The Regulation of Some Enzymes Involved in Ammonia Assimilation by *Rhizobium japonicum*

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SUMMARY

The concentrations of three enzymes active in ammonia assimilation by *Rhizobium japonicum* (glutamate dehydrogenase, aspartate aminotransferase, alanine aminotransferase) were influenced considerably by the N-source in the culture medium. Relatively high concentrations of these enzymes were present when *R. japonicum* was grown in a medium containing NH_4Cl + 2-oxoglutarate. Relatively low amounts of these enzymes were found in bacteria grown in the presence of various amino acids. Active protein and RNA synthesis were necessary for the induction of the enzymes by NH_4Cl + 2-oxoglutarate.

INTRODUCTION

It has been known for some time that ammonia formed during symbiotic nitrogen fixation is rapidly incorporated into amino acids (e.g. glutamate, aspartate, alanine; Aprison, Magee & Burris, 1954), but relatively little is known about the enzymes which catalyse these reactions in soybean nodules. We showed (Fottrell & Montgomery, 1968) that several enzymes active in ammonia assimilation, including glutamate dehydrogenase, aspartate aminotransferase and alanine aminotransferase were present in the bacteroids of soybean nodules. In the present report the mechanism involved in the regulation of these enzymes was investigated in *Rhizobium japonicum* (symbiont of soybeans).

METHODS

Rhizobium japonicum strain 392 (obtained from C. L. Masterson, Agricultural Institute, Wexford, Irish Republic), which is a slow-growing strain, was grown in a defined medium (Evans & Lowe, 1964) with different N-sources as indicated in Table 1. Cultures were grown in 4-l. Erlenmeyer flasks, from an inoculum of standard size, on an orbital shaker at 28°. Growth was estimated by measuring the extinction at 562 m μ .

At a suitable stage of growth, bacteria were harvested by centrifugation, washed with 0.9% (w/v) NaCl and suspended (1 g. bacteria/1.5 ml. buffer) in 0.06 M-phosphate buffer (pH 7.2) containing 1 mM-glutathione (reduced) + 0.1% (w/v) Triton X-100. The bacteria were disrupted with an MSE 60 W ultrasonic disintegrator for 1.5 min. at less than 5°, then centrifuged at 34,000 g for 90 min. and the clear supernatant fluid used for the enzyme assays. Enzymes were assayed spectrophotometrically at 340 m μ as described by Bergmeyer (1965). Assays were also made for glutamine synthetase and carbamyl phosphate synthetase by the methods of Woolfolk, Shapiro & Stadtman (1966) and Metzenberg *et al.* (1957), respectively. Specific enzyme activity

was expressed in International Units/g. protein (Bergmeyer, 1965). Protein was assayed by a modified biuret reaction (Gornall, Bardawill & David, 1949).

Net synthesis of glutamate and aspartate was shown by using ^{14}C -2-oxoglutarate ($3.2 \mu\text{C}/\mu\text{M}$) (Radiochemical Centre, Amersham, Buckinghamshire, England) as described by Weiss (1967). Glutamate and aspartate were separated by thin-layer chromatography on cellulose with isopropanol + formic acid + water (40 + 2 + 10, by vol.) for 3 hr. Autoradiographs were prepared with Kodak Industrex X-ray Film.

Table 1. *The effect of various nitrogen sources in the medium on enzymes in Rhizobium japonicum*

Nitrogen source	Conc. (mM)	Enzyme		
		Glutamate dehydrogenase	Aspartate amino-transferase	Alanine amino-transferase
		Specific activity (I. units/g. protein)		
KNO_3	7.0	20.1	22.0	18.6
	35.0	22.4	32.3	20.7
	70.0	35.5	48.7	40.2
NH_4Cl	14.0	15.3	40.8	28.1
$(\text{NH}_4)_2\text{SO}_4$	4.0	19.3	30.6	26.4
$\text{NH}_4\text{Cl} +$ 2-oxoglutarate	{ 14.0 }	96.8	230.5	142.2
	{ 2.5 }			
L-Glutamate	3.0	0.0	98.2	37.0
L-Glutamine	3.0	12.0	27.2	7.2
L-Ornithine	5.0	8.2	15.8	5.6
L-Alanine	9.0	26.4	58.2	1.0
L-Aspartate	3.0	0.0	0.0	22.4

RESULTS

The amounts of glutamate dehydrogenase, aspartate aminotransferase and alanine aminotransferase were influenced by the N-source (Table 1). One of the most interesting findings was the relatively high amounts of enzyme in bacteria grown with $\text{NH}_4\text{Cl} + 2$ -oxoglutarate. In contrast, glutamate dehydrogenase was not detected in bacteria grown with glutamate or aspartate. Aspartate aminotransferase was absent when the medium contained aspartate and alanine aminotransferase activity was not detected in bacteria grown with alanine. Further measurements showed that glutamine synthetase activity was very low in bacteria grown with KNO_3 (7 mM) or NH_4Cl (0.014 M), but was increased over 100-fold in bacteria grown with glutamate (3 mM). Carbamyl phosphate synthetase activity, in contrast, was relatively high in bacteria grown with nitrate, but was absent from bacteria grown with ornithine (5 mM) or alanine (9 mM).

To determine the mechanism involved in the regulation of glutamate dehydrogenase and aspartate aminotransferase, bacteria were grown in a medium containing aspartate (3 mM) for 48 hr. The bacteria were harvested under sterile conditions, washed with sterile 0.9% (w/v) NaCl and inoculated into new medium containing 2-oxoglutarate (2.5 mM) + NH_4Cl (0.014 M), in place of aspartate. Various concentrations of actinomycin D, puromycin or cycloheximide were added to replicate cultures in the new medium; control cultures contained no antibiotic. The results (Table 2) showed that enzyme induction was inhibited when either antibiotic was included in the medium.

With the exception of actinomycin D, which inhibited growth by 22% at 1 µg./ml. medium, there was no inhibition of growth by puromycin, cycloheximide or actinomycin D at the concentrations given in Table 2. Alanine amino-transferase activity was investigated only with actinomycin D; the results were very like those obtained for the other two enzymes. On the basis of these results we concluded that active protein and RNA synthesis were necessary for the induction of all three enzymes in *Rhizobium japonicum*.

Table 2. *Effects of inhibitors of RNA and protein synthesis on enzyme induction in Rhizobium japonicum*

Inhibitor	Concentration (µg./ml.)	Glutamate dehydrogenase	Aspartate aminotransferase
		Enzyme activity: % of control (without antibiotic)	
Actinomycin D	0.10	83.6	98.7
	0.25	61.4	70.3
	0.50	43.2	58.5
	0.75	0.0	31.0
	1.0	0.0	0.0
Puromycin	1.00	91.5	102.2
	2.00	49.0	21.7
	2.50	0.0	0.0
Cycloheximide	1.00	20.8	97.8
	2.00	0.0	73.6
	3.00	0.0	27.3
	5.00	0.0	0.0

DISCUSSION

The results show that enzymes probably active in the assimilation of ammonia in soybean nodules, were influenced to a considerable degree in *Rhizobium japonicum* by the N-source in the growth medium. As regards assimilation of fixed nitrogen by legume nodules it was interesting that glutamate dehydrogenase, aspartate amino-transferase and alanine aminotransferase were induced in cultures of *R. japonicum* by $\text{NH}_4\text{Cl} + 2\text{-oxoglutarate}$. Ammonia is the first stable intermediate formed in soybean bacteroids during nitrogen fixation (Bergersen, 1965) and 2-oxoglutarate would probably be supplied by the host legume since very little isocitrate dehydrogenase activity was detected in the bacteroids in our experiments. It seems that in bacteroids of soybean nodules, glutamate, aspartate and alanine are important intermediates in the assimilation of fixed nitrogen.

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