Expression of nodule-specific uricase in soybean callus tissue is regulated by oxygen

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In soybean root nodules the enzyme uricase is expressed concomitantly with nodule development. The initial expression of this protein does not depend on active nitrogen fixation, as demonstrated by analysis of uricase activity in effective and ineffective root nodules. However, the maximal level of uricase activity is determined by the infecting *Rhizobium japonicum* strain. Sterile root cultures and callus tissue, devoid of the microsymbiont, were incubated at varying oxygen concentrations and analyzed for uricase activity. The specific activity of uricase was increased by lowering the oxygen concentration, with the highest activity obtained around 4–5% oxygen. The increase in uricase activity was due to increased uricase synthesis, as demonstrated by *in vivo* labelling of callus culture followed by immunoprecipitation with antibodies raised against highly purified nodule uricase.

Key words: nodulin/*Glycine max*/root culture/urate oxidase/partial anaerobiosis

Introduction

Certain legumes including soybean and cowpea synthesize ureides (allantoin and allantoic acid) from currently fixed nitrogen in nodules, and use these compounds for transport and storage of nitrogen (Herridge *et al.*, 1978; McClure and Israel, 1979; Atkins *et al.*, 1980). The synthesis of ureides, which occurs in the root nodules, is a result of *de novo* purine synthesis and subsequent oxidative degradation (Fujihara and Yamaguchi, 1978; Schubert, 1981). The purine catabolic pathway (Figure 1) involves five enzymatic activities, 5'-nucleotidase, purine nucleosidase, xanthine dehydrogenase, uricase and allantoinase (Triplett *et al.*, 1980; Schubert, 1981; Christensen and Jochimsen, 1983). Uricase (urate: oxidoreductase, EC 1.7.3.3) converts uric acid to allantoin and has been demonstrated to be nodule specific; it is designated uricase II. In addition another activity degrading uric acid has been found in uninfected roots, uricase I (Bergmann *et al.*, 1983), however, this activity requires a co-factor and is due to the combined action of a diamine oxidase and a peroxidase (Tajima *et al.*, 1983). In soybean root nodules uricase occurs preferentially in the uninfected cells (Bergmann *et al.*, 1983; Hanks *et al.*, 1983) and is localized in peroxisomes (Hanks *et al.*, 1981; Nguyen *et al.*, 1985).

Time-course studies during nodule development indicate which enzymatic activities are linked to the phenomenon of nitrogen fixation. Such studies have shown that uricase and xanthine dehydrogenase are expressed simultaneously (Schubert, 1981; Reynolds *et al.*, 1982) and differently from leghemoglobin, a characteristic protein in root nodules.

To obtain an indication of the bacterial signals possibly involved in regulating uricase expression, we have analyzed nodule for-

Fig. 1. Pathways for the formation of ureides in soybean nodules. The reactions involved in the degradation of IMP (inosine 5'-monophosphate) and XMP (xanthosine 5'-monophosphate) are identified by the names of the respective enzymes. Purine *de novo* indicates a series of reactions where individual enzymes have not been identified in soybean, except for 5-phosphoribosyl-pyrophosphate amidotransferase (Reynolds *et al.*, 1984).

mation caused by bacterial mutants, resulting in deficient nitrogen fixation: so-called ineffective nodules. From these studies it can be concluded that active nitrogen fixation is not a prerequisite for the expression of nodule-specific proteins. However, the specific activity of uricase is affected and constitutes <50% of that in effective nodules.

The purpose of this report is to identify a possible bacterial signal that might control gene expression in the host plant. We have examined the regulation of uricase in an artificial system, namely soybean callus tissue, compared with the natural symbiotic relationship in the root nodule. Working with callus tissue makes it possible to detect *de novo* synthesis of uricase, due to the low level of enzyme activity found in such tissue. Our hypothesis is based on the assumption that bacterial invasion and proliferation in plant root cells creates a situation with an increased demand for oxygen for bacterial respiration. The lowered oxygen availability might result in synthesis of enzymes characteristic of anaerobic or partial anaerobic conditions. The data presented here confirm our hypothesis.

Results

The appearance of uricase in soybean root nodules

The appearance of uricase activity in effective (Fix⁺) and ineffective (Fix⁻) root nodules was compared. Results are shown in Figure 2. The specific activity of uricase in effective (110) nodules increased during nodule development from a root level

Fig. 2. Specific activity of uricase in extracts prepared from nodules harvested at the indicated times after infection. Infections were performed with three different *R. japonicum* strains: (●) *R. japonicum* 110: nod⁺, fix⁺; (○) A3: nod⁺, fix⁻; (▽) HS124: nod⁺, fix⁻, leghemoglobin⁻. Extracts were prepared and assay for uricase was carried out as described in Materials and methods.

of ~1 unit to 150 units. The maximal specific activity was reached ~3 weeks after infection. However, the maximal specific activity obtained with ineffective nodules was one half (A3) and one third (HS124) the activity in Fix⁺ nodules. The activity rapidly decreased in Fix⁻ nodules, which was followed by disappearance of the uricase 33-kd subunit (data not shown). The patterns of appearance of uricase activity in Fix⁻ nodules were not significantly different when nodules synthesizing leghemoglobin (A3) were compared with nodules unable to do so (HS124).

Similar results have been obtained with other pairs of effective and ineffective root nodules (unpublished).

Regulation of uricase activity in sterile root cultures

To test the regulation of uricase activity in plant tissue devoid of the microsymbiont, sterile root cultures were exposed to varying oxygen concentrations. After 48 h of incubation uricase activity was determined radiometrically. The results given as specific enzyme activity are shown in Figure 3. The highest specific activity, 2.5 times the activity with normal aerobic (20% oxygen) atmosphere, is obtained with root culture exposed to 5% oxygen in the gas phase.

Regulation of uricase activity in callus tissue

The observation with root cultures was extended by examination of uricase activity in undifferentiated callus tissue, exposed to varying oxygen concentrations. After 48 h of incubation uricase activity was determined. The results are shown in Figure 4. The maximum specific activity was obtained at 4% oxygen in the gas phase. The pattern obtained with uricase was compared with that obtained with alcohol dehydrogenase (Figure 5). The specific activity of alcohol dehydrogenase, an established indicator of anaerobiosis, increased with decreasing oxygen concentration and reached its peak activity around 1% oxygen; a pattern significant-

Fig. 3. Specific activity of uricase in extracts of sterile soybean root culture exposed to various oxygen concentrations. Extracts were prepared and enzyme activity determined as described in Materials and methods. Data are the average of duplicate experiments.

Fig. 4. Specific activity of uricase in soybean callus tissue exposed to various oxygen concentrations. Incubation at different oxygen concentrations, preparation of extracts and enzyme determinations was carried out as described in Materials and methods. (●) Callus tissue incubated in the dark, average of triplicate experiments, (○) callus tissue incubated in light, average of duplicate experiments.

ly different from that obtained with uricase.

In vivo synthesis of uricase

To test whether the increase in specific activity of uricase observed with callus tissue was due to modification of pre-existing enzyme, or to *de novo* synthesized protein, callus tissue was incubated with [³⁵S]methionine and the gas phase adjusted as above. Specific labelling of uricase protein was determined after im-

Fig. 5. Specific activity of alcohol dehydrogenase in extracts pre-treated as in Figure 4. Enzyme activity was determined spectrophotometrically. The means of three experiments are reported.

munoprecipitation using antibodies raised against nodule-specific uricase (Figure 6).

The 33-kd subunit of nodule uricase was labelled equally intensely at 1.5 and 8% oxygen in the gas phase, indicating a possible optimum within these concentrations. The serum also reacted with an unknown antigen (probably a contaminant in the uricase preparation) that showed a labelling pattern different from that of the uricase subunit.

Discussion

Uricase catalyzes the oxidative conversion of uric acid into allantoin. In soybean (and cowpea) the main transport forms of fixed nitrogen from the root nodules are the ureides, allantoin and allantoic acid. These compounds are the final products of purine synthesis and subsequent catabolism. Uricase is located in the terminal part of this metabolic pathway (Figure 1). During soybean nodule development uricase is induced some 50-fold or more. Expression of uricase seems to be a result of the presence of *Rhizobia* bacteria within the nodule tissue. This induction is not dependent initially on a functional nitrogenase activity, since uricase is observed both in effective and ineffective nodules, in agreement with nodulin-35 (Legocki and Verma, 1979) later identified as the 33-kd subunit of uricase (Bergmann *et al.*, 1983). The overall appearances, however, differ in Fix^+ and Fix^- nodules (Figure 2), in agreement with the general observation of reduced expression of nodulins in ineffective nodules (Legocki and Verma, 1980; Noel *et al.*, 1982).

The disappearance of uricase activity from Fix^- nodules indicates that expression is not due to a permanent transformation, but has to be sustained somehow by an active bacterial metabolism. It should be noted that this disappearance is not due to a general nitrogen starvation in plants with Fix^- nodules, since all plants are watered with a mineral solution containing 1 mM KNO_3 in order to sustain normal plant growth and metabolism, especially in plants infected with ineffective mutants. Examination of the nodules formed by infection with A3 and HS124 also shows that uricase expression is independent of the expression of the most prominent nodule protein, leghemoglobin, since A3 nodules contain normal amounts of leghemoglobin, while leghemoglobin is undetectable in HS124 nodules.

Expression of uricase controlled by oxygen

Fig. 6. Autoradiogram of [^{35}S]methionine-labelled immunoprecipitated proteins from soybean callus tissue. Callus was exposed to different oxygen concentrations for 48 h in the presence of [^{35}S]methionine. Specific immunoprecipitation was carried out as described in Materials and methods. Separation of denatured proteins was carried out on a 10% SDS-polyacrylamide gel. Lane 1, total TCA-precipitated proteins from callus incubated at 0% oxygen concentration. Lanes 2–6, immunoprecipitated protein from callus incubated at 0, 0.3, 1.5, 8 and 20% oxygen, respectively. Lane 7, total TCA-precipitated proteins from callus incubated at 20% oxygen. Lane 8, ^{14}C -methylated protein mixtures (mol. wt. markers). [^{35}S]Methionine incorporation was not equally efficient at the different oxygen concentrations. At 0, 0.3, 1.5 and 8%, incorporation represented 2, 37, 51 and 105% of that obtained at aerobic (20%) concentrations.

Since *Rhizobia* are microaerobic bacteria, the presence of bacteria within the plant tissue must result in an increased demand for oxygen in such tissues. We hypothesized that a diminished oxygen concentration within the nodules might cause an increased expression of certain genes among these uricase since the enzymatic reaction is dependent on oxygen. The expression of uricase in root cultures and in callus tissue was demonstrated to depend on the available oxygen concentration (Figures 3 and 4). Lowering oxygen concentrations increased uricase specific activity, with maximal activity obtained around 4–5% oxygen in the gas phase. This increase in activity did not depend on light (Figure 4).

The increase in uricase specific activity is not due to increased synthesis of diamine oxidase (uricase I), since this activity is dependent on a co-factor, cadaverine (Tajima *et al.*, 1983) and all assays were performed on G-25 dialyzed extracts.

By measuring, under similar conditions, alcohol dehydrogenase, an established indicator of anaerobiosis (compare Figures 4 and 5), a difference in optimal expression between uricase and alcohol dehydrogenase was observed, indicating uricase expression to be maximal at an intermediate oxygen concentration.

The elevated level of uricase was shown to be due to increased synthesis of the uricase enzyme, as shown in Figure 6, by *in vivo* labelling of the 33-kd subunit of uricase, detected by immunoprecipitation. The autoradiogram revealed maximal synthesis at 1.5 and 8% oxygen, indicating an optimal oxygen concentration for expression within these values in agreement with the above results. Expression of an unknown antigen (unrelated to uricase) at the indicated oxygen concentrations served as an internal control (upper band lanes 5–6, Figure 6), showing that

elevated expression at lowered oxygen concentrations is not a general phenomenon.

The main uricase activity is localized in the uninfected cells of soybean root nodules (Bergmann *et al.*, 1983; Hanks *et al.*, 1983). Assuming a close resemblance between such uninfected cells and the callus and root cells used in this study, the signal controlling uricase expression in the intact nodule might be oxygen concentration.

By the isolation of cDNA and genomic clones encoding uricase (Nguyen *et al.*, 1985), it is now possible to establish whether the oxygen regulation is exerted at the transcriptional level.

A series of proteins (nodulins), among which leghemoglobins are the most prominent and best studied, have been shown to be expressed specifically in soybean root nodules (Legocki and Verma, 1980). Nodulins have been divided into three classes (Fuller *et al.*, 1983) of which the second class includes enzymes necessary for assimilation of nitrogen. Uricase (nodulin-35) therefore belongs to class II nodulins. Another protein belonging to this class is a nodule-specific form of glutamine synthetase from *Phaseolus* (Lara *et al.*, 1983). However, expression of this enzyme as well as glutamine synthetase from soybean is dependent on active nitrogen fixation (Werner *et al.*, 1980). Thus, it seems that low oxygen concentrations might be only one of several (bacterial) signals required for expression of nodulins. In future work we would like to analyze whether other nodulins are expressed as a consequence of lowered oxygen concentration within the root nodules.

Materials and methods

Chemicals

Radiochemicals [L - 35 S]methionine, [2 - 14 C]uric acid and 14 C-methylated protein mixture (mol. wt. markers) were obtained from the Radiochemical Centre, Amersham, UK. Polyethyleneimine cellulose thin-layer plates (Polygram CEL 300 PEI) were from Machery-Nagel, Düren, FRG.

Biological materials

Seeds of soybean (*Glycine max* var. Evans and Mandrin) were from Agricultural Exports Inc., Hudson, IA. Seeds used for sterile root cultures were treated as described by Reinert and Yeoman (1982). Primary roots from seedlings were transferred to a defined root medium (Reinert and Yeoman, 1982) and incubated in the dark at 25°C. Tissue lines were derived from soybean (var. Mandrin) stem tissue and soybean (var. Evans) root tissue. Established callus cultures were propagated at 25°C, 3000 lux on solidified Miller medium (Miller, 1968) containing 1 mg/l naphthylacetic acid, and subcultured every 3 weeks. Growth of soybean plants and infection with *Rhizobium japonicum* bacteria was performed as previously described (Christensen and Jochimsen, 1983). *R. japonicum* strains used for infection, 110 (*spc4*, *nod*⁺, *fix*⁺), A3 (*spc4*, *nifD*::Tn5, *nod*⁺, *fix*⁻) (Hahn and Hennecke, 1984) and HS124 (*nod*⁺, *fix*⁻, leghemoglobin⁻) (Noel *et al.*, 1982), were derivatives of the wild-type strain 311b110 and obtained from the respective authors.

Incubations at various oxygen concentrations

Excised pieces of callus tissue (200–500 mg) or sterile roots (100–250 mg) were aseptically transferred to serum bottles. The bottles were closed and the gas phase replaced by dinitrogen. Different oxygen concentrations were established by replacing a defined amount of dinitrogen with an equal volume of pure oxygen. The resulting oxygen concentrations were determined using a Gow Mac series 572 gas chromatograph with TCD detector, mounted with a molecular sieve 5A column and argon as carrier gas. Plant material with adjusted gas phase was incubated at 25°C, light/dark, respectively, for 48 h.

Preparation of extracts

Plant tissues were homogenized and treated as previously described (Christensen and Jochimsen, 1983).

Enzyme assays

Spectrophotometric determination of uricase (EC 1.7.3.3.) in root nodules was performed according to Christensen and Jochimsen (1983). Uricase activity in callus and root extracts was determined essentially as described by Hanks *et al.* (1981) except that the assay contained 10 μ M CuSO₄.

Alcohol dehydrogenase (EC 1.1.1.1.) activity was determined according to

Freeling (1973). Protein concentration was determined according to the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as a standard.

In vivo labelling

Callus tissue was cut into cube-shaped explants (100 mg) and transferred to serum bottles containing solidified Miller medium with 1 mg/l naphthylacetic acid. Subsequently 25 μ Ci of [35 S]methionine solution (sp. act. 500–1000 Ci/mmol) was carefully pipetted onto the top of the tissue. Incubation at different oxygen concentrations was performed as described above.

Preparations of protein extracts

After 48 h incubation, extracts of tissue explant were prepared as described above. The resulting supernatant was used for determination of total [35 S]methionine incorporation by trichloroacetic acid precipitation, and for specific immunoprecipitation of uricase following the technique described by Kessler (1975). Electrophoresis of denatured extracts was performed on SDS-10% acrylamide-0.2% bis-acrylamide slab gels using the discontinuous buffer system of Laemmli (1970).

Purification of nodule uricase

Nodule uricase was purified to constant specific activity using a protocol analogous to Bergmann *et al.* (1983). Our protocol involved (NH₄)₂SO₄ precipitation to 35%, DEAE- and CM-Sephadex chromatography, followed by G-150 gel filtration. From 56 g of 6-week-old nodules 10 mg of apparently homogenous protein, as judged by native PAGE and SDS-PAGE, was obtained.

Preparation of antisera

Antiserum against purified uricase was prepared by repeated injections of a New Zealand white rabbit i.m. with 1 mg/ml portions of uricase mixed with equal volumes of Freund's complete adjuvant. Antiserum was collected and purified using DEAE-Sephadex chromatography, dialyzed and used without further treatment. In order to test that the obtained antiserum was directed against uricase, an immunotitration was performed. Purified uricase was inhibited by the antiserum obtained, when enzyme and serial dilutions of antiserum were mixed directly in the assay cuvette.

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