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Localization of xanthine dehydrogenase in cowpea root nodules: Implications for the interaction between cellular compartments during ureide biogenesis

(ureides/purine synthesis/allantoin/allantoic acid/nitrogen metabolism)

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ABSTRACT **Immunocytochemistry was used to assess the location of xanthine dehydrogenase (EC 1.1.1.204) in the infected region of nodules of cowpea (*Vigna unguiculata* [L.] Walpers cv. Queen Anne Blackeye). Polyclonal antibodies raised against purified cowpea xanthine dehydrogenase were used to localize this enzyme at the electron microscopic level. Sparse nonspecific labeling was observed after treatment of nodule sections with preimmune serum. Although immune serum cross-reacted with the ground cytoplasm of both infected and uninfected cells, significantly more labeling was observed in the uninfected cells. No labeling above background was observed in peroxisomes, mitochondria, proplastids, endoplasmic reticulum, cytoplasmic or peribacteroid membranes, peribacteroid spaces, or bacteroids. The enzyme is soluble and not present in any organelle or membrane. The greater concentration of xanthine dehydrogenase in the uninfected cells suggests that xanthine or a precursor to xanthine, rather than uric acid, is the intermediate that moves from infected to uninfected cells during ureide biogenesis.**

Xanthine dehydrogenase (XDH; EC 1.1.1.204) is an essential enzyme in the biogenesis of the ureides allantoin and allantoic acid in cowpea and soybean nodules (1–3). The production of ureides in nodules is correlated with N_2 fixation in tropical legumes of the tribe *Phaseoleae*, where up to 90% of the fixed nitrogen is assimilated in nodules in ureide form (4).

The metabolic pathway of ureide production has been studied primarily in soybean and cowpea nodules (4). Ureides are formed by ammonia assimilation through glutamine synthetase and glutamate synthase, followed by purine synthesis and oxidation (4). The infected region of the nodule, where the steps in the pathway occur, contains uninfected as well as infected cells. In soybean, there are 1.6 times as many uninfected cells in the infected region as there are infected cells, and every infected cell is in contact with at least one uninfected cell (5).

Although it is known that the early steps in the pathway occur in the infected cells and the final steps occur in the uninfected cells, it is not yet clear where the intermediate steps take place. Some evidence suggests that purine synthesis, particularly the first enzyme of that pathway, is present in the plastids of infected cells (4, 6, 7). In contrast, uricase, which converts uric acid to allantoin, is present exclusively in the enlarged peroxisomes of the uninfected cells (8–13). Thus, determining the intercellular location of additional enzymes in the pathway leading to ureide biogenesis may enable us to identify that intermediate of the pathway which is transported from the infected to the uninfected cells.

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The inter- and intracellular locations of XDH have been sources of some controversy. The localization of XDH was determined at the electron microscopic level using immunocytochemistry. XDH is shown to be localized in the ground cytoplasm of both infected and uninfected cells. No labeling above background was found in any organelle or membrane.

MATERIALS AND METHODS

Seeds of cowpea (*Vigna unguiculata* [L.] Walpers cv. Queen Anne Blackeye) were inoculated with *Rhizobium* spp., var. "EL" (Nitragin, Milwaukee, WI). The plants were grown as described (13). The polyclonal antibodies reactive with cowpea XDH used in these experiments were prepared and characterized as described (14–16). The antibodies used in this study react specifically with XDH (15). Samples were prepared, fixed, dehydrated, embedded, sectioned, and labeled as described by VandenBosch and Newcomb (17) with the following exceptions. Samples were incubated at 4°C overnight during the fixation process followed by dehydration at 0°C. Crude immune and preimmune sera were diluted 1:50 in 10 mM Tris·HCl, pH 7.4/150 mM NaCl/0.05% Tween 20/1% bovine serum albumin. Sera were incubated with the grids for 1 hr. The diameter of the protein A-gold particles was 20 nm. Post-staining of sections, electron microscopy, and determination of labeling density were carried out as described by VandenBosch and Newcomb (17). The number of gold particles per μm^2 in the ground cytoplasm of sections treated with preimmune serum was assumed to represent background.

RESULTS

Intense specific labeling of XDH was observed in the ground cytoplasm of both infected and uninfected cells (Fig. 1). The amount of XDH labeling in infected and uninfected cells was determined. The number of gold particles in the ground cytoplasm of uninfected and infected cells was 59.3 ± 2.8 particles per μm^2 and 30.8 ± 2.2 particles per μm^2, respectively. The amount of labeling in the ground cytoplasm of both cell types with preimmune serum was 9.1 ± 1.1 particles per μm^2. These values are significantly different at the 1% level of significance. The error figures represent the standard error about the mean. Thus, after subtracting the background counts, labeling of XDH in the uninfected cells was 2.3 times that in the infected cells per unit area of ground cytoplasm.

No labeling was observed in the enlarged peroxisomes of uninfected cells (Fig. 1*A*). Furthermore, no XDH-specific labeling was observed in mitochondria or plastids or on either cytoplasmic or peribacteroid membranes. An artifactual origin for the labeling would seem to be conclusively ruled out

Abbreviation: XDH, xanthine dehydrogenase.

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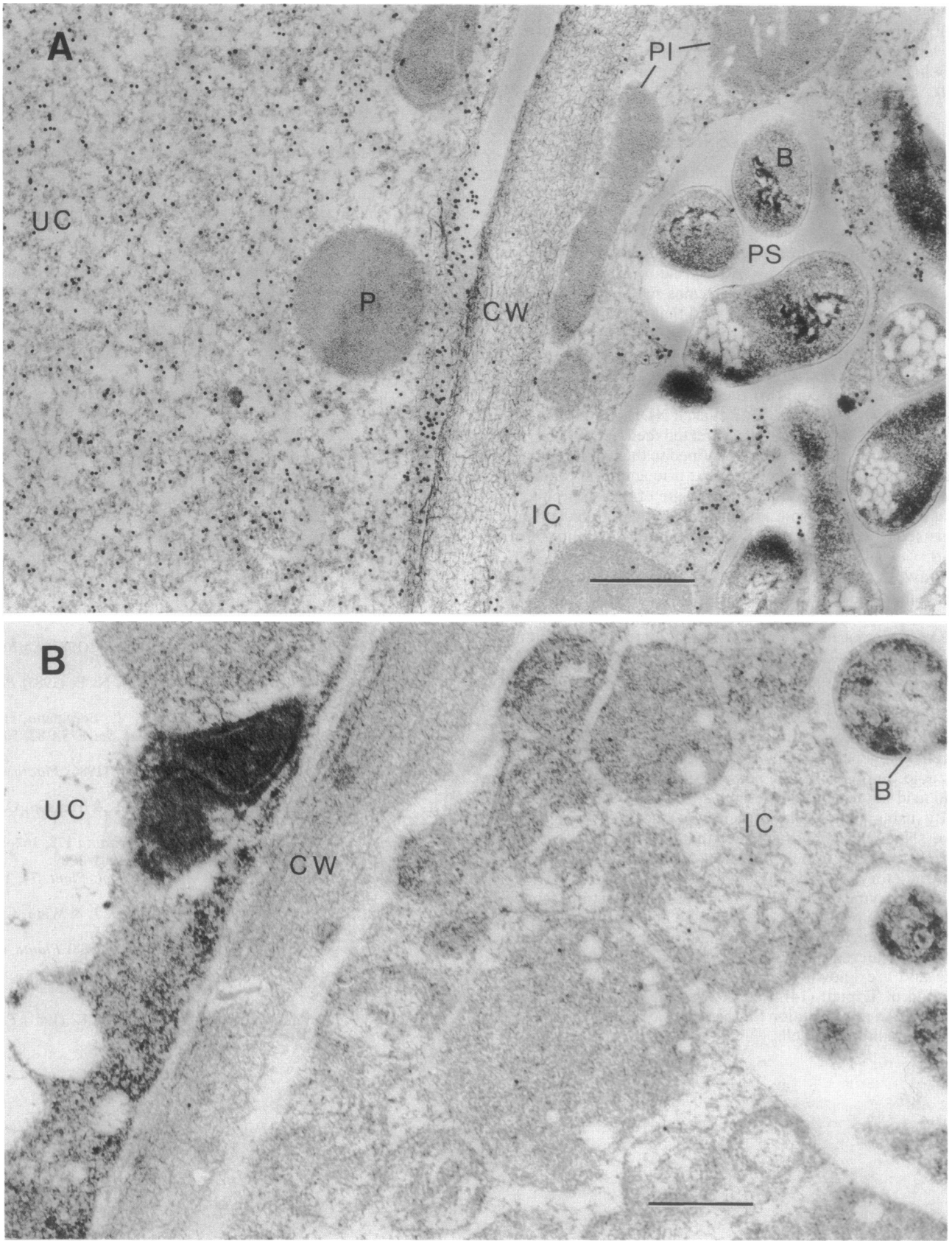

FIG. 1. Immunogold labeling for XDH in infected and uninfected cells in root nodules of cowpea. Gold particles are 20-nm protein A-gold. (*A*) Immunogold labeling of XDH in infected (IC) and uninfected (UC) cells of the infected region of a 4-week-old cowpea nodule. Note that labeling of the uninfected cell is greater than that of the infected cell. B, *Rhizobium* bacteroid; CW, cell wall; P, peroxisome; Pl, plastid; PS, peribacteroid space. (×37,500; bar = 0.5 μm.) (*B*) Preimmune labeling in infected (IC) and uninfected (UC) cells of the infected region of a 4-week-old cowpea nodule. Note sparse nonspecific labeling. B, *Rhizobium* bacteroid; CW, cell wall. (×37,500; bar = 0.5 μm.)

by the lack of label in the bacteroids, peribacteroid spaces, cell walls, or intercellular spaces (Fig. 1*A*). A small amount of labeling was observed with preimmune serum but this labeling was not confined to any particular cell structure (Fig. 1*B*).

DISCUSSION

Knowing the cellular location of XDH is important because it can help identify the intermediate in the ureide pathway that moves from infected to uninfected cells. Based on the results of protoplast fractionation, Boland and Schubert (4, 6) concluded that the infected cells appear to be the primary site of ammonia assimilation and purine synthesis, although maintenance levels of these enzymes may be present in all cells. Shelp *et al.* (7) also observed that the major portion of the activity of enzymes associated with *de novo* purine synthesis is in the infected cell fraction. If XDH is present in the uninfected cells, xanthine or an even earlier precursor of ureide biogenesis could be the intermediate transported between the two cell types. However, if XDH were found to be localized exclusively in the infected cells, then *de novo* purine synthesis could not be confined to the uninfected cells. Furthermore, it would require that uric acid, the product of the XDH reaction, be the intermediate of ureide production that is transported from the infected to the uninfected cells. This seems unlikely in view of the limited solubility of uric acid.

Previous efforts to localize XDH have been contradictory. By using a histochemical stain, Triplett (14) found XDH activity in the infected cells. Based on that observation and the presence of uricase activity in the peroxisomes of uninfected cells (8–12), he suggested that uric acid was the intermediate transported between the two cell types. However, we believe that histochemical staining at the light microscopic level lacks sufficient resolution for XDH to be detected if it is present also in the smaller uninfected cells, given their generally thin layer of cytoplasm and large central vacuole. In this case, the possibility that these cells produce uric acid could not be ruled out.

By using immunofluorescence at the light microscopic level, Nguyen *et al.* (18) claimed that XDH was present in the peroxisomes of uninfected cells. This work, however, is contradictory to previous work showing that XDH is a soluble enzyme (3, 19). We interpret the peroxisomal labeling proposed by Nguyen *et al.* (18) to be nonspecific labeling of plastids, since their labeled organelles appear to be too large to be considered peroxisomes, whereas plastids are of this size and are known to give nonspecific labeling. Like the method of Triplett (14), that of Nguyen *et al.* (18) probably lacks sufficient resolution for the detection and localization of XDH in uninfected cells.

The labeling results presented here illustrate that XDH is present in the ground cytoplasm of both infected and uninfected cells. Although XDH is not nodule-specific, an elevated level of XDH is present in nodules (14, 16). The higher concentration of XDH observed in the uninfected cells implies that these cells may be the site of xanthine hydroxylation for ureide biogenesis in nodules and that xanthine or a precursor to xanthine, rather than uric acid, is the intermediate transported from the infected to the uninfected cells. The concentration of XDH in the infected cells may be more or less similar to that found in the ground cytoplasm of cells in other parts of the plant.

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