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Glutamate Oxaloacetate Transaminase in Pea Root Nodules[1]

Participation in a Malate/Aspartate Shuttle between Plant and Bacteroid

Michiel A. Appels and Huub Haaker*

Department of Biochemistry, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

ABSTRACT

Glutamate oxaloacetate transaminase (L-glutamate:oxaloacetate aminotransferase, EC 2.6.1.1 [GOT]), a key enzyme in the flow of carbon between the organic acid and amino acid pools in pea (*Pisum sativum* L.) root nodules, was studied. By ion exchange chromatography, the presence of two forms of GOT in the cytoplasm of pea root nodule cells was established. The major root nodule form was present in only a small quantity in the cytoplasm of root cells. Fractionation of root nodule cell extracts demonstrated that the increase in the GOT activity during nodule development was due to the increase of the activity in the cytoplasm of the plant cells, and not to an increase in activity in the plastids or in the mitochondria. The kinetic properties of the different cytoplasmic forms of GOT were studied. Some of the K_m values differed, but calculations indicated that not the kinetic properties but a high concentration of the major root nodule form caused the observed increase in GOT activity in the pea root nodules. It was found that the reactions of the malate/aspartate shuttle are catalyzed by intact bacteroids, and that these reactions can support nitrogen fixation. It is proposed that the main function of the nodule-stimulated cytoplasmic form of GOT is participation in this shuttle.

The genus *Rhizobium* consists of bacteria that are able to induce nodules on roots of members of the *Leguminosae*. Carbon metabolism in the root nodule must fulfill two major tasks. The bacteroids must be supplied with oxidizable substrates, and newly fixed nitrogen must be assimilated and transported to other parts of the plant. It is generally accepted that C_4-dicarboxylic acids are the major carbon source for the bacteroids to support nitrogen fixation, which are formed by the plant from sucrose (9). The key reaction is the carboxylation of phosphoenolpyruvate catalyzed by phosphoenolpyruvate carboxylase (EC 4.1.1.31). The formed oxaloacetate is reduced by NADH to malate catalyzed by malate dehydrogenase (EC 1.1.1.37).

Ammonia formed by the action of nitrogenase (EC 1.18.6.1) is excreted by the bacteroids into the host-cell cytoplasm (5). In the cytoplasm of the plant cell, ammonia is assimilated in glutamate by the action of glutamine synthetase (EC 6.3.1.2). The formed glutamine is metabolized further and, depending on the type of plant, the newly fixed N is transported as an amide or ureide by way of the xylem of the host plant (23).

The role of GOT[2], EC 2.6.1.1, in the organic acid and amino acid metabolism is complex. It is known that, during nodule development, the specific activity of GOT in the cytoplasm of root nodule cells increases (11, 20), and this is associated with the increase in activity of isoforms. Scott *et al.* (24) (for a review see ref. 23) proposed a pathway for asparagine synthesis, the predominant amino acid of amide transporting legumes. In this pathway, GOT catalyzes the transamination of oxaloacetate to aspartate, the precursor for asparagine synthesis. It is also suggested that GOT participates in a malate/aspartate shuttle between the bacteroids and the cytoplasm of the plant cell (1, 14, 27). In this series of reactions, GOT catalyzes the reverse reaction, namely, the formation of oxaloacetate from aspartate. *In vivo* ^{14}C-labeling studies with alfalfa (*Medicago sativa* L.) nodules support the last option. It was shown that exogenously supplied ^{14}C-aspartate was rapidly metabolized to malate, succinate, and fumarate (26). The conversion of ^{14}C-aspartate to ^{14}C-asparagine was much lower and was stimulated by the addition of amino-oxyacetate, a specific inhibitor of GOT.

To give an answer to the question of the role of the different forms of GOT present in the cytoplasm of root nodule cells, particularly their role in the carbon metabolism related with the formation of oxidizable substrates for the bacteroids and their role in the ammonia assimilation, the kinetic properties of the different forms of GOT were studied.

Furthermore, it was tested whether the proposed malate/aspartate shuttle could operate reversibly between the symbionts and if the shuttle contributes to the transfer of reducing equivalents to the bacteroids linked to nitrogen fixation.

MATERIALS AND METHODS

Growth Conditions of the Plants and Fractionation Procedures

Root nodules were produced under controlled conditions on pea (*Pisum sativum* L.) cv Rondo by inoculation with

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[2] Abbreviations: GOT, glutamate oxaloacetate transaminase; MDH, malate dehydrogenase; pO_2, oxygen partial pressure; K_{eq}, equilibrium constant.

Rhizobium leguminosarum strain PRE as described previously (2). Root nodules were ground gently in a chilled beaker with a pestle with isolation buffer (approximately 1 mL/g fresh material) under argon. The isolation buffer consisted of 50 mM Tes/KOH, 0.46 M sucrose, 50 mM glucose, 5 mM DTT, and 1 mM EDTA, pH 7.4 at 4°C, and was made anaerobic with argon. The crude nodule extract was filtered through two layers of miracloth (Calbiochem), and the filtrate was centrifuged by accelerating the centrifuge up to 3,000*g*. After reaching 3,000*g*, centrifugation was stopped immediately. The supernatant was centrifuged further for 5 min at 5,000*g*. The pellet, containing the bacteroids, was resuspended gently in washing buffer (50 mM Tes/KOH, 0.47 M sucrose, 50 mM glucose, 1% (w/v) BSA (fatty acid-free), pH 7.4, and was washed twice. The bacteroids were suspended in washing buffer at a protein concentration of 6 to 10 mg protein · $mL.^{-1}$. The 5,000*g* supernatant was used to prepare the soluble cytoplasmic plant proteins or mitochondria. The soluble cytoplasmic plant proteins were prepared by 80-min centrifugation at 60,000*g*. The mitochondria were precipitated by 15-min centrifugation at 10,000*g* and washed twice with washing buffer. The soluble proteins from bacteroids, bacteria, uninfected roots, and meristematic tissue were prepared as described earlier (2). The method of Sedmark and Grossberg (25) was used to estimate the protein concentration. BSA was used as standard.

Ion Exchange Chromatography

Desalted samples were analyzed on a Q-Sepharose column (bed volume 16 mL; fast protein liquid chromatography system of Pharmacia) equilibrated with 50 mM Tris/HCl, pH 7.4. Approximately 2% of the protein load capacity of the column was utilized. The proteins were eluted at 2.0 mL/min with a NaCl gradient. The increase in the NaCl concentration was 1 mM/mL. Fractions of 7.0 mL were collected and analyzed for GOT activity. The recovery of GOT activity was 85 ± 10%. Re-runs of the different fractions gave only one peak at exactly the same NaCl concentration, indicating that the different fractions were homogeneous with respect to the GOT activity.

Localization Studies

Nodule extract was prepared as described above. The bacteroids were removed from the homogenate by centrifugation for 5 min at 5,000*g*. One milliliter of the supernatant was applied to a continuous sucrose gradient (23–55% w/v) containing 50 mM Tes/KOH and 5 mM DTT, pH 7.4. The gradient was layered on a sucrose cushion of 80% (w/v). Gradients were centrifuged 5 h at 100,000*g* at 4°C. Fractions (1.5 mL) were collected and sonicated for 20 s with an amplitude of 26 μm and a frequency of 23 kHz in a Soniprep 150 Ultrasonic disintegrator (MSE). After centrifugation, the supernatant was examined for GOT, triose-phosphate isomerase (EC 5.3.1.1), and fumarase (EC 4.2.1.1) activities. Triose-phosphate isomerase was used as a cytoplasmic marker enzyme and fumarase as a mitochondrial marker enzyme.

Enzyme Assays

The nitrogenase activity (EC (1.18.6.1) and MDH (EC 1.1.1.37) activity were measured as described earlier (2). The O_2 uptake rate under conditions of nitrogen fixation was determined as described by Laane *et al.* (16).

Oxaloacetate decarboxylase activity (EC 4.1.1.3) was measured in a reaction mixture containing 50 mM Tes/NaOH, 5 mM $MgCl_2$, 1.5 mM oxaloacetate, 0.1 mM NADH, and 1 unit/mL lactate dehydrogenase (EC 1.1.1.27), final pH 7.5 at 25°C.

Malic enzyme activity (EC 1.1.1.39) was measured in a reaction mixture containing 5 mM malate, 0.5 mM NAD^+ or $NADP^+$, 6 mM $MnCl_2$, 70 mM KCl, and 50 mM Tes/NaOH, final pH 7.4 at 25°C. The reaction was terminated by addition of $HClO_4$, centrifuged, and neutralized, whereupon the reaction mixture was examined for pyruvate by an enzymatic assay with lactate dehydrogenase. Enzyme activity was linear with protein concentration and in time for at least 10 min.

The GOT activity was measured routinely in a MDH-linked reaction system. The incubation mixture contained 50 mM K_2HPO_4, 45 mM L-aspartate, 10 mM 2-oxoglutarate, 0.1 mM NADH, and 7.5 units/mL MDH, final pH 7.4 at 25°C. The rate of disappearance of NADH was monitored at 340 nm before and after addition of substrate. The former rate served as a measurement of background NADH oxidation (if present), which was subtracted from the rate of substrate-dependent activity. Addition of pyridoxal-5′-phosphate to the reaction system did not stimulate the enzyme activity.

The kinetic experiments were performed as described by Henson and Cleland (12). The reaction mixture for measuring the formation of L-glutamate and oxaloacetate contained 50 mM K_2HPO_4, 45 mM L-aspartate, and 10 mM 2-oxoglutarate, final pH 7.4. For the reverse reaction, the incubation mixture consisted of 50 mM K_2HPO_4, 100 mM L-glutamate, and 0.95 mM oxaloacetate, final pH 7.4 at 25°C. The keto tautomer of oxaloacetate is the enzymatically active form of oxaloacetate. The measured reaction rates showed no initial lag; this observation indicates that the enolization rate exceeded the reaction rates under the conditions used. The concentrations of the substrates, particularly 2-oxoglutarate and oxaloacetate, were low enough to prevent substrate inhibition. The product inhibition constants for the keto acids were calculated from the appropriate Haldane relationships (12), using the estimated K_m values, a K_{eq} of 2.51, and the obtained product inhibition constants for glutamate and aspartate. The K_{eq} is expressed considering aspartate and 2-oxoglutarate as products. The substrate concentrations were determined by enzymatic analysis with the appropriate enzymes.

Enzyme Control Analysis

The rate equation derived by Henson and Cleland (12) was used to calculate the initial change in the velocity ($\delta v/\delta S$) caused by infinitesimal changes in the substrate concentration. This was done by calculation of the tangential slope of the plot of v *versus* S at a chosen value of S (13). In the calculations, a physiological concentration of 2.9 mM glutamate and 3.2 mM aspartate was used (5, 19).

Malate/Aspartate Shuttle

The bacteroids were isolated from the nodules as described above. We found that washing the bacteroid preparations twice was enough to remove the last traces of mitochondria, as measured by the *a* of mitochondrial cytochrome *aa*3 at 600 nm. Bacteroids do not contain a cytochrome absorbing around 600 nm. The incubation medium contained 50 mM Tes/KOH, 5 mM $MgSO_4$, 0.2 mM myoglobin, 2.5% (w/v) fatty acid-free BSA, and 16% (w/v) sucrose, pH 6.8 at 30°C. The substrate concentrations were 5 mM. The changes in the external concentrations of the substrates and the nitrogenase activity were estimated during a 20- to 30-min period at 30°C. The incubations were terminated by placing the assay bottles in ice, after which the bacteroids were removed by centrifugation. Malate and 2-oxoglutarate were determined with the appropriate enzyme assay, and the amino acids were determined with a Biotronik LC 6000E analyzer equipped with a Durrum DC 6A ion exchanger (physiological run). The changes in concentrations as well as the nitrogenase activity exhibited a linear relationship with the incubation time. The maximal rate of respiration of the bacteroids catabolizing the different substrates was measured with a Gilson water-jacketed oxygraph equipped with a polarographic Clark electrode at O_2 concentrations above 150 μM.

RESULTS

GOT Activities Present in Root and Root Nodule Cells

The total GOT activity of root nodules per g fresh tissue weight was 11 times larger than the activity found in roots (respectively, 8.8 ± 0.7 against 0.79 ± 0.09 unit/g fresh tissue, $n = 6$). If bacteroids were removed from an extract of root nodule cells by a low-speed centrifugation step, the GOT activity in the cytoplasmic plant fraction of root nodules and in the sonicated bacteroid fraction were in the ratio of 4:1; the ratio of the specific GOT activities was 8:1 (1.15 ± 0.04 against 0.15 ± 0.02 unit/mg protein, $n = 4$). The specific GOT activity in the cytoplasmic plant fraction of root nodules (1.15 ± 0.04 unit/mg protein) was about 2 times higher than the activity found in the cytoplasmic fraction of uninoculated roots (0.65 ± 0.03 unit/mg protein, $n = 6$). For the localization studies, the supernatant obtained after low-speed centrifugation was fractionated with a continuous sucrose density gradient. Nearly 90% of the GOT activity was recovered in the cytoplasmic fractions. The remaining 10% was found mainly in the mitochondrial fractions. Nearly the same distribution of GOT activity between mitochondrial fractions and cytoplasmic fractions was found for extracts of uninoculated roots (not shown).

Table I. *Increase in GOT and Nitrogenase Activities during Pea Nodule Development*

Days after Inoculation	GOT Activity		Nitrogenase Activity
	$\mu mol \cdot min^{-1} \cdot g^{-1}$ fresh wt	$\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein	$nmol\ C_2H_4 \cdot min^{-1} \cdot g^{-1}$ fresh wt
5	0.79 ± 0.04[a]	0.36 ± 0.02	0
11	0.83 ± 0.04	0.36 ± 0.02	0.6 ± 0.06
13	1.35 ± 0.06	0.51 ± 0.03	3.7 ± 0.04
15	2.01 ± 0.10	0.56 ± 0.04	23.3 ± 2.6
18	4.45 ± 0.22	0.92 ± 0.07	55.5 ± 6.6

[a] Each value represents the mean of three replicates ± 1 SE.

GOT of the cytoplasmic fraction of root nodule cells and of the cytoplasmic fraction of cells of primary roots of uninfected plants were separated by ion exchange chromatography. The elution profile of GOT activity in the cytoplasm of root nodule cells is shown in Figure 1. A minor peak eluted at 85 mM NaCl (root nodule fraction I) and accounted for 18% of the activity recovered. The main fraction eluted at 130 mM NaCl; this peak (root nodule fraction II) accounted for 79% of the activity eluted. A fraction with minute activity was observed at 185 mM NaCl (root nodule fraction III). The elution profile of GOT activity in root cytoplasm consisted also of three peaks that eluted at 80, 130, and 185 mM NaCl; the peaks correspond to 71, 21, and 8% of the activity eluted, respectively.

The increase of the GOT nitrogenase activity was followed during nodule development (Table I). Structural analysis of infected roots of pea revealed that, 8 d after inoculation, nodule meristem was developed and that cell differentiation already occurred (10). At d 10, nodule-like structures became

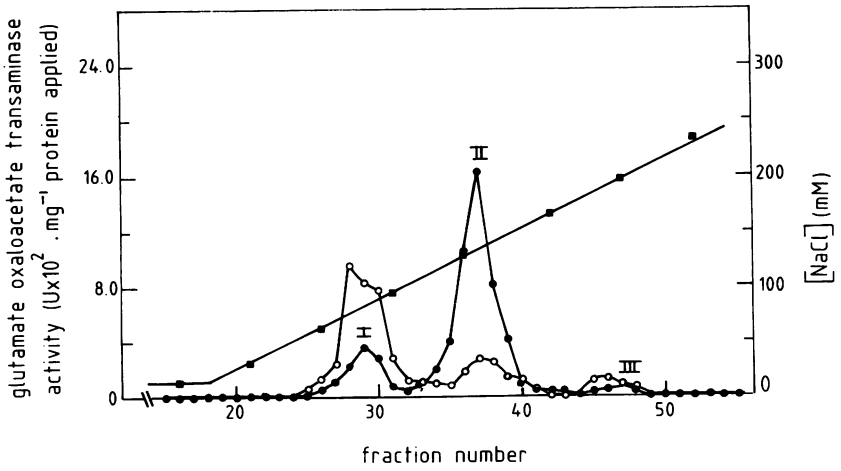

Figure 1. Elution profile of GOT activity from Q-Sepharose of the cytoplasmic plant fraction of pea root nodules harvested 19 d after inoculation (●—●) and of the cytoplasmic fraction of uninoculated roots harvested 19 d after sowing (○—○). One unit of activity (U) is defined as that forming 1 μmol of product min^{-1}. NaCl concentration was determined in the fractions (■—■). Representative of eight determinations.

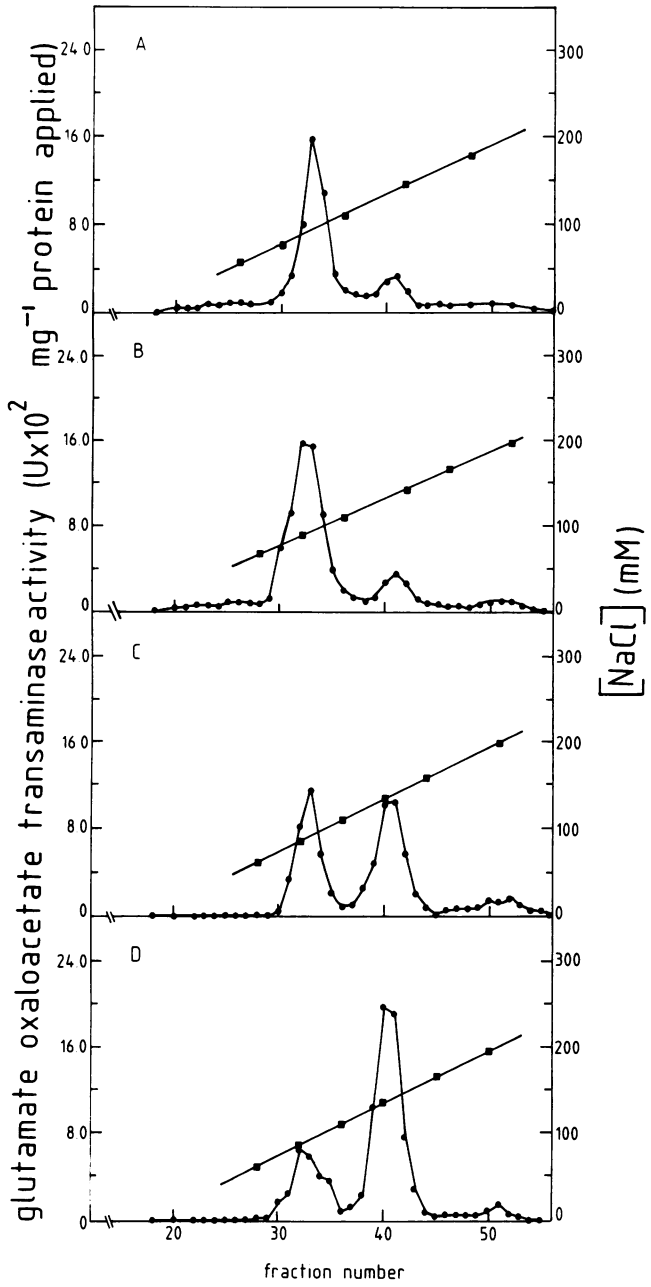

Figure 2. Elution profile of GOT activity from Q-Sepharose of root fragments with developing nodules harvested at different times after inoculation. The root fragments were harvested at 5 d (A), 10 d (B), 13 d (C), and 18 d (D) after inoculation. The root fragments consisted of 2.5-cm pieces of the main roots where nodules normally appear. The root fragments were extracted and fractionated as described for nodules. One unit of activity (U) is defined as that forming 1 μmol of product min^{-1}. NaCl concentration was determined in the fractions (■—■). Representative of three determinations.

Table II. *Kinetic Constants for the Different GOT Fractions*

Kinetic Constant[a]	Root Nodule Fraction I	Root Nodule Fraction II	Root Fraction I
K_a (glutamate)	9.4 (0.6)[b]	27 (3)	7.9 (0.4)
K_b (oxaloacetate)	0.076 (0.003)	0.14 (0.01)	0.019 (0.002)
K_p (2-oxoglutarate)	0.26 (0.01)	0.23 (0.01)	0.21 (0.01)
K_q (aspartate)	1.22 (0.03)	4.9 (0.4)	0.84 (0.03)
K_{ia} (glutamate)	8.3 (1)	24 (3)	6.9 (0.5)
K_{ib} (oxaloacetate)	0.014	0.062	0.0079
K_{ip} (2-oxoglutarate)	0.54	0.60	0.27
K_{iq} (aspartate)	0.54 (0.04)	6.4 (0.4)	0.51 (0.12)
K_{eq}	2.60	2.42	2.51
V_1/V_2[c]	2.42	2.85	1.46
V_1	0.56	2.99	0.70
V_2	0.23	1.05	0.48

[a] The K_m and K_i values are in mM; V_1 and V_2 are expressed in μmol · min^{-1} · (mg cytoplasmic protein)$^{-1}$. [b] The values in parentheses are the SE. [c] V_1 is the maximum velocity for the formation of aspartate and 2-oxoglutarate; V_2 is the maximum velocity in the reverse reaction.

macroscopically visible. Nitrogen fixation was detectable at d 11. Until 11 d after inoculation, no increase in GOT activity was observed in the cytoplasmic fraction of root fragments containing developing nodules. At 13 d after inoculation, there was a steady increase in nitrogen fixation activity and a simultaneous increase in the specific and total GOT activity. The change from the root elution profile to the root nodule profile was followed during nodule development (Fig. 2). At d 5 and 10, the root pattern was observed (Fig. 2, A and B). The change from root to root nodule pattern started at d 13 (Fig. 2C). At that time, the root nodule fraction comprised 49% of the total activity eluted. The activity of root nodule fraction II increased over the subsequent days (Fig. 2D). This was caused by the increased size of the root nodules as compared with roots. Since root fragments were analyzed, the contribution of the root form to the total GOT activity in the extracts prepared at d 18 (Fig. 2D) was more than in isolated root nodules (Fig. 1).

Kinetic Properties of GOT Present in Different Fractions of Root and Root Nodule Cells

The kinetic data, obtained for the different GOT fractions, are consistent with a Ping Pong Bi Bi mechanism. The K_m values for oxaloacetate, glutamate, and aspartate of GOT in root nodule fraction II (0.14, 27, and 4.9 mM, respectively, Table II) were larger than those of the main root fraction (0.019, 7.9, and 0.84 mM, respectively). Only the K_m values for 2-oxoglutarate of the three different fractions were comparable. Of all K_m values, only the K_m and K_i values for oxaloacetate of root nodule fraction I differed from the K_m and K_i values determined for root fraction I.

The equilibrium constant of the reaction catalyzed by the different GOT fractions was calculated from the K_m values and from the ratio of the V_{max} values in both directions (Table II), using the appropriate kinetic Haldane relationship (7). The calculated K_{eq} for the three fractions was 2.5 ± 0.1 when the reaction is considered as the formation of aspartate and

2-oxoglutarate. This observation indicates the internal consistency between the obtained kinetic constants derived from a number of different experiments (7).

The product inhibition by glutamate and aspartate was determined to complete the rate equation. The results of the inhibition experiments showed that glutamate and aspartate act as competitive inhibitors towards each other. Glutamate was a classic noncompetitive inhibitor towards 2-oxoglutarate, whereas aspartate functioned in the same way towards oxaloacetate. These observations are consistent with a Ping Pong Bi Bi mechanism (8). The inhibition constant of the enzyme-glutamate complex of root nodule fraction II (24 ± 3 mM, Table II) was approximately 3 times larger than the values estimated for the root nodule fraction I (8.3 ± 1 mM) and that of the root fraction (6.9 ± 0. 5mM). Also the inhibition constant of the enzyme-aspartate complex of root nodule fraction II was larger than the values obtained for the other two fractions (Table II).

To determine the possible contribution of the different GOT isoenzymes in the GOT activity in root nodule cells, an enzyme control analysis (13) of the GOT isoenzymes was performed. The initial change in the reaction rate caused by an infinitesimal change in a substrate concentration has been determined at physiological concentrations. The concentrations used for glutamate (2.9 mM) and aspartate (3.2 mM) have been measured in root nodules (5, 19). A physiological concentration of oxaloacetate was assumed to be 0.02 mM, which implies that the 2-oxoglutarate concentration has to be 0.0455 mM to achieve equilibrium (K_{eq} = 2.51). The results of the enzyme control analysis are presented in Table III.

The response of root nodule fraction II to changes in the substrate concentration was 5 times larger than the response of root nodule fraction I. The reason for this is that the amount of this enzyme was larger than that of the other GOT fractions. The response of the reaction rate to changes in the concentrations of the amino acids is small when compared with the response to changes in the concentrations of the keto acids.

The Operation of a Malate/Aspartate Shuttle between Bacteroids and Plant Cytoplasm

In mammalian cells, cytoplasmic and mitochondrial GOT function in the malate/aspartate shuttle to transport reducing equivalents from the cytoplasm to the mitochondria. To examine whether a similar shuttle operates between the plant cytoplasm and bacteroids, bacteroids were incubated with different substrates. *In vivo*, bacteroids are enclosed by the peribacteroid membrane, which is a permeability barrier for substrates (28). Isotonically vortexing removes the peribacteroid membrane (28), but this treatment did not stimulate the rate of oxidation of the substrates tested (data not shown). This indicates that the peribacteroid membrane of pea bacteroids contains carriers for malate, aspartate, glutamate, and 2-oxoglutarate. The incubations were performed at different O_2 concentrations to test the effect of the substrates on nitrogen fixation. The optimum O_2 concentration required for maximal nitrogenase activity is dependent on the rate of respiration of the bacteroids with a particular substrate. The larger the rate of respiration, the higher the pO_2 at the maximum of the nitrogenase activity. The optimum pO_2 for nitrogenase activity with glutamate was 40% of that for malate or the combination of malate plus glutamate. The substrates 2-oxoglutarate and aspartate required nearly the same pO_2 for maximal nitrogenase activity. This was about 30% of the pO_2 maximal nitrogenase activity with malate as a substrate. Maximal nitrogenase activity with an endogenous substrate requires a low pO_2 (20% of the malate-driven nitrogenase activity). When the substrate combination 2-oxoglutarate plus aspartate was used, the maximal nitrogenase activity was measured at a higher pO_2. The simultaneous increase in nitrogenase activity was variable from preparation to preparation and varied between 50 and 90% of the malate-driven nitrogenase activity.

The consumption of both malate and glutamate was enhanced by combined addition (Table IV). Only under this condition were aspartate and 2-oxoglutarate formed together with an increase in the alanine concentration. The combined addition of 2-oxoglutarate and aspartate to the incubation medium also stimulated the consumption of both compounds and the production of glutamate, malate, and alanine. If a malate/aspartate shuttle functions between the cytoplasm of the plant cells and the bacteroids, it is necessary that MDH and GOT are present both in the bacteroids and in the cytoplasm of the plant cells. GOT activities were established in the bacteroids (0.15 ± 0.02 unit/mg protein, n = 5) and in the cytoplasm of root nodule cells (1.15 ± 0.04 unit/mg protein, n = 6). MDH activity was estimated to be 3.8 ± 1.7 unit/mg protein in the bacteroids and 31 ± 5 unit/mg protein in the cytoplasm of root nodule cells.

The bacteroids were also analyzed for enzyme activities that catalyze the conversion of malate or oxaloacetate to pyruvate. This activity is necessary for the oxidation of C_4-dicarboxylic acids in the citric acid cycle. With a standard assay, oxaloacetate decarboxylase (EC 4.1.1.3) could not be detected, but malic enzyme activity (EC 1.1.1.39) was established. The specific activity of a bacteroid extract was 197 ± 14 nmol pyruvate·min^{-1}·mg^{-1} protein (n = 3). When malate and NAD^+ were omitted from the reaction mixture, the activity was lower than 2 nmol pyruvate·min^{-1}·mg^{-1} protein. Without Mn^{2+} or K^+, the activity was 88 ± 20 or 52 ± 19 nmol pyruvate·min^{-1}·mg^{-1} protein, respectively. When NAD^+ was replaced by $NADP^+$, the activities were 16% of the activities with NAD^+. The low K_m for malate, namely 0.71 ± 0.12 mM, makes a physiological role for this enzyme possible, as suggested by McKay *et al.* (17).

Table III. *Quantitative Responses of the Reaction Rate to Causative Change in a Substrate Concentration δv/δS under Physiological Conditions*

Response[a]	Root Nodule Fraction I	Root Nodule Fraction II	Root Fraction I
δv/δ (glutamate)	0.0048	0.025	0.0079
δv/δ (oxaloacetate)	0.70	3.6	1.1
δv/δ (2-oxoglutarate)	0.32	1.7	0.52
δv/δ (aspartate)	0.0044	0.023	0.0072

[a] $\delta v/\delta S$ is expressed as 10^{-3}·min^{-1}·(mg protein)$^{-1}$·1.

Table IV. *Changes in the Concentration of Malate, Glutamate, 2-Oxoglutarate, Aspartate, and Alanine in the Incubation Mixture of Bacteroids under Conditions of Nitrogen Fixation*

Consumption and production are assigned to negative and positive, respectively.

Substrate	pO_2 at Optimum of Nitrogenase Activity	Nitrogenase Activity	Consumption/Production of				
			Glutamate	Aspartate	Malate	2-Oxoglutarate	Alanine
	%	*nmol·C_2H_4 formed·min^{-1}·mg^{-1} protein*			*nmol·min^{-1}·mg^{-1} protein*		
Malate	7.9	17.7	0	0	−14.0	0	0
	(0.54)[a]	(3.1)			(3.1)		
Glutamate	3.2	9.3	−2.1	0	0	0	0
	(0.65)	(2.1)	(1.4)				
Malate + glutamate	7.9	18.5	−26.8	+2.1	−22.1	+7.5	+11.0
	(0.65)	(3.2)	(6.0)	(0.7)	(6.8)	(1.1)	(2.1)
2-Oxoglutarate	2.4	7.4	0	0	0	−9.8	0
	(0.32)	(3.1)				(2.9)	
Aspartate	2.7	6.2	+5.3	−2.2	0	0	0
	(0.65)	(2.1)	(1.8)	(1.5)			
2-Oxoglutarate + aspartate	5.6	15	+58	−52	+4.4	−50	+3.2
	(1.45)	(3.5)	(17)		(1.0)	(15)	(0.7)

[a] Each value represents the mean of three replicates; 1 SE in parentheses.

DISCUSSION

The Nodule-Stimulated GOT

The observations reported here indicate that in pea, just as in lupin (20), soybeans (21), and alfalfa (11), there is a nodule-stimulated GOT. This is based on the observation that the GOT activity in the cytoplasm of root nodule cells could be separated by ion exchange chromatography into a minor peak (18%, referred to as root nodule fraction I) and a major peak (79%, referred to as root nodule fraction II, see Fig. 1). Analysis of the cytoplasm of uninfected roots by the same method revealed an elution profile with different proportions of the peaks. The first peak corresponded to 71% of the activity recovered, the second to only 21%. The kinetic properties of the various GOT fractions differ, which is indicative of different (iso)enzymes. The elution profile of GOT activity of root fragments with developing nodules, harvested at different times during nodule development, showed an increasing contribution of root nodule fraction II to the total activity recovered (Fig. 2). The amount of root nodule fraction II remained low until the onset of nitrogen fixation at day 11 (Fig. 2). This fact points to a possible involvement of this enzyme activity in C and N metabolism, linked with nitrogen fixation and not linked with the cell differentiation occurring earlier during nodule development. It is possible that root nodule fraction II is identical with root fraction II. For this reason, the designation "nodule-stimulated" is preferred to "nodule-specific."

The possibility that the presence of cytoplasmic root nodule fraction II is due to bacterial or bacteroid contamination was excluded. At the sucrose concentration used (0.47 M), the osmotic stability of bacteroids and bacteria is preserved. Bacterial and bacteroid GOT were distinguishable from the root nodule fraction II by the elution characteristics from the ion exchange column (data not shown). Furmarase—a marker enzyme for bacteroids and mitochondria—could only be determined in minute quantities in the cytoplasmic nodule fraction. It is therefore concluded that no major part of the bacteroids or bacteria was collapsed during extraction and fractionation of the nodule cells.

It can also be excluded that root nodule fraction II is of meristematic origin. If root nodule fraction II originates from meristematic tissue, the nodule-stimulated form should already be expressed at the beginning of nodule development, which was not the case.

The identity of root nodule fraction I is not clear. As discussed for root nodule fraction II, it is clear that fraction I is not from bacteroid, bacterial, mitochondrial, or plastid origin. Root nodule fraction I and root fraction I are very similar to each other in elution characteristics from the ion exchange column, native molecular mass, and several kinetic properties (Table II). However, both enzymes can be distinguished by the K_m value for oxaloacetate and by a different V_{max} ratio.

Kinetic Properties of GOT Present in Nodule Cytoplasm

The rate of catalysis near equilibrium of the different forms of GOT found in the root and root nodules depends on the intrinsic kinetic properties of the enzyme, on the enzyme concentration, and on the concentrations of substrates, products, and effectors in question. The quantitative responses of the reaction rate to a causative change of a substrate concentration ($\delta v/\delta S$) were calculated at physiological concentrations (Table III). Under V_{max} conditions, root nodule fraction II catalyzes the transaminase reaction in the direction of oxaloacetate and glutamate 5 times faster than root nodule fraction I and 3 times faster than root fraction I. At nonsaturating

substrate concentrations and with all of the four substrates present, the differences in the rates of catalysis between root nodule fraction I, root nodule fraction II, and root fraction I are 1:5.2:1.6, respectively. This indicates that the relatively high K_m values for glutamate, oxaloacetate, and aspartate (Table II) of root nodule fraction II have only a minor effect on the rate of catalysis at physiological, nonsaturating substrate concentrations. Consequently, only the high enzyme concentration of root nodule fraction II is the cause of the relatively large response of the reaction rate to changes in the substrate concentrations.

The Presence of a Malate/Asparate Shuttle

Bacteroids within the infected cells of a legume are enclosed by a plant-encoded membrane, the peribacteroid membrane. This membrane is a possible site of transport regulation. A bacteroid preparation will always contain a mixed population of bacteroids, *i.e.* bacteroids enclosed with the peribacteroid membrane, and intact bacteroids without a peribacteroid membrane. The bacteroid preparations used in this study have been prepared at a high osmotic value (0.47 M sucrose), and pellets were gently homogenized to prevent breakage of the peribacteroid envelopes. Therefore, we expect that, analogous to the *R. leguminosarum* preparations of Brewin *et al.* (6), the majority of our bacteroids are surrounded by an intact peribacteroid membrane. This membrane was removed by vortexing (28). If the peribacteroid membrane is a transport barrier for a particular substrate, one expects a stimulation of uptake and of the rate of oxidation. The rate of oxidation of the different substrates and substrate combinations as presented in Table IV was not stimulated by vortexing the bacteroids. This indicates that the peribacteroid membrane is not a barrier for the substrates tested. In this respect, the permeability of the pea peribacteroid membrane is different from that of soybean, in which vortexing stimulates uptake of glutamate (18), and a removal of the peribacteroid membrane by an osmotic shock stimulates the oxidation of glutamate and 2-oxoglutarate (29). Our observation that pea bacteroids can oxidize glutamate and aspartate confirms the observations of Salminen and Streeter (22) and Bergersen and Turner (4). However, it is not clear whether an intact peribacteroid membrane was present in these studies, because the osmotic value was low during the isolation of the bacteroids.

Experiments with the substrate combinations demonstrate that a malate/aspartate shuttle can operate reversibly between the bacteroids and the cytoplasm of root nodule cells. The experiments also indicate that transport of malate, glutamate, aspartate, and 2-oxoglutarate across the peribacteroid membrane and cytoplasmic membrane is not limiting the rate of oxidation, since the combined addition stimulates the consumption of the substrates involved (Table IV). Both the stoichiometry of the consumption and the formation of the different compounds indicate that uptake and exchange mechanism operate at the same time. It can, therefore, not be excluded that glutamate and aspartate are only transported by an exchange system and not by a uniport system. The same might be true for the transport of 2-oxoglutarate, and thus transport via 2-oxoglutarate malate exchange.

The sums of carbon consumed and produced are not equal. Since bacteroids contain large amounts of poly-β-hydroxybutyrate, the metabolism of which is connected with nitrogen fixation (4, 15), it is possible that, during the incubations, storage materials like poly-β-hydroxybutyrate have been synthesized or degraded. The lack of knowledge of the different pathways operative in the bacteroids makes it difficult to interpret the results quantitatively.

The formation of alanine, which was observed by the addition of the substrate combinations (Table IV), can be explained. Under both conditions (malate/glutamate and aspartate/2-oxoglutarate), glutamate and malate will be present in the bacteroids. In the last case, glutamate and oxaloacetate are formed by the action of GOT and malate by the action of MDH. Pyruvate is formed from malate by the action of malic enzyme, the presence of which has been established in the bacteroids. Glutamate pyruvate transaminase (EC 2.6.1.2) present in the bacteroids (unpublished results) will catalyze the transamination of pyruvate to alanine by glutamate.

A functional malate/aspartate shuttle might be necessary for the aspartate formation. It was shown by Snapp and Vance (26) that [^{14}C]aspartate, when added to root nodules, is rapidly converted to C_4-dicarboxylic acids. This experiment indicates that GOT in root nodules catalyzes the formation of oxaloacetate from aspartate and not the transamination of oxaloacetate to aspartate. To maintain a certain aspartate concentration, other reactions must produce aspartate. This might be the shuttle. Aspartate can be formed inside the bacteroid by the transamination of oxaloacetate by glutamate. Aspartate can be subsequently transported to the cytoplasm of the root nodule cell. In this way, the aspartate concentration in the cytoplasm of root nodule cells can be kept high, despite the high rate of conversion into oxaloacetate. Aspartate can be withdrawn from the shuttle for asparagine biosynthesis, and the carbon lost from the shuttle can be replenished by malate.

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