing of glutamate for synthesis of amides or ureides.

Extra key words: amination, immunofluorescence, mitochondria, symbiotic nitrogen fixation.

Introduction

The primary product of symbiotic nitrogen fixation, ammonia (Bergersen 1965; Kennedy 1966*a*), is excreted from the *Rhizobium* bacteroids into the plant cell cytoplasm. Previous studies have shown that glutamine *plus* glutamate are the primary amino products from $^{15}N_2$ (Kennedy 1966*b*) and that other nitrogenous compounds are formed from these (Ohyama and Kumazawa 1980).

Free ammonia may be assimilated into glutamate, in legume nodules, by either glutamine synthetase (GS, EC 6.3.1.2) coupled with glutamate synthase (GOGAT, EC 1.4.1.13) (Miflin and Lea 1976) or by direct reductive amination of 2-oxoglutarate by glutamate dehydrogenase (GDH, EC 1.4.1.2). The former pathway (GS/GOGAT) is regarded as the primary means of ammonia assimilation in nitrogen-fixing systems (Scott *et al.* 1976; Rawsthorne *et al.* 1980). Such a role for GS/GOGAT is supported by analysis of $^{15}N_2$ assimilation in soybean nodules (Ohyama and Kumazawa 1980). However, even these data do not exclude GDH from an important supporting role in ammonia assimilation in legume nodules. Definitive proof excluding GDH from any such role, such as a localization not exposed to newly fixed ammonia, has not been presented. A knowledge of the intracellular location of GDH is important in reaching a decision as to its role. In this paper we describe the localization of GDH in the nodules of soybean at the cell level using an indirect immunofluorescence technique and also by immuno-electron microscopy using protein A-gold.

Lupin nodule GDH is an enzyme with a very high catalytic rate (Stone *et al.* 1979) and even the substantial activity found in lupin nodules corresponds to only a very small concentration of the GDH protein. The total catalytic activity (ammonia-saturated) of GDH is similar to that of GS in legume nodules, but the concentration of GDH (molecules of

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enzyme per unit volume of tissue) is over two orders of magnitude less. For example, in lupin nodules, GS is about 2–3% of nodule protein by weight (Chen and Kennedy 1985), similar to its concentration in soybean nodules (McParland *et al.* 1976). GDH, on the other hand, is about 0·01–0·02% of the protein in infected lupin nodule cells (Stone *et al.* 1979).

Assuming the specific activity and catalytic rate of soybean nodule GDH is similar to that of lupin nodules, only of the order of 100 molecules of GDH would be expected to occur in each ultrathin section of about 50 nm per plant cell of 30 μm diameter. Furthermore, only a small proportion of these could be expected to be suitably exposed on the surface of the section for antibody labelling. Even if all GDH were concentrated in mitochondria and full antigenicity retained, only about two molecules could be expected to be labelled in each mitochondrion. Thus, labelling of GDH can be anticipated to be more difficult, on any background of non-specific labelling, than that for more prolific antigens such as leghaemoglobin (Robertson *et al.* 1984) or glutamine synthetase.

Material and Methods

Plant Material

The fresh soybean (*Glycine max*) nodules were taken from the roots of soybean plants (cv. *Forrest*) grown in soil in a greenhouse 4–8 weeks after inoculation of the seedlings with *Bradyrhizobium japonicum* CB 1809.

Preparation of Specific Antibody (AbGDH)

GDH was purified from lupin nodules (Stone *et al.* 1979) and an anti-GDH (AbGDH) prepared in rabbit as described earlier (Tchan *et al.* 1981). The AbGDH was clearly demonstrated by extensive immunoelectrophoresis to be monospecific, producing a single immunoprecipitin peak. The range of cross-reaction of this AbGDH has been shown to be restricted to the GDH of higher plants (Kennedy *et al.* 1985). The titre of the AbGDH was measured by the extent of inhibition of GDH activity, assayed as described previously (Kennedy *et al.* 1985). Fifty per cent inhibition of 1 mU lupin nodule GDH was produced by reaction with 0·28 μl of antiserum in 1·0 ml of reaction mixture, full inhibition appearing within 5 min incubation at 30°C.

The AbGDH was shown to be cross-reactive with soybean nodule GDH as described earlier (Kennedy *et al.* 1985).

Purification of Immunoglobulins

Immunoglobulins (IgG) were prepared from the AbGDH, goat antirabbit immunoglobulin (GAR-IgG) (125 units, Calbiochem–Boehringer crude, lyophilized) and normal rabbit serum (NRS) using Protein A Sepharose CL-4B (Pharmacia), following the procedure of Goding (1976).

Immunofluorescence Techniques

FITC-labelling of GAR-IgG

The GAR-IgG was labelled with fluorescein isothiocyanate using alkaline carbonate buffer, pH 9·5, at 4°C, according to the method described by Goding (1976). The free dye was removed by passage through a Sephadex G-25 column. The FITC/protein (F/P) molar ratio of labelling of GAR-IgG was measured using the equation reported by Kawamura (1969). In this experiment the F/P ratio of GAR-IgG-FITC was 1·5.

Preparation of frozen sections

Fresh soybean nodule tissues were cut to 4–8 μm thickness with a freezing microtome (IEC Minotome) and collected onto clean glass slides. These were immediately treated with a drop of 70% (v/v) ethanol and dried in air.

Immunolabelling procedure

The indirect immunolabelling procedure used was according to Wang (1980) as follows:

Test: (i) Slides of sections were washed with phosphate-buffered saline [PBS; 0·05 M sodium phosphate pH 7·4 containing 0·85% (w/v) NaCl], for 5 min.

(ii) Each slide was treated with 20 μl AbGDH-IgG (diluted with PBS) and then incubated at 37°C for 50 min in a water-saturated chamber. They were then washed with PBS for 10 min.

(iii) Each slide was treated with 20 μl of GAR-IgG-FITC (1·4 mg ml^{-1}, diluted with PBS) and then incubated at 37°C for 25 min in a moist chamber. Next, it was washed with PBS for 10 min. Finally, two drops of a solution containing sodium phosphate buffer (0·05 M) and glycerol (1 : 1, pH 8·5) were placed on each slide and covered with cover glasses.

Control: (i) The same as test (i) above.

(ii) The same as test (ii) above, but using NRS-IgG at the same concentration, instead of AbGDH-IgG.

Observation of sections

Slides of the sections were examined with an Olympus BH series microscope (×40 objective, ×3·3 eye piece) equipped with an RFL episcopic fluorescence illuminator, using an HBO 100 W mercury vapour lamp with two BG-12 exciter filters and a Y-495 barrier filter. Photographs were taken with an Olympus PM6 camera using Kodak Ektachrome daylight film (ASA 400). Exposure times for recording of fluorescence were about 1 min.

Immunogold Techniques

Preparation of colloidal gold

Colloidal gold was prepared by methods based on Frens (1973) and Roth (1983) using reduction with trisodium citrate. Particles of 17 nm diameter were formed when 100 ml of 0·01% (w/v) $HAuCl_4$ was reduced with 3 ml of 1% (w/v) citrate solution. All glassware used with colloidal gold was scrupulously cleaned with detergent and double-distilled water, siliconized by washing with undiluted LKB-Silane and rinsed in double-distilled water. Solutions were prepared using membrane-filtered (0·4 μm) double-distilled water.

Preparation of protein A-gold complexes

Gold particles were adsorbed with staphylococcal protein A (Pharmacia) according to the method of Roth (1983). Using indicator paper, the pH of the gold solution was adjusted to the isoelectric point of protein A (pH 6·5) by addition of 0·2 M K_2CO_3. Estimation of the minimal amount of protein A needed for stabilization of colloidal gold was made using the method of Roth and Binder (1978) and a slight excess of protein added.

Removal of uncomplexed protein A and unstable gold particulates was achieved by centrifugation at 5°C for 30 min at 30 000 r.p.m. (60 000 *g*) using a Ti-50 rotor (Beckman L2-65). After resuspension of the soft pellet in Na-phosphate (0·01 M), pH 7·2 containing 0·85% (w/v) NaCl (PBS) and 0·5 mg ml^{-1} human serum albumin (HSA Fraction V, Sigma) the protein A-gold was recentrifuged in an SW27 head for 60 min at 26 000 r.p.m. (45 000 *g*), resuspended in PBS containing HSA and 0·2 mg ml^{-1} NaN_3 and stored at 4°C. Washing in a horizontal swing-out centrifuge tube substantially improved the total yield of protein A-gold compared with using an angle-head rotor, apparently by minimising aggregation from sedimentation on the centrifuge tube wall.

Tissue Procedures

The schedule for fixation, dehydration and embedding of nodule tissue in Lowicryl K4M resin (Chem. Werke Lowi) for immunocytochemistry was adapted slightly from the method of Wells (1985) as follows:

Fixation

Fresh tissues from soybean nodules were cut into slices of approximately 1 mm thickness and fixed in a large volume of 2% paraformaldehyde (w/v) and 0·2% glutaraldehyde (v/v) in 0·1 M Na-phosphate buffer, pH 7·2, for 2 h at 25°C.

Dehydration

(a) 30% ethanol 1 h at 4°C
(b) 50% ethanol 1 h at −20°C
(c) 70% ethanol 1 h at −35°C
(d) 95% ethanol 1 h at −35°C
(e) 100% ethanol 1 h at −35°C
(f) 100% ethanol 1 h at −35°C

Infiltration with Lowicryl K4M resin

(a) 1 : 1 resin : ethanol 1 h at −35°C
(b) 2 : 1 resin : ethanol 1 h at −35°C
(c) 3 : 1 resin : ethanol 1 h at −35°C
(d) 100% resin 1 h at −35°C
(e) 100% resin overnight at −35°C
(f) 100% resin 8 h at −35°C

Polymerization

(a) 24 h with indirect ultraviolet irradiation at −40°C

(b) 48 h with indirect ultraviolet irradiation (long-wave) at room temperature.

Sections about 50 nm thick were cut using a diamond knife in an LKB Ultratome and were placed on parlodion 200 mesh copper grids (Polysciences) coated with carbon.

Immunolabelling of Sections

The immunolabelling procedure was modified slightly from the method of Robertson *et al.* (1984). Sections were immersed in HSA (20 mg ml^{-1}) in Tris-buffered saline (TBS) containing 10 mM Tris–HCl pH 7·4, 9 mg ml^{-1} NaCl, 0·2 mg ml^{-1} NaN_3, 0·5 mg ml^{-1} polyethylene glycol (MW 6000), for 1 h at 37°C. Sections were then immersed in AbGDH-IgG diluted with HSA (2 mg ml^{-1}) in TBS for 1 h at 37°C; washed with gentle agitation in several changes of HSA (2 mg ml^{-1}) in TBS at room temperature; immersed in protein A-gold complex (diluted with HSA, 2 mg ml^{-1}, in TBS) for 1 h at 37°C; washed with gentle agitation in several changes of TBS containing HSA (2 mg ml^{-1}) at room temperature, followed by the same solution containing 0·1% Triton X-100, and finally washed with double-distilled water.

In the control test, either AbGDH-IgG was omitted or normal rabbit serum IgG (NRS-IgG) was employed at the same protein concentration. Sections were post-stained briefly with 2% (w/v) aqueous uranyl acetate, followed by the lead citrate reagent of Reynolds (1983) and then examined in a Philips EM 201 electron microscope.

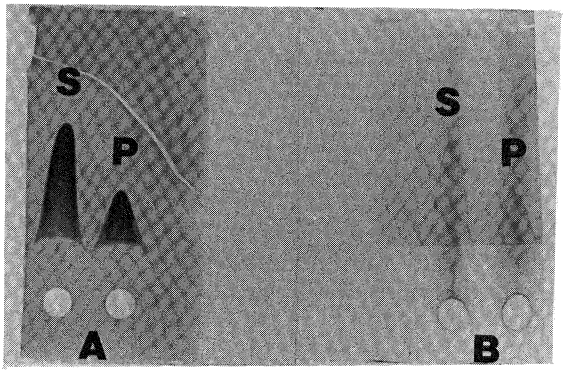

Fig. 1. Rocket immunoelectrophoresis of soybean-nodule extracts against AbGDH (lupin). Both soluble (S) and non-bacteroid particulate (P) fractions were applied in wells in an antibody-free zone and electrophoresed into gel containing AbGDH. Precipitation peaks given by an enzyme activity stain (A) and Coomassie protein stain (B) are shown. Immunoelectrophoretic conditions were as described previously (Kennedy *et al.* 1985).

Results and Discussion

Antibody Cross-reaction with Soybean Nodule GDH

In Fig. 1 is shown the cross-reaction of soybean nodule GDH with monospecific AbGDH to lupin nodule GDH. GDH activities in the soluble cytosol and that solubilized after freezing of the particulate fraction (excluding bacteroids) were cross-reactive. Earlier, we showed the cross-reaction of this AbGDH with GDH from a range of legumes and wheat (Kennedy *et al.* 1985), though no immunoelectrophoresis was performed with soybean nodule GDH. The immunological identity of the GDH antigens from both soluble and particulate fractions was shown in other tests and is also indicated in the enzyme-stained reaction of Fig. 1.

The gel plates showed only a single peak of precipitated antigen in crude nodule extract and this corresponded exactly with a GDH-specific enzyme stain using iodonitrotetrazolium salt to visualize the reaction. Thus the monospecific AbGDH to lupin nodule GDH was also monospecific with soybean nodules and suitable for immunocytochemistry.

Immunofluorescence Labelling

Initial attempts at labelling frozen sections of soybean nodule tissue were made using whole antiserum to GDH. While these tests yielded successful results under some conditions,

they sometimes produced non-specific labelling with control serum. However, results consistently indicating specific binding were obtained when purified AbGDH-IgG was used.

After staining of frozen sections of the soybean nodules using AbGDH-IgG, followed by GAR-IgG-FITC, it was found that the specific fluorescence was located mainly around the circumference of the infected cell in soybean nodules (Fig. 2*B*), particularly near air spaces.

In the control tests there was no bright-green FITC-specific fluorescence visible in the sections, but only a yellow autofluorescence (Fig. 2*C*).

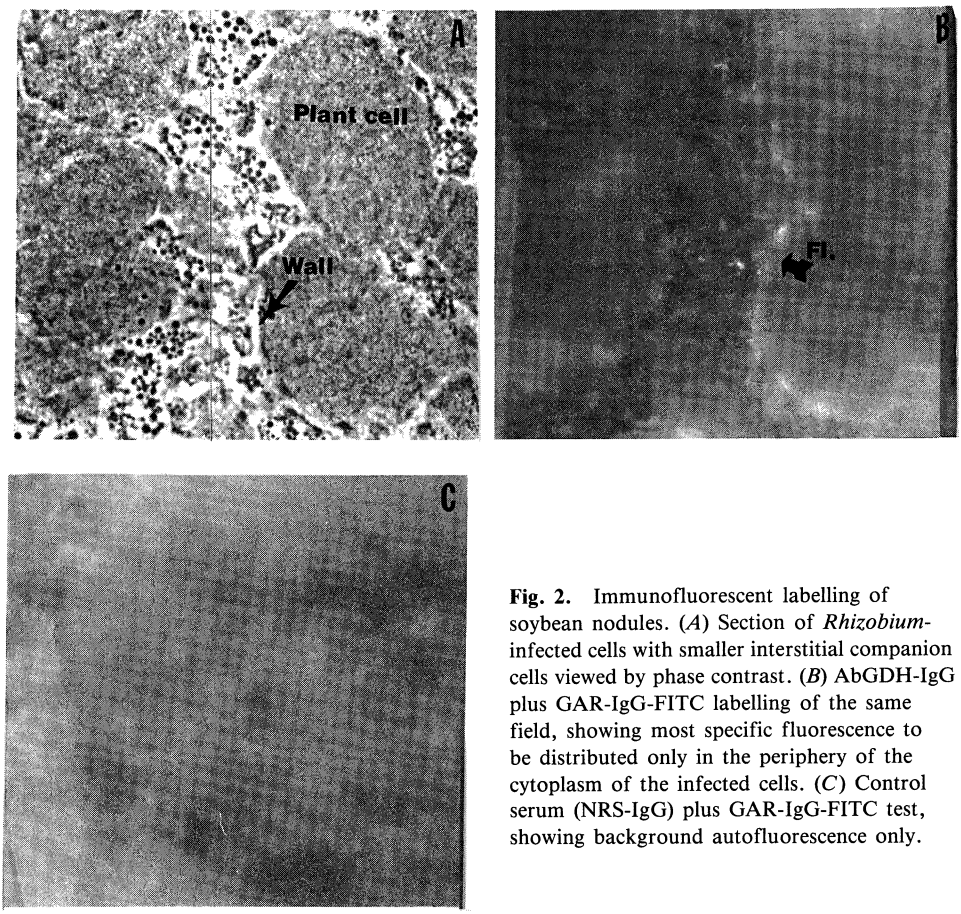

Fig. 2. Immunofluorescent labelling of soybean nodules. (*A*) Section of *Rhizobium*-infected cells with smaller interstitial companion cells viewed by phase contrast. (*B*) AbGDH-IgG plus GAR-IgG-FITC labelling of the same field, showing most specific fluorescence to be distributed only in the periphery of the cytoplasm of the infected cells. (*C*) Control serum (NRS-IgG) plus GAR-IgG-FITC test, showing background autofluorescence only.

It would be expected that specific fluorescence should appear where the GDH enzyme was located. Therefore, in the sections of soybean nodules, GDH appears to be in the cytoplasm concentrated near the outer region of the nodule cells. It is in this area that mitochondria are found in infected soybean nodule cells (Bergersen and Goodchild 1973). The results are therefore consistent with the conclusion that GDH is located in the mitochondria of the soybean nodule cells, although they do not prove this exact location.

The greater degree of fluorescence at the periphery of an infected cell adjacent to an uninfected cell shown in Fig. 2 probably reflects the greater likelihood of occurrence of mitochondria where higher oxygen pressure is available. We have found in electron micrographs of nodule sections that mitochondria in infected cells are rare, even at the cell periphery, where distant from air spaces, and the distribution of fluorescence also followed this pattern.

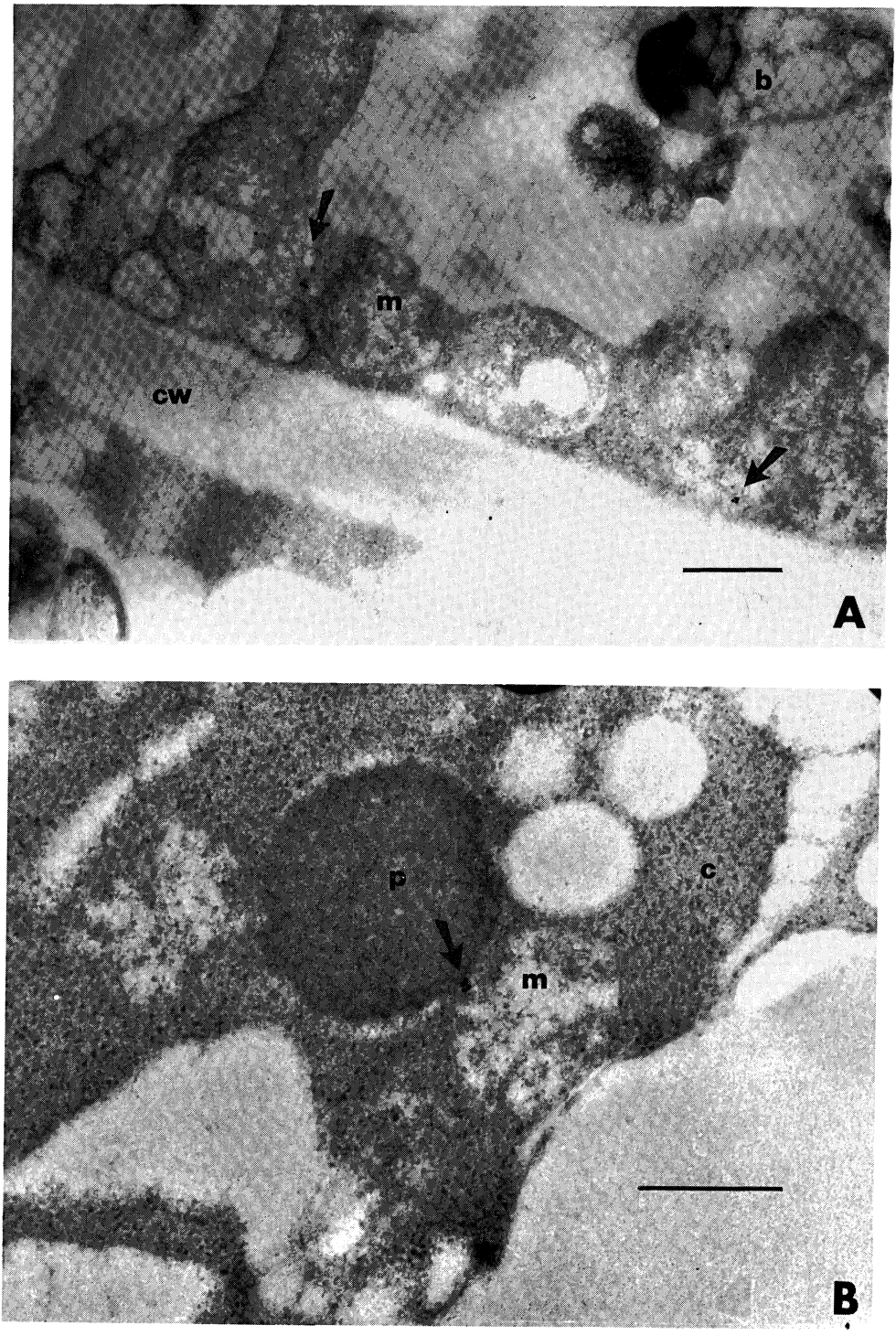

Fig. 3. Protein A-gold/AbGDH-IgG labelling of soybean nodule cells. (*A*) Peripheral zone of infected cell, showing mitochondria (m) packed against the cell wall (cw) with adjacent peribacteroid zones containing *Rhizobium* bacteroids. Colloidal gold visible on mitochondria is indicated by arrows. (*B*) Uninfected cell, showing four gold particles in a mitochondrion but with none in adjacent cytoplasm (c) or a peroxisome (p).

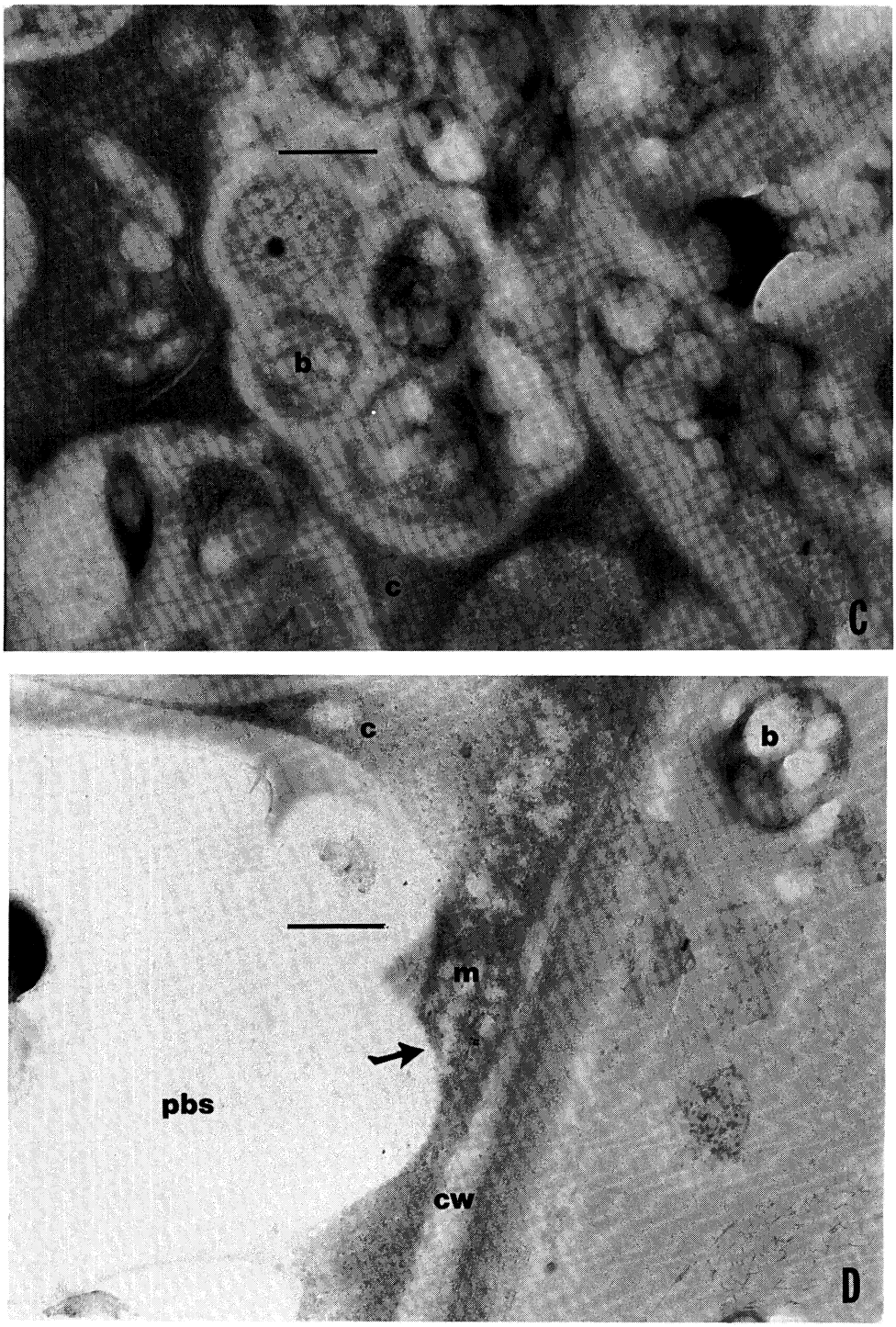

Fig. 3. Protein A-gold/AbGDH-IgG labelling of soybean nodule cells. (*C*) Central zone of an infected cell, showing virtual absence of label in bacteroids, peribacteroid spaces (pbs) and the adjacent cytoplasm. (*D*) Peripheral zone of infected cells, with gold only in a mitochondrion. Scale bar, 0·5 μm.

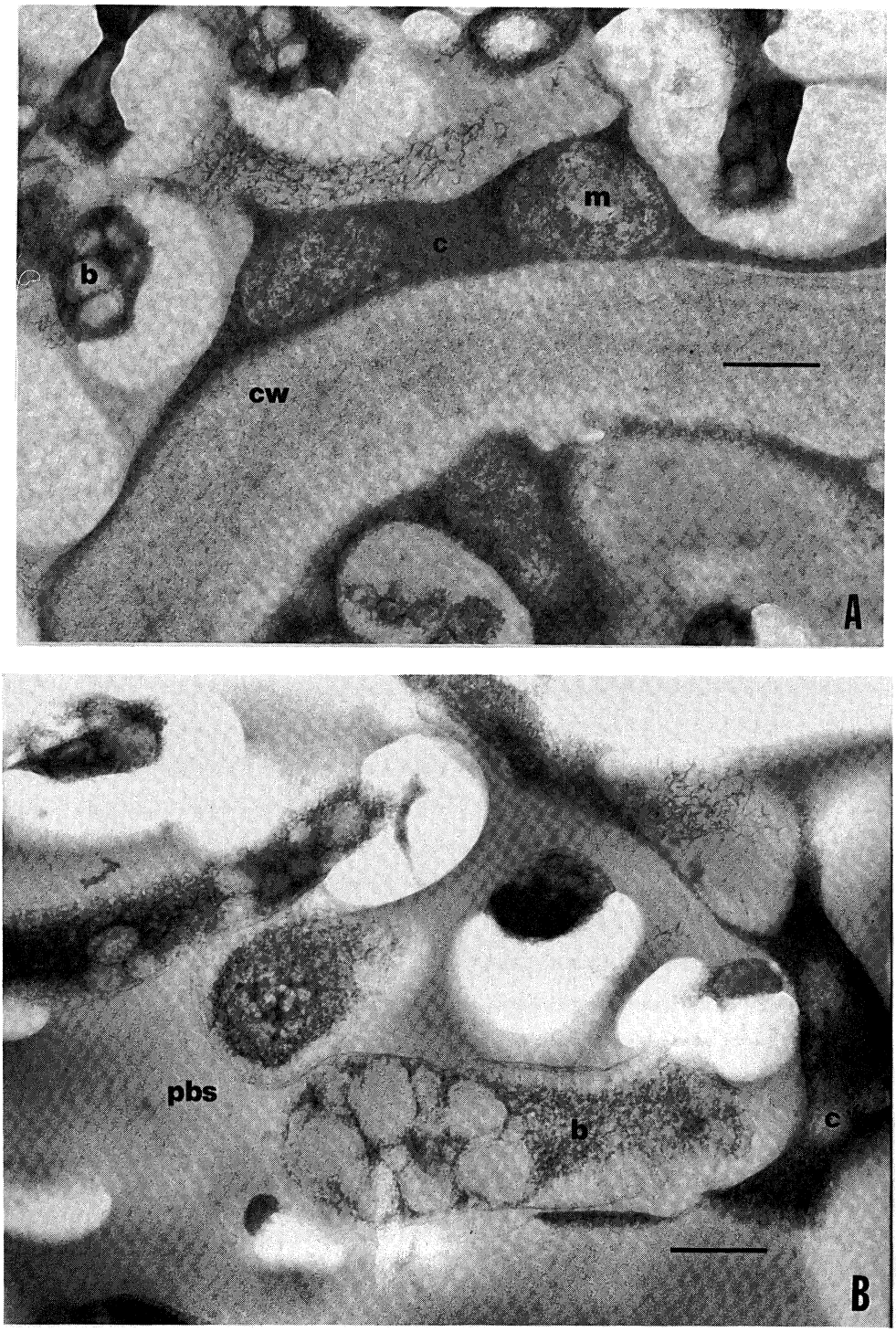

Fig. 4. Protein A-gold/normal rabbit serum IgG labelling of soybean-nodule cells. (*A*) Peripheral zone of infected cell. (*B*) Central zone of infected cell. Scale bar, 0·5 μm.

Immunogold Electron Microscopy

Satisfactory preservation of the antigenicity and the ultrastructure in thin sections of soybean nodule tissues embedded with K4M was obtained (Fig. 3). Immuno-specific labelling by protein A-gold particles was localized mainly in the mitochondria on the periphery of *Rhizobium*-infected nodule cells (Figs 3*A*, 3*B*, 3*D*).

Very few gold particles were found in the bacteroids and peribacteroid envelopes and the interior cytoplasm of the plant cells (Fig. 3*C*). The mitochondria of uninfected cells were also labelled (Fig. 3*B*). In the NRS-IgG controls, there were few gold particles in the sections (Figs 4*A*, 4*B*). In accordance with the principle of specific immunological reaction of antibody with antigen and specific binding of protein A to rabbit IgG, the protein A-gold particles indicate the sites where GDH molecules are located.

Table 1*A*. Immunogold labelling of components of soybean-nodule cells

The area of each category of cell structure was computed by tracing photographs, followed by the weighing of cut-outs of photocopies of tracings. The photocopy paper was shown to be of uniform weight distribution; areas shown are actually as weights of paper (g)

	Bacteroids	Mitochondria	Peribacteroid space	Cytosol
	Test series (29 photographs)			
Occurrences of category	155	105	—	—
Area	6·21	6·98	17·52	14·27
Au number	5	166	16	12
Au/area (intensity)	0·81	23·78	0·91	0·84
	Control series (16 photographs)			
Total occurrences	71	52	—	—
Area	4·65	2·77	11·9	6·58
Au number	1	2	3	1
Au/area	0·22	0·72	0·25	0·15

Table 1*B*. Expected occurrence of GDH antigens in 50 nm sections

MW of lupin nodule GDH	270 000 (Stone *et al.* 1979)
Nodule concentration of GDH	2 μg cm^{-3} ($4{\cdot}5 \times 10^{12}$ molecules cm^{-3})
Volume of cell (γ = 15 μm)	$1{\cdot}4 \times 10^{-8}$ cm^3
Molecules of GDH per cell	$6{\cdot}3 \times 10^4$
Number of sections per cell	600
Molecules GDH per cell per section	105
Molecules GDH exposed per cell	20
Cell perimeter (γ = 15 μm)	90 μm
Perimeter in typical photograph	3–4 μm
Expected number of exposed GDH molecules per photograph	ca. 1–10[A]
Observed	5

[A] Dependent on the proportion of the perimeter exposed to air spaces.

An anlysis of the distribution of colloidal gold in sections of nodule cells with AbGDH-IgG tests and controls (minus AbGDH-IgG with NRS-IgG) is given in Table 1. Shown in the table, the occurrence of gold in mitochondria was several times greater than in all other

nodule cell locations, such as soluble cytosol, peribacteroid envelopes and the bacteroids. Expressed as the number of gold particles per unit area of section, this difference was considerably enhanced.

Usually, gold particles occurred singly, although the likelihood of multiple occurrences was greater in mitochondria. No such clumping was observed other than in mitochondria even though a greater total number of gold particles would have occurred dispersed (as non-localized background) over the much greater area of the cytoplasm, the bacteroids and the peribacteroid spaces. This suggests that multiple occurrences reflect multiple occurrences of the antigen rather than clumping of protein A-gold.

Mitochondria were distinguished from other organelles by their characteristic size and some evidence of internal membrane structure. Bergersen and Goodchild (1973) have shown by electron microscopy of soybean nodules that mitochondria are by far the most common organelles in the cytoplasm, but are restricted to the periphery of infected cells in the vicinity of air spaces.

In the sections of these soybean nodules the GDH enzyme was therefore distributed mainly in the mitochondria. The very few protein-A gold particles on the other areas of the nodules appear to be from non-specific background binding, somewhat greater in the Ab-GDH stained sections than the NRS-IgG stained sections. The background with protein-A gold alone was negligible.

Statistical Analysis of Gold-labelling Pattern

The low numbers of gold particles found on mitochondria are not impressive when individual photographs are viewed. Considering the total areas involved, more gold particles were found on some of the other areas of the nodule sections. However, the number of gold particles per unit area of mitochondria was found to be many times that on any other area of the sections (see Table 1).

Both the Chi-square test and analysis of variance indicated that the likelihood of a purely random concentration of gold on mitochondria had a probability of less than 0·001 (see Table 2).

An analysis of the expected occurrence of GDH molecules on individual mitochondria is also given in Table 1. The observed occurrence is in fairly good agreement with the expected occurrence. In other work using lupin nodules prepared by the same procedure, we have observed a prolific labelling by immunogold of sections with an antibody prepared to GS (Chen, Kennedy and Wang, unpublished), indicating that preservation of antigenicity by the procedure is good. We have found that GDH is a more stable antigen than GS in cell-free extracts and therefore consider that the low occurrence of gold labelling of GDH observed here is consistent with its actual occurrence in nodules.

General Discussion

Purified GDH from lupin nodules has properties suiting it to a role in ammonia assimilation (Stone *et al.* 1979), but only at relatively high ammonia concentrations when compared with GS (Chen and Kennedy 1985). GDH is also capable of oxidative deamination of glutamate and its role could be in a reprocessing of glutamate to release ammonia for further amide or ureide synthesis, following its initial synthesis by GS/GOGAT. GDH is not subject to obvious regulation by metabolites (Stone *et al.* 1979) and it will probably have its role dictated by prevailing concentrations of glutamate, NAD^+, NADH, 2-oxoglutarate and ammonia at its site of action.

The present results indicate clearly that GDH is localized distant from most nitrogenase activity, since in infected cells it appears to be restricted to the mitochondria at the perimeter, separated from most *Rhizobium* bacteroids. A localization in the peribacteroid space, for instance, might have favoured a primary role of GDH in ammonia assimilation. Since it is

Table 2*A*. Statistical analysis of gold labelling—Chi-square test

Category	Occurrences				χ^2
	Observed		Expected		
	Test	Control	Test	Control	
Bacteroids					
Zero	25	15	25·8	14·2	
Not zero	4	1	3·2	1·8	
					0·59
Mitochondria					
Zero	0	14	8·7	5·3	
Not zero	26	2	17·3	10·7	
					34·12
Peribacteroid spaces					
Zero	17	13	19·3	10·7	
Not zero	12	3	9·7	5·3	
					2·38
Cytosol					
Zero	23	15	24·5	13·5	
Not zero	6	1	4·5	2·5	
					1·64

Note: This test shows that only in the case of mitochondria was there a significant difference in the observed number of occurrences of gold particles (zero or not zero on a particular photograph) between the test and control series. The proportion of photographs with gold on bacteroids, peribacteroid spaces and cytosol was apparently greater in the test series with AbGDH-IgG than with control NRS-IgG; however, this occurrence was not statistically different from that of the control series (a χ^2 value greater than 3·8 indicates significance at 0·05 level of probability).

Table 2*B*. Statistical analysis of gold labelling—Analysis of variance
Variate: Au number per unit area (intensity)

Source of variation	Degrees of freedom	Sum squares	Sum squares (%)	Mean square	Variance ratio
Series: (test *v* control)	1	59·39	7·77	59·39	36·48***
Intensity: (high *v* low)	1	275·17	36·01	275·17	169·04***
Series. Intensity	1	154·10	20·17	154·10	94·66***
Low intensity/category	2	0·17	0·02	0·08	0·05ns.
Series. (Low intensity/ category)	2	0·14	0·02	0·07	0·04ns.
Residual	169	275·11	36·01	1·63	
Totals	176	764·08	100·00		

Note: The intensity data were transformed by adding 1·0 to the variates for categories on each photograph to eliminate zeros and taking the square root before analysis of variance using the Genstat program on an IBM microcomputer. The analysis shows highly significant differences between the test series and the control series, between labelling of mitochondria and the mean intensity elsewhere (high *v* low), and that there was a significant interaction between series and intensity of labelling. This variation was derived almost entirely from the labelling of mitochondria in comparison with the uniformly low intensity of labelling of bacteroids, peribacteroid space and cytoplasm (see Table 1*A*). ***$P < 0·001$; ns., not significant.

not so localized, its involvement in assimilation must be as a reserve activity, if at all, acting to assimilate the overflow of ammonia from GS/GOGAT.

A distribution at the periphery of the cell distant from nitrogenase would not *per se* prevent ammonia assimilation by GDH, in view of the extreme rapidity of ammonia diffusion across the cytoplasm (Kennedy and Geering 1984). Even if GS activity were distributed throughout the cytoplasm, it would be unable to prevent such ammonia diffusion to the periphery of the cell.

As suggested in the introduction, the small number of GDH molecules expected in a particular ultrathin section presented a problem in obtaining a clear demonstration of the localization of GDH with immunogold electron microscopy. Because of this, the complementary results obtained by fluorescence microscopy take on special importance. In the case of the fluorescent specimens, there were much larger fields (40 times), thicker sections (100 times) and the likelihood of breakage of organelles such as mitochondria, allowing diffusion and a greater probability of reaction with specific antibodies during staining. Thus, much more significant fluorescence from perhaps 10 000 times as many GDH molecules reacting in a given microscope field is still consistent with the low rate of labelling obtained with immunogold.

No other case of the need for statistical analyses in connection with immunogold labelling of antigens is known to the authors. The success of this analysis suggests that this is an approach that could be used with other antigens occurring in tissues with very low frequency.

An earlier report showed that the cytochemical activity (using tetrazolium salts) of GDH in the mitochondria of cells from lupin nodules was high (Ratajczak *et al.* 1979). Multiple forms of GDH were shown by cell fractionation techniques to be located in pea-shoot mitochondria (Nauen and Hartmann 1980). In French beans, also, a mitochondrial localization for GDH was indicated for nodule cells by density-gradient fractionation (Awonaike *et al.* 1981).

In agreement with these findings, our results with immunological techniques demonstrate directly that in infected cells the GDH enzyme is concentrated in the mitochondria of the cytoplasm near the cell walls. Too few occurrences of proplastids were observed to form any definite conclusions about the relation of GDH to these organelles. Possibly, a few unlabelled proplastids were concluded to be mitochondria, acting to bias our results against the mitochondrial localization. Accepting the exclusively mitochondrial localization, any hypothesis that soybean nodule GDH participates in ammonia assimilation rather than in the reprocessing of glutamate to release ammonia requires that conditions in mitochondria at least temporarily favour the amination reaction of GDH and that newly fixed ammonia can penetrate the mitochondria. Studies to test these aspects of this hypothesis employing $^{15}N_2$ labelling are in progress.

Acknowledgments

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