Indole-3-acetic Acid Catabolism by Soybean Bacteroids

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(Received 19 June 1974; revised 11 October 1974)

SUMMARY

Bacteroid preparations from nodules of soybean were able to destroy aerobically substantial quantities of indole-3-acetic acid (IAA). Kinetic studies indicate the involvement of two processes. The supernatant derived during preparation of bacteroids had an active IAA oxidation during the first 30 min. Successive washings of bacteroids still retained some activity although it was reduced. A high level of IAA destruction was always observed with washed bacteroids and the reaction proceeded linearly for at least 4 h. Glucose gave a slight stimulation but succinate, pyruvate and fumarate at low concentrations (approx. 25 mM) were strong inhibitors. No IAA catabolism occurred with washed bacteroids at a PO_2 of 30 mmHg or less, but it increasingly occurred with further increase in oxygen partial pressure up to 150 mmHg. No difference was observed between bacteroids with or without active nitrogenase.

INTRODUCTION

Significant quantities of indole-3-acetic acid (IAA) occur in the nodules of different legumes (Pate, 1958; Dullaart, 1967). Mennes (1972) reported IAA degradation in nodules of *Lupinus* and suggested that bacteroids were not involved in this activity. Earlier results showed that different strains of free living *Rhizobium* could destroy IAA (Rigaud, 1969; Dullaart, 1970) and it was of interest to investigate the possible role of bacteroids in this catabolism. We report an IAA-oxidase activity in preparations of soybean bacteroids; the effects of substrates and oxygen tensions were also investigated.

METHODS

Nodules. Soybeans (*Glycine max* Merr., cultivar Altona) inoculated with *Rhizobium japonicum* CC705 (syn. Wisconsin 505), were grown in a glasshouse (temperature range 20 to 27 °C) in plastic pots in a 2:1 mixture of washed vermiculite and sand. The composition (g/l) of the culture medium was: KH_2PO_4 , 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; KCl, 0.2; $CaSO_4 \cdot 2H_2O$, 0.12; $Na_2FeEDTA$, 0.025; $Na_2MoO_4 \cdot 2H_2O$, 0.004; and microelements as recommended by Heller (1953). Nodules were harvested about 40 days after sowing (nodule age 31 to 32 days).

Bacteroid suspensions. Nodules (about 15 to 35 g fresh wt) were crushed aerobically in Bergersen & Turner's (1970) mixture, except that sodium ascorbate was omitted. The bacteroid preparations with active nitrogenase were prepared anaerobically under argon with a modified Sorvall Omni-mixer according to Bergersen & Turner (1973). After filtration of the homogenate, a first centrifugation (300 g) removed plant cell debris. Bacteroids were separated by a second centrifugation (6800 g), washed twice with a phosphate buffer

Fig. 1. General scheme for the preparation of soybean bacteroids. Oxidation of IAA was tested at different steps (●).

(0.1 M, pH 7) containing sucrose (0.3 M), and resuspended in phosphate buffer. This method was regarded as the initial preparation of bacteroids.

In addition, these bacteroids were sometimes washed with citrate-phosphate buffer (0.1 M) or phosphate buffer (25 to 100 mM) at several pH values. Figure 1 gives a general scheme for the preparation of bacteroids and shows the successive steps when the degradation of IAA was tested.

IAA-oxidase activity. Glass vials (25 ml) were used, containing IAA (0.3 mM) in 2 ml of phosphate buffer (25 mM, pH 6), sometimes with added substrates such as glucose, sodium succinate or sodium fumarate at various concentrations. Reactions in duplicate were initiated by the addition of bacteroids (20 to 30 mg dry wt/assay) or a volume of supernatant derived from this quantity. The flasks, plugged with cotton wool, were agitated on a reciprocating shaker at 100 rev./min at 30 °C. To stop the reaction, 0.1 ml of NaOH (1 M) was added.

To obtain anaerobic conditions or low oxygen tensions, the vials were capped with rubber seals, evacuated, and filled with nitrogen or with nitrogen and oxygen at different partial pressures. The reactions were started by the injection of bacteroids.

The IAA concentrations were estimated colorimetrically (Pilet & Collet, 1962).

Nitrogenase activity. The assays were carried out in 18 ml Warburg vessels as described by Rigaud *et al.* (1973). The activity was measured by C_2H_2 reduction (Hardy *et al.* 1968) using a Pye Unicam gas chromatograph fitted with a column of Porapak R.

Fig. 2. Time course of IAA oxidation by initial preparations of bacteroids. Incubation mixtures (2 ml) contained IAA (0.3 mM) and bacteroids (20 mg dry wt) in a phosphate buffer (0.1 M, pH 7), and were shaken at 100 rev./min at 30 °C. Vertical bars represent ± 1 standard deviation.

Fig. 3. Effect of (a) the number of washings, and (b) the nature and pH of buffers, upon IAA degradation by the supernatants. After initial preparation, bacteroids were washed for 3 min (a) 2, 4 or 6 times with phosphate buffer pH 7 (100 mM), or (b) twice with (▲) phosphate buffer (100 mM), or (●) citrate-phosphate buffer (100 mM). Incubation mixtures (2 ml) contained IAA (0.3 mM) and supernatant derived from 22 mg dry wt of bacteroids, and were shaken at 100 rev./min for 30 min. Vertical bars represent ± 1 standard deviation.

RESULTS

IAA degradation by initial preparations of bacteroids

Active degradation of IAA (54 nmol/assay) was obtained for 4 h without an energy substrate. The time course (Fig. 2) indicates three steps: a high initial rate, a short lag time, and a subsequent rate which is lower than the initial rate and remains constant for at least 3 h.

IAA-oxidase activity in the supernatants

The supernatant 1 remaining after the initial preparation induced the disappearance of about 20 nmol IAA in 2 h. There was a decrease of IAA oxidation after successive washings with phosphate buffer (supernatant 2), although even after six washings substantial activity persisted in supernatant 3 (Fig. 3a). The influence of the buffer characteristics (constituents, pH and ionic strength) on the activity in these supernatants was investigated. Least IAA oxidation was obtained with 0.1 M-phosphate buffer, pH 5.5 and 6 (Fig. 3b). More activity was found at higher pH and with citrate-phosphate buffer pH 4 to 6. Phosphate concentration had little effect over the range 100 to 25 mM, at pH 6, although least activity was obtained at 25 mM.

Fig. 4. Time course of IAA oxidation (*a*) by supernatant 4 and (*b*) by washed bacteroids. Incubation mixtures (2 ml) contained IAA (0.3 mM) and 27 mg dry wt of (*a*) supernatant 4 or (*b*) washed bacteroids in 25 mM-phosphate buffer pH 6. The vessels were shaken at 30 °C.

Fig. 5. The effect of the concentrations of (*a*) IAA, and (*b*) glucose, sodium succinate and sodium fumarate, upon IAA degradation by washed bacteroids. (*a*) Incubation mixtures (2 ml) contained IAA at different concentrations and bacteroids (30 mg dry wt) in 25 mM-phosphate buffer, pH 6. (*b*) The bacteroids (24 mg dry wt), resuspended in 25 mM-phosphate buffer, pH 6, were incubated, for a final volume of 2 ml, with 0.3 mM-IAA plus (▲) succinate, (■) fumarate, or (●) glucose. Assayed at 30 °C with shaking (100 rev./min). Vertical bars represent ± 1 standard deviation.

In later experiments, after the initial preparation, the bacteroids were washed by shaking (100 rev./min) for 30 min at 30 °C in 25 mM-phosphate buffer pH 6. The time course of IAA oxidation by the supernatant 4, in these conditions, is shown in Fig. 4(*a*). Degradation was rapid during the first 30 min but the rate declined after 1 h. No activity was observed during the following 7 h.

IAA degradation by washed bacteroids

IAA was destroyed by bacteroids at a linear rate for up to 4 h (Fig. 4*b*). To estimate the activity of bacteroids alone, the first value was determined after 1 h. The reaction proceeded linearly for 4 h, and for further experiments we used the value of IAA degraded between the 2nd and the 5th hour.

The rate of IAA oxidation increased with increasing IAA concentration up to 0.35 mM (Fig. 5*a*). For the following assays 0.3 mM-IAA was used.

The effects of different substrates upon IAA oxidation by washed bacteroids are shown in Fig. 5(*b*). A slight stimulation was observed with increasing concentrations of glucose

Fig. 6. Effect of oxygen partial pressures upon IAA degradation by washed bacteroids. The vessels contained 25 mM-phosphate buffer, pH 6, plus IAA (0.3 mM), washed bacteroids (20 mg dry wt) and glucose (50 mM), and were shaken (100 rev./min) at 30 °C. The other gas present was argon.

(20 %); the optimal effect was reached with 100 mM. Higher levels of glucose, as well as sucrose up to 300 mM, did not substantially increase IAA degradation, suggesting that an osmotic effect was not involved. In contrast, relatively small concentrations of succinate or fumarate (25 mM) strongly inhibited IAA oxidation by washed bacteroids (Fig. 5*b*); pyruvate gave similar results.

Oxidation of IAA by washed bacteroids was oxygen-dependent. For low oxygen tensions, up to 40 mmHg, a little IAA destruction was observed (Fig. 6). Higher partial pressures sharply increased this activity, reaching a maximum at 120 mmHg.

In contrast with the preceding experiments with bacteroids prepared in aerobic conditions, we also used an anaerobic preparation procedure to prevent nitrogenase destruction. In both cases the results were similar in the range of O_2 partial pressures up to 38 mmHg, which did not inactivate nitrogenase for this strain of bacteria (Rigaud *et al.* 1973). With O_2 as terminal electron acceptor, succinate and fumarate have been reported to be efficient substrates supporting nitrogen fixation by these bacteroids (Bergersen & Turner, 1967). In our experiments, because these compounds inhibited IAA-oxidase activity (Fig. 5*b*) we used glucose as substrate, despite its low efficiency for nitrogen fixation.

DISCUSSION

A substantial destruction of IAA is observed on incubation with soybean bacteroids. The time course of this reaction (Fig. 2) suggests that two different processes may be involved in this catabolism. The high initial rate observed during the first 30 min suggests an enzymic activity not directly dependent on the bacteroids themselves. This is supported by the ability of supernatants alone to destroy IAA strongly during the initial 30 min, the reaction ending after 1 h (Fig. 4*a*). Successive washings of the bacteroids (Fig. 3*a*) reduced IAA oxidation activity, in agreement with the results of Mennes (1972), but more than 60 % of the original activity was still present in the sixth washing solution. A relationship was observed between activity in the supernatants and the characteristics of the washing buffers. Possibly this activity derives from the plant organelles. In our experiments this seems unlikely because plant cell debris was removed by several steps of centrifugation (see Methods) and the bacteroids were washed several times. On the other hand, this activity could come

from particles bound to the bacteroids as proposed by Mennes (1972), or from the bacteroids themselves by leakage.

A second phase of IAA oxidation in which bacteroids alone are involved was observed after 1 h. The existence of a similar lag phase was reported by Rigaud (1969) with free living *Rhizobium* and this might be due to the slow penetration of IAA into the bacteroids. Under our experimental conditions no relationship was observed between oxidation of IAA and nitrogenase activity. To prevent an inhibition of IAA oxidase, substrates which stimulate nitrogen fixation by bacteroids (succinate, fumarate or pyruvate) were omitted, and this certainly limited nitrogenase activity.

A low oxygen partial pressure reduced the oxidation of IAA by bacteroids. A similar oxygen requirement for IAA oxidase was reported by Rigaud (1969) with free living *Rhizobium*. The low oxygen tensions likely to occur in the nodules could limit the degradation of IAA by the bacteroids.

Mennes (1972) reported that lupin bacteroids are devoid of IAA-oxidase activity; the use of fresh nodules and the protection of IAA oxidase from non-specific binding with phenolic products during the preparation procedure, as suggested by Sequeira (1973), achieved here with polyvinylpyrrolidone could partly explain the lack of agreement with our results.

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