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Short Communication

Phosphoserine Aminotransferase in Soybean Root Nodules[1]

DEMONSTRATION AND LOCALIZATION

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ABSTRACT

Phosphoserine aminotransferase activity was detected in the plant and bacteroid fractions from soybean (*Glycine max*) root nodules. Both total and specific activities increased in the plant fraction during nodule development. Serine-pyruvate aminotransferase activity was not detectable in the plant or bacteroid fractions of these nodules. Sucrose density gradient fractionation indicated a proplastid localization for phosphoserine aminotransferase. The data presented support a role for this enzyme in carbon supply to purine biosynthesis in the pathway of ureide biogenesis in soybean nodules.

The synthesis of ureides in soybean root nodules requires ammonia assimilatory enzymes, purine biosynthetic and purine oxidative enzymes. As well as having a requirement for assimilated nitrogen, the purine biosynthetic pathway demands a significant carbon input (*i.e.* five carbons for every four nitrogens) in the forms of methenyl·FH_4[3], formyl·FH_4, glycine, and HCO_3^-. Phosphoglycerate dehydrogenase (2) and serine hydroxymethyltransferase (7) have been purified from soybean nodules and their kinetic properties studied. These enzymes have been localized in the proplastid fraction of soybean nodules (1). The presence of these two activities, together with the existence of a phosphoserine pool in ureide-transporting nodules (10), has led to the proposal that the source of carbon for purine biosynthesis is in a pathway via phosphohydroxypyruvate and phosphoserine with the production of glycine and FH_4 from serine (1, 10, 11). This pathway (the 'phosphorylated' pathway) requires the presence of a phosphoserine aminotransferase activity:

Glutamate + phosphohydroxypyruvate ⇌ 2-oxoglutarate + phosphoserine

Another route for serine biosynthesis in plants uses serine-pyruvate aminotransferase in the glycerate pathway (9):

Alanine + hydroxypyruvate ⇌ serine + pyruvate

This paper reports on the activity of these enzymes in soybean root nodules and demonstrates the localization of phosphoserine aminotransferase in these nodules.

MATERIALS AND METHODS

Materials. [3-^{14}C]Serine was obtained from Amersham and 3a20 counting cocktail was purchased from Research Products International, Prospect, IL. All other chemicals and enzyme preparations were obtained from Sigma.

Methods. Soybean (*Glycine max* [L.] Merr. cv Williams) seeds were germinated for 48 h on wet paper towels. The seeds were then planted, radicle down, and inoculated with *Rhizobium japonicum* (3Ib143). The plants were grown in the absence of N and maintained as described previously (12). Nodules were harvested at appropriate times and the bacteroid and plant fraction prepared (14), using an extraction buffer which consisted of 50 mM Tricine (pH 8.0), 0.4 M sucrose, 5 mM DTE, 50 μg ml^{-1} pyridoxal-P. The plant fraction was centrifuged through Sephadex G-25 (8), and aliquots used for enzyme assay. Spinach leaves were extracted and used as a positive control for the detection of SPAT activity. Fresh spinach leaves were homogenized for 25 s in a Waring Blendor in a buffer containing 40 mM Tricine (pH 7.8), 2 mM $MgCl_2$, 1 mM EDTA, 14 mM 2-mercaptoethanol, 1 mM benzamidine, 1 mM Σ-aminocaproic acid, and 5 μm leupeptin (1:1 grinding medium to g fresh weight of leaves). The homogenate was squeezed through a layer of microcloth and four layers of cheesecloth. The final protein concentration was 3.23 mg protein/ml of homogenate after centrifugation.

PSAT (EC 2.6.1.52) was assayed using a method based on that of Walsh and Sallach (16), in which glutamate dehydrogenase is used to determine 2-oxoglutarate production in the reaction. The reaction mixture contained in a volume of 550 μl, 3.2 mM phosphohydroxypyruvate, 20 mM glutamate, 50 μg ml^{-1} pyridoxal-P, 40 mM K-phosphate (pH 7.6), 10 mM NaF, and enzyme solution. Reactions were stopped at various times by the addition of 50 μl of 3 N $HClO_4$. After centrifuging the supernatant was decanted, neutralized by the addition of 100 μl of 2 N KOH, and the insoluble $KHClO_3$ removed by centrifuging. An aliquot (600 μl) was then put into a 1 ml cuvette along with 150 μl of a mixture of 400 mM NH_4Cl and 2 mM NADH, and 200 μl of 1 M K-phosphate (pH 7.6). Lactate dehydrogenase (27 U) was added to remove any hydroxypyruvate present in the reaction mixture as this keto acid can also serve as a substrate for glutamate dehydrogenase. When the absorbance change at 340 nm was

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[3] Abbreviations: methenyl·FH_4, N^5, N^{10}-methenyltetrahydrofolate; formyl·FH_4, N^{10}-formyltetrahydrofolate: SPAT, serine-pyruvate aminotransferase; PSAT, phosphoserine aminotransferase.

zero, the 2-oxoglutarate concentration was determined by the absorbance change on the addition of 25 U of glutamate dehydrogenase. Provided phosphoglycerate dehydrogenase was quantitatively removed from the crude extract using affi-gel blue, the reaction could be stopped using aminooxyacetic acid and the 2-oxoglutarate concentration then determined. However, this method tended to underestimate activity due to some association of PSAT activity with the gel.

SPAT (EC 2.6.1.51) was assayed using a method based on that of Cheung *et al.* (4). The reaction mixture contained in a volume of 500 μl, 60 mM sodium borate (pH 8.2), 50 μg ml^{-1} pyridoxal-P, 7.5 mM alanine, 4.4 mM β-hydroxypyruvate, and enzyme solution. Reactions were stopped at various times by the addition of 50 μl 4.5 N $HClO_4$. Following centrifuging, 125 μl of 1 N KOH was added to a 350 μl aliquot of the supernatant. This was then centrifuged and 300 μl of this supernatant was diluted 1:1 with 1 M K-phosphate (pH 7.6); 500 μl of this was then used to estimate the pyruvate concentration (4).

Phosphoglycerate dehydrogenase (EC 1.1.1.95) was assayed according to Boland and Schubert (2); fumarase (EC 4.2.1.2) was measured by following fumarate production at 240 nm in the presence of 50 mM malate; 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) activity in the bacteroids was measured by 3-hydroxybutyrate-dependent $NADH^+$ reduction (5). Serine hydroxymethyltransferase (EC 2.1.2.1) was assayed by the method of Taylor and Weissbach (15). TCA precipitated protein was resuspended in 0.5 N NaOH and its concentration determined by the method of Bradford (3).

Extracts from 1.6 g of nodules from 21-d-old plants were fractionated on sucrose density gradients as described by Hanks *et al.* (5). The grinding medium contained 0.4 M sucrose, 0.1 M Tricine (pH 7.8), 10 mM KCl, 10 mM EDTA, 1 mM $MgCl_2$, 25 μg ml^{-1} pyridoxal-P, 2.5 mM DTE, and 10 mg ml^{-1} fatty acid-free BSA. The nodule extract (in 5 ml) was layered on a 30 ml linear gradient of 0.75 to 2.0 M sucrose in 20 mM tricine (pH 7.8), 2.5 mM DTE, 25 μg ml^{-1} pyridoxal phosphate over a 1.5 ml bottom layer of 2.3 M sucrose. The gradients were centrifuged in a Beckman model 13-50 centrifuge with a SW 28 rotor at 24,300 rpm (106,609*g* at r_{max}) for 3 h after slow acceleration consisting of 5 min at 5,000 rpm, 10 min at 10,000 rpm, 10 min at 15,000 rpm, and 5 min at 20,000 rpm. The gradient was collected in 1.5 ml fractions by upwards displacement using an Isco gradient fractionator and the density of the fractions determined using a Bausch-Lomb refractometer.

RESULTS AND DISCUSSION

Demonstration of Phosphoserine Aminotransferase Activity in Soybean Nodules. PSAT activity was detected in the plant fraction of soybean root nodules (Table I). The specific activity increased 2.5-fold between 11 and 25 d after inoculation. This increase was similar in magnitude to that already reported for phosphoglycerate dehydrogenase and serine hydroxymethyltransferase (12), the other enzymes in the proposed phosphorylated pathway for glycine synthesis in nodules. PSAT activity also appeared to be associated with the bacteroid fraction of the nodule (Table I), and represented 21% of the total activity present in 18 d-old nodules.

The glycerate pathway enzyme SPAT, was not detected in either the plant or the bacteroid fraction of d 18 nodules, nor was the enzyme demonstrated in the plant fraction of d 11 or d 25 nodules. SPAT is present in the leaves of higher plants (4) and so an extract of spinach leaves was assayed for this activity as a positive control to check the efficacy of the assay system. SPAT activity was detected in spinach leaves at levels comparable to those already reported (4). The data are consistent with there being either none, or extremely low levels, of SPAT in the nodule and serine synthesis occurring via the phosphorylated pathway.

Localization of Phosphoserine Aminotransferase in Soybean Nodules. The gradient centrifugation separated mitochondria, proplastid, and bacteroid enzymes as indicated by the marker enzymes, fumarase, phosphoglycerate dehydrogenase, and 3-hydroxybutyrate dehydrogenase, respectively (Fig. 1). Phosphoglycerate dehydrogenase was used as a marker for the proplastid fraction in preference to triose P isomerase (1). The presence of this activity at the top of the gradient is probably due to plastid breakage.

PSAT activity was located, together with serine hydroxymethyltransferase activity, with the marker for the proplastid fraction (Fig. 1). Both phosphoglycerate dehydrogenase and serine hydroxymethyltransferase have previously been reported to have a proplastid localization (1). The recoveries from the gradient of PSAT (52%) and serine hydroxymethyltransferase (42%) were similar to that of the marker, phosphoglycerate dehydrogenase (46%). SPAT activity was not detectable in any of the gradient fractions.

There is an apparent anomaly between the presence of PSAT activity in the bacteroid fraction prepared by differential centrifugation (Table I) and the absence of an activity peak with the bacteroids isolated by sucrose density gradient centrifugation (Fig. 1). This can be explained by proplastid contamination of bacteroids prepared by differential centrifugation, or by association of the enzyme from lysed proplastids with the peribacteroid membrane. Contamination of bacteroids by a proplastid enzyme has been reported previously with detection of the proplastid isoenzyme of aspartate aminotransferase ($AAT-P_2$) in a bacteroid fraction from lupin nodules (13). Bacteroids prepared from these nodules were shown by EM to be largely enclosed by peribacteroid membranes and to be still contaminated by some proplastids (13). Osmotic shock procedures which removed the peribacteroid membrane, and which would also lyse any contaminating and osmotically sensitive proplastids, removed all traces of the $AAT-P_2$ isoenzyme from this bacteroid fraction (13).

Role for Phosphoserine Aminotransferase in Ureid Biogenesis. A role for PSAT in ureide biogenesis has been suggested by earlier work using both comparative (10) and time course (12) studied. Comparative studies have correlated the presence of a nodule phosphoserine pool with ureide production. Time course studies utilized cabinet-grown soybeans, allowing measurement of enzyme and metabolite changes concomitant with ureide production over a 21 d period. Allantoic acid has been measured in the nodules and stems of such plants following induction of the purine oxidative enzymes involved in ureide production (12);

Table I. *Phosphoserine Aminotransferase Activity in Soybean Nodules*

Plant Age (Time after Inoculation)	Fraction	Phosphoserine Aminotransferase Activity	
d		*nmol min^{-1} mg^{-1} protein*	*nmol min^{-1}*
11	Plant cytosol	5.0	21
18	Plant cytosol	11.7	219
	Bacteroid	8.0	59
25	Plant cytosol	12.3	225

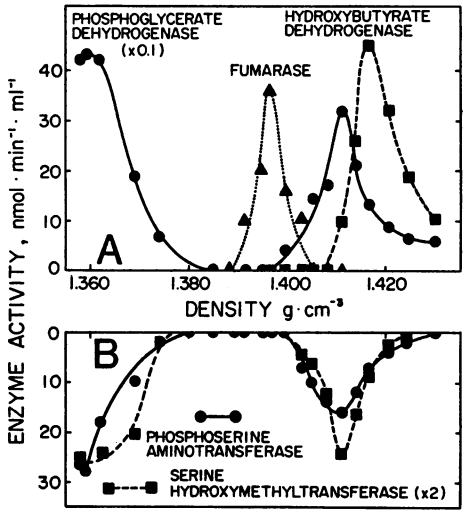

FIG. 1. A, Distribution of marker enzymes for mitochondria, proplastids, and bacteroids of soybean nodules following sucrose density gradient centrifugation. Phosphoglycerate dehydrogenase × 0.1 (proplastid marker) (●); fumerase (mitochondrial marker) (▲); and 3-hydroxybutyrate dehydrogenase (bacteroid marker) (■). B, Phosphoserine aminotransferase (●) and serine hydroxymethyltransferase (■) distribution on the sucrose density gradient.

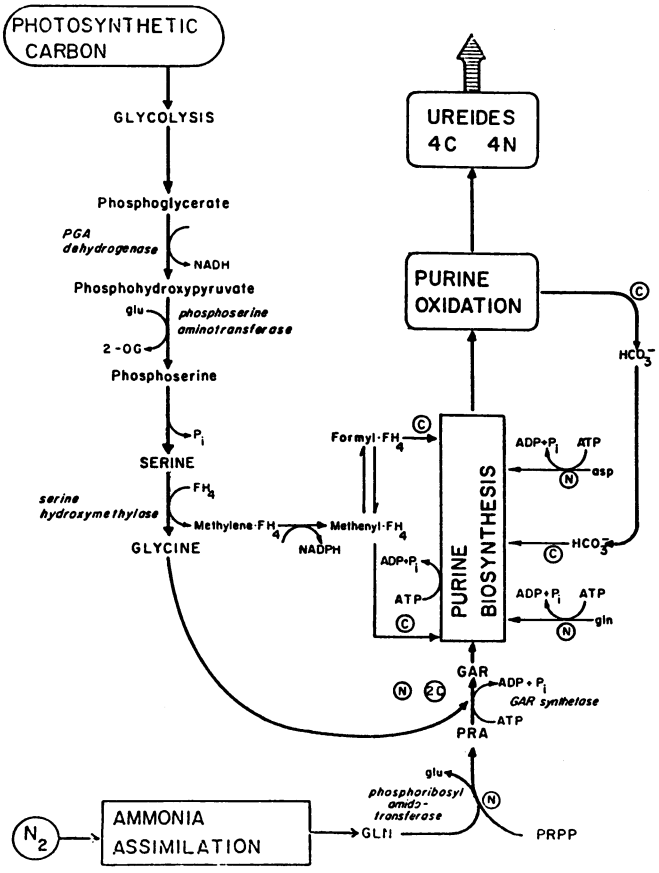

FIG. 2. Scheme showing nitrogen and carbon supply routes to purine biosynthesis in soybean nodules.

these enzyme activities are established by about 17 d after inoculation in this system (see Fig. 2B, Ref. 12).

The demonstration of PSAT activity in nodules at a specific activity similar to other enzymes of ureide biogenesis, including glutamate synthase, 5-phosphoribosylpyrophosphate amidotransferase, and xanthine dehydrogenase (12), together with its localization in the proplastid fraction argue strongly for a role for this enzyme in ureide biogenesis in soybean nodules (Fig. 2), and underlines the importance of the phosphorylated route for serine/glycine production in the nodule.

Enzymes proposed to be involved in carbon supply to purine synthesis (12) include two enzymes of the phosphorylated pathway leading to glycine production—serine hydroxymethyltransferase and phosphoglycerate dehydrogenase. These enzymes fall into a separate group which are not so strongly induced as other enzymes involved in ureide synthesis (see Fig. 2C, Ref. 12). Their levels are already established and may not increase until the glycine and one-carbon demand increase as ureide biogenesis commences (Fig. 2). PSAT falls neatly into this grouping of enzymes, showing a similar pattern of increase to the other activities of the phosphorylated pathway. This further supports a role for PSAT and a phosphorylated route to serine as it fits into the metabolic pattern already established for enzymes involved in carbon supply to purine biosynthesis (12).

The phosphorylated route for serine synthesis is reportedly associated with meristematic or rapidly proliferating plant tissues (4). For example, in pea seeds only PSAT activity is detectable and in the apical meristem the PSAT activity is 50% higher than SPAT activity (4). The developing soybean nodule is certainly a rapidly proliferating tissue and so fits into this model. Because of our inability to demonstrate SPAT activity, it seems unlikely that the glycerate pathway (9) makes a significant contribution to the synthesis of serine and glycine in the soybean root nodule.

The possibility exists that PSAT may have an important regulatory role in this pathway moving carbon from glycolysis to purine biosynthesis. In spinach chloroplasts (6) PSAT is strongly inhibited by serine and shows nonlinear reaction kinetics in the presence of this inhibitor. This is of particular interest because of the high serine levels in soybean nodules (10) and the fact that kinetic studies of phosphoglycerate dehydrogenase (2) and serine hydroxymethyltransferase (7) did not show regulatory properties for these enzymes.

It will be of interest to investigate further the properties of PSAT and of the enzymes of one-carbon metabolism in soybean nodules with a view to understanding the regulation of carbon supply to purine biosynthesis in ureide biogenesis.

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LITERATURE CITED

1. BOLAND MJ, JF HANKS, PHS REYNOLDS, DG BLEVINS, NE TOLBERT, KR SCHUBERT 1982 Subcellular organisation of ureide biogenesis from glycolytic intermediates and ammonia in nitrogen-fixing soybean nodules. Planta 155: 45–51
2. BOLAND MJ, KR SCHUBERT 1983 Phosphoglycerate dehydrogenase from soybean nodules—partial purification and some kinetic properties. Plant Physiol 71: 658–661
3. BRADFORD MM 1976 A rapid and sensitive method for the determination of microgram quantities of protein utilising the principle of protein-dye binding. Anal Biochem 72: 248–254
4. CHEUNG GP, IY ROSENBLAUM, HJ SALLACH 1968 Comparative studies of enzymes related to serine metabolism in higher plants. Plant Physiol 43: 1813–1820
5. HANKS JF, NE TOLBERT, KR SCHUBERT 1981 Localization of enzymes of ureide biosynthesis in peroxisomes and microsomes of nodules. Plant Physiol 68: 65–69
6. LARSSON C, E ALBERTSSON 1979 Enzymes related to serine synthesis in spinach chloroplasts. Physiol Plant 45: 7–10
7. MITCHELL MK, PHS REYNOLDS, DG BLEVINS 1985 Serine hydroxymethyltransferase from soybean root nodules: Purification and kinetic properties. Plant Physiol. In press
8. NEAL MW, JR FLORINI 1973 A rapid method for desalting small volumes of solution. Anal Biochem 55: 328–330
9. RABSON R, NE TOLBERT, PC KEARNEY 1962 Formation of serine and glyceric acid by the glycolate pathway. Arch Biochem Biophys 98: 154–163
10. REYNOLDS PHS, MJ BOLAND, DG BLEVINS, KR SCHUBERT, DD RANDALL

1982 Enzymes of ammonia assimilation in legume nodules: A comparison between ureide- and amide-transporting plants. Physiol Plant 55: 255–260
11. REYNOLDS PHS, MJ BOLAND, DG BLEVINS, DD RANDALL, KR SCHUBERT 1982 Ureide biogenesis in leguminous plants. Trends Biochem Sci 7: 366–368
12. REYNOLDS PHS, MJ BOLAND, DG BLEVINS, KR SCHUBERT, DD RANDALL 1982 Enzymes of amide and ureide biogenesis in developing soybean nodules. Plant Physiol 69: 1334–1338
13. REYNOLDS PHS, KJF FARNDEN 1979 The involvement of aspartate aminotransferases in ammonium assimilation in lupin nodules. Phytochemistry 18: 1625–1630
14. ROBERTSON JG, KJF FARNDEN, MP WARBURTON, JM BANKS 1975 Induction of glutamine synthetase activity during nodule development in lupin. Aust J Plant Physiol 2: 265–272
15. TAYLOR RT, H WEISSBACH 1965 Radioactive assay for serine hydroxymethylase. Anal Biochem 13: 80–84
16. WALSH DA, HJ SALLACH 1966 Comparative studies on the pathways for serine biosynthesis in animal tissues. J Biol Chem 241: 4068–4076