Carbon Dioxide Fixation by Lupin Root Nodules

I. CHARACTERIZATION, ASSOCIATION WITH PHOSPHOENOLPYRUVATE CARBOXYLASE, AND CORRELATION WITH NITROGEN FIXATION DURING NODULE DEVELOPMENT

Received for publication December 7, 1976 and in revised form March 1, 1977

JOHN T. CHRISTELLER, W. A. LAING, AND WILLIAM D. SUTTON
Plant Physiology Division, Department of Scientific and Industrial Research, Palmerston North, New Zealand

ABSTRACT

In vivo CO2 fixation and in vitro phosphoenolpyruvate (PEP) carboxylase levels have been measured in lupin (Lupinus angustifolius L.) root nodules of various ages. Both activities were greater in mature nodule tissue than in either primary or secondary root tissue, and increased about 3-fold with the onset of N2 fixation. PEP carboxylase activity was predominantly in the bacteroid-containing zone of mature nodules, but purified bacteroids contained no activity. Partially purified PEP carboxylases from nodules, roots, and leaves were identical in a number of kinetic parameters. Both in vivo CO2 fixation activity and in vitro PEP carboxylase activity were significantly correlated with nodule acetyl-CoA reduction activity during nodule development. The maximum rate of in vivo CO2 fixation in mature nodules was 7.9 nmol hour−1 mg fresh weight−1, similar to rates of N2 fixation and reported values for amino acid translocation.

The results suggest that the oxaloacetate used as the primary "carbon skeleton" acceptor for ammonia assimilation and amino acid synthesis in lupin nodules is provided via the PEP carboxylase reaction rather than through the tricarboxylic acid cycle. The source of PEP is presumably glycolysis, while the major source of CO2 is inferred to be respiration.

The symbiotic association which develops when legume roots are infected with an appropriate strain of Rhizobium is able to convert atmospheric N2 into ammonia. The ammonia is then assimilated into amino acids which are used for plant growth (1).

The pathway for ammonia assimilation has been elucidated for lupin and other legumes (9, 11, 12).

In lupin, asparagine is the main compound exported from the nitrogen-fixing nodule tissue, together with smaller amounts of aspartate, glutamate, glutamine, and threonine (11, 13). Both asparagine and glutamate can derive their carbon skeletons from oxaloacetate, while glutamate and glutamine can derive their carbon from α-ketoglutarate (9). If it is accepted that oxaloacetate is the carbon skeleton and asparagine the aminated product, then the over-all stoichiometry for nitrogen assimilation in the lupin nodule requires 1 molecule of oxaloacetate to be converted to 1 molecule of asparagine per dinitrogen molecule fixed (13).

Oxaloacetate and α-ketoglutarate are tricarboxylic acid cycle intermediates. However, any consumption of acids of the tricarboxylic acid cycle for ammonia assimilation would ultimately result in a shortage of oxaloacetate, and since oxaloacetate is the acetyl-CoA acceptor, this would lead to a build-up of acetyl-CoA and the input of the tricarboxylic acid cycle would stop (16).

An alternative source of oxaloacetate is therefore needed.

One possible source is the reaction catalyzed by PEP carboxylase (EC 4.1.1.31).

The PEP carboxylase in roots is located in the cytoplasm (4), as are also the root and nodule enzymes for ammonia assimilation (9, 11-13). In addition, it has been shown that PEP carboxylase is present in the root nodules of broad bean, and that bean nodules take up CO2 (8).

In this paper we present results showing that both in vitro PEP carboxylase activity and in vivo CO2 fixation are found in detached lupin root nodules at rates suggesting that PEP carboxylase may be the main source of oxaloacetate for ammonia assimilation.

MATERIALS AND METHODS

Lupinus angustifolius L. cv. Bitter Blue, inoculated with Rhizobium strain NZP 2257, was grown and harvested as previously described (14).

For each experiment, detached nodules from at least three plants were mixed and divided into a sufficient number of samples of 50 to 200 mg fresh wt for separate duplicate measurements of acetyl-CoA reduction and PEP carboxylase activity and quadruplicate measurements of CO2 fixation.

Acetylene reduction activity was assayed as before (14).

In vivo CO2 fixation was measured by incubating detached nodules at 25°C in a 25-ml Erlenmeyer flask containing a small piece of moist filter paper. The assay was initiated by the injection of 10 μl of 14CO2 (Radiochemical Centre, Amersham; specific activity 5-10 Ci/mol) through the serum cap, and stopped by adding 1 ml of 1 M HCl and placing the samples on ice. The samples were then ground in a glass homogenizer, aliquots dried at 90°C, and acid-stable radioactivity determined by scintillation counting as previously described (3). CO2 fixation/mg fresh wt of nodule, calculated as incorporated acid-stable radioactivity/mg divided by the measured specific radioactivity of the CO2 surrounding the nodules during the assay, was linear with time for at least 30 min.

PEP carboxylase activity was assayed using aliquots of a crude homogenate prepared by grinding nodules in at least 20 vol of 50 mM tris-Cl, 10 mM MgCl2, 5 mM dithiothreitol, pH 8. The assay medium contained 50 mM tris-Cl, 10 mM MgCl2, 5 mM dithiothreitol, 1 mM NADH, 10 mM NaH14CO3, 2 mM PEP and excess malic dehydrogenase, pH 8. The reaction, in 0.5 ml total volume, was initiated with extract (10-25 μl) and terminated after 5 min with 0.1 ml 1 M HCl. The mixture was dried at 90°C and acid-stable radioactivity determined as above.

Ribulose bisphosphate carboxylase (EC 4.1.1.39), PEP carboxykinase (EC 4.1.1.49), and carbonic anhydrase (EC 4.2.1.1) were extracted and assayed as described by Bowes et al. (2), Hatch (7), and Rickli (10), respectively.

1 Abbreviation: PEP, phosphoenolpyruvate.
purified bacteroids were obtained by sucrose gradient centrifugation using the method of Sutton and Mahoney (personal communication). Nodule tissue was homogenized in 10 volumes of ice-cold 0.5 M mannitol, 50 mM tris-HCl (pH 7.4) and filtered through two layers of Miracloth. A 1-ml aliquot of the filtered homogenate was layered over a 20-ml gradient at 12 to 30% (w/v) sucrose in 50 mM tris-HCl (pH 7.4) and centrifuged for 15 min at 2,500 rpm in a swing-out rotor at 5°C. The red, leghemoglobin-containing supernatant layer was separated from the opaque, bacteroid-containing zone about halfway down the tube by puncturing the tube with a hot needle and collecting 1-ml fractions from the bottom.

Purification of PEP carboxylase was performed as described by Ting and Osmond (15).

Respiration rates in detached nodules were estimated by IR measurements of the CO₂ produced in a closed system. All nodule activities were expressed as nmol hr⁻¹ mg fresh wt⁻¹.

RESULTS

When mature nodules were assayed for in vitro activity of various carboxylases, no ribulose bisphosphate carboxylase or PEP carboxykinase activities were detected, but high levels of PEP carboxylase were found. We then investigated the patterns of development of this enzyme and in vivo CO₂ fixation in relation to the induction of acetylene reduction activity in developing nodules.

The previously reported pattern of induction of acetylene reduction activity (14) was confirmed (Fig. 1A). The activity increased rapidly over days 12 to 14 after inoculation to reach a maximum rate of 15 nmol hr⁻¹ mg fresh wt⁻¹. The corresponding patterns of development of in vivo CO₂ fixation and in vitro PEP carboxylase are shown in Figure 1B. Both activities increased over the same period as observed for acetylene reduction. However, while the in vitro PEP carboxylase activity increased from a minimum of 80 nmol hr⁻¹ mg fresh wt⁻¹ to a maximum of 300 nmol hr⁻¹ mg fresh wt⁻¹, in vivo CO₂ fixation activity had corresponding rates of only 0.6 to 1.6 nmol hr⁻¹ mg⁻¹. Thus, the rate of PEP carboxylase activity in vitro was much greater than nodule acetylene reduction activity, while the in vivo CO₂ fixation rate was much smaller.

The acetylene reduction activity of day 10 nodules was nearly zero, but there was significant PEP carboxylase and CO₂ fixation. We measured the in vitro PEP carboxylase activity in primary and secondary root tissues, which have no acetylene reduction activity, and found rates ranging from 10 to 71 nmol hr⁻¹ mg fresh wt⁻¹ (Table I). We inferred that the PEP carboxylase and CO₂ fixation activity of young nodules may be a reflection of their root cortical origin.

We carried out a series of experiments on localization of PEP carboxylase, in which the cortex and bacteroid-containing regions of nodules were separated by dissection and then were homogenized and assayed separately. Cortex tissue had activity comparable with that of secondary roots, while the activity of the bacteroid-containing tissue was 4- to 6-fold greater (Table II). However, when bacteroids were purified from nodule homogenates by sucrose gradient sedimentation, they had no detectable PEP carboxylase activity either before or after sonication. Most of the applied activity was recovered in the plant cytoplasmic fractions at the top of the gradient (Table II).

It has been reported that PEP carboxylase enzymes from different plant organs have different in vitro properties consistent with different mechanisms for regulating PEP carboxylase activity in vivo (15). In an attempt to determine whether the lupin nodule PEP carboxylase is regulated by the same mechanisms as the lupin root and leaf PEP carboxylases, we purified all three enzymes and compared some of their properties in vitro. The results (Table III) showed no substantial differences between the enzymes from the three tissues, other than in a previously unreported ability of Ba²⁺ to supply the cation cofactor requirement, which has not been investigated in detail.

Figure 2 shows that throughout the nodule development period there were strong correlations between in vitro PEP carboxylase activity and nodule acetylene reduction activity (r = 0.93) and between acetylene reduction and in vivo CO₂ fixation (r = 0.88). Although these correlations, together with the localization of PEP carboxylase, were consistent with a role for PEP carboxylase-mediated CO₂ fixation in ammonia assimilation, our measured CO₂ fixation rates were insufficient for the predicted stoichiometry. We investigated the rate of CO₂ fixation as a function of external CO₂ concentration around the nodule, using both immature (10 and 11 day) and mature (20 and 21 day) nodules (Fig. 3). The data show that differences in in vivo CO₂ fixation between young and old tissue persisted over all CO₂ concentrations used. Whereas the standard assays were performed in 0.333% CO₂, the concentrations necessary to obtain half-maximal rates were 0.48% CO₂ for young nodules and 1.1% CO₂ for old nodules. The maximal rates at saturating CO₂ calculated by a least squares analysis of Lineweaver-Burk plots.

![FIG. 1. Development of acetylene reduction activity, in vitro PEP carboxylase activity, and in vivo CO₂ fixation. Each point represents the mean of 8 to 16 separate determinations using nodule samples obtained over a period of months from a succession of plantings. The bars on the points represent ± 1 SEM. The curves were fitted by eye.](image-url)

<table>
<thead>
<tr>
<th>Table I. Distribution of PEP carboxylase in Lupin nodules.</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Age of nodule</td>
<td>1st root</td>
</tr>
<tr>
<td>Fresh CO₂ fixation, nmol hr⁻¹ mg fresh wt⁻¹</td>
<td>46</td>
</tr>
<tr>
<td>PEP carboxylase activity, nmol hr⁻¹ mg fresh wt⁻¹</td>
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Table II. Distribution of PEP carboxylase in nodules

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fraction</th>
<th>10/11</th>
<th>20/21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td></td>
<td>101</td>
<td>-</td>
</tr>
<tr>
<td>Mixed cortex</td>
<td></td>
<td>44</td>
<td>76</td>
</tr>
<tr>
<td>Mixed, bacteroid-free</td>
<td></td>
<td>309</td>
<td>-</td>
</tr>
<tr>
<td>Mixed, bacteroid-rich</td>
<td></td>
<td>238</td>
<td>446</td>
</tr>
<tr>
<td>Nodule</td>
<td></td>
<td>372</td>
<td>-</td>
</tr>
</tbody>
</table>

To determine the Km(CO2) values calculated for partially purified PEP carboxylase were less than the equivalent of 100 μl/l CO2 (0.01%) in the gas phase above the reaction mixture (Table III). Thus, PEP carboxylase in the nodules should be saturated easily by CO2 even in air levels (0.03%). Since our data show a response of in vivo CO2 fixation to CO2 concentration up to 2 to 4% CO2, it appears that diffusion of 14CO2 into the nodule tissue must strongly limit the rates of observed CO2 fixation (see Appendix).

Carbonic anhydrase has been postulated to facilitate diffusive transport across tissue (5). The relative activities of this enzyme were determined in vitro using homogenates of mature and immature nodules, and it was found that mature nodules had a 12.5-fold greater carbonic anhydrase activity/unit fresh wt than immature nodules. Sutton and Jepsen (personal communication) have explored the induction of nodule carbonic anhydrase in more detail, with similar results. Since immature nodules were saturated with CO2 at a lower external CO2 concentration than mature nodules (Fig. 3), a cursory examination of our results does not support a role for the high levels of carbonic anhydrase in mature nodules in facilitating diffusive transport. However, this analysis is complicated as lupin nodules, which are roughly spherical, have a 3- to 4-fold greater volume at 20/21 days than at 10/11 days. Consequently, diffusion pathways would be longer in the older nodules, and the surface area to volume ratio would be smaller.

The measured respiratory CO2 production of 10/11 day nodules was 160 ± 28 nmol hr⁻¹ mg fresh wt⁻¹ (mean ± se from nine samples), while that of day 20/21 nodules was 64 ± 5 nmol hr⁻¹ mg fresh wt⁻¹ (mean ± se from 13 samples). These rates were considerably higher than the maximal observed rates of in vivo CO2 fixation.

DISCUSSION

If 1 molecule of oxaloacetate is converted to 1 molecule of asparagine for each molecule of dinitrogen fixed, and if PEP carboxylase provides the oxaloacetate, then 1 CO2 molecule should be fixed per dinitrogen fixed.

Our data allow an indirect estimate of the true rate of CO2 fixation in nodule tissue. We assume that the very high rates of respiratory CO2 release significantly dilute radioactive CO2 inside the nodule and that the internal specific activity approaches that outside the nodule only at very high external CO2 concentrations. The maximal rates at saturating external CO2 estimated in Figure 3 should therefore approximate the true in vivo CO2 fixation rates (see Appendix). The values were 1.1 and 7.9 nmol hr⁻¹ mg fresh wt⁻¹ for 10/11- and 20/21-day-old nodules, respectively. The maximum observed rate of acetate reduction was
15 nmol hr\(^{-1}\) mg fresh wt\(^{-1}\) in 20/21-day-old nodules. Assuming a ratio of 3 acetylene to 1 \(N_2\) in fixation (6), we obtain 5 nmol hr\(^{-1}\) mg fresh wt\(^{-1}\) for nodule \(N_2\) fixation. This value is in reasonable agreement with published rates of asparagine transport from nodulated lupin roots (13), which give a calculated value for asparagine synthesis in nodules of 8 nmol hr\(^{-1}\) mg fresh wt\(^{-1}\) (K. J. F. Farnden, personal communication). Both values fall very close to the maximal rate of \(CO_2\) fixation in similar tissue. We conclude that if the observed \(CO_2\) fixation is by PEP carboxylase, it is adequate to provide all of the oxaloacetate needed for ammonia assimilation.

The significant correlations of \textit{in vivo} \(CO_2\) fixation activity and \textit{in vitro} PEP carboxylase activity with acetylene reduction activity during nodule development further strengthen the hypothesis that the processes are linked.

Our analysis does not imply that internal \(CO_2\) concentrations in nodule tissue under physiological conditions must be high to sustain high rates of \(CO_2\) fixation, since PEP carboxylase saturates \textit{in vitro} at very low levels of \(CO_2\). The high rates of respiration measured suggest that the internal \(CO_2\) concentration is probably high and that respiration is normally the major source of the \(CO_2\) fixed into oxaloacetate by PEP carboxylase. High carbonic anhydrase levels do not seem to be needed in order to facilitate the transport of \(CO_2\) from the outside air to the site of \(CO_2\) fixation.

The measured rates of PEP carboxylase activity \textit{in vitro} were at all times considerably greater than the rates of \textit{in vivo} \(CO_2\) fixation, even at high external concentrations of \(CO_2\). This difference may reflect limitations on PEP carboxylase by low concentrations of PEP in the nodule, or it may be due to regulatory mechanisms controlling the relative rates of PEP carboxylase and pyruvate kinase.

**APPENDIX**

A simple model has been derived relating the \textit{in vivo} \(CO_2\) uptake of nodules as measured by radioactive \(CO_2\) to the external \(CO_2\) concentration. The model assumes that the nodules are in steady-state and homogeneous with respect to over-all metabolism of \(CO_2\), that no radioactive \(CO_2\) is respired during the 5-min period of the assay, that PEP carboxylase is saturated with \(CO_2\), and that \(CO_2\) must pass across a uniform diffusive barrier to enter and leave the nodules. A scheme of the model is shown:

\[
\begin{align*}
\text{nodule tissue} & \quad \text{atmosphere} \\
C_{i} & \quad \text{P} & \quad r & \quad C_{b} \\
C_{i}^{*} & \quad \text{P}^{*} & \quad r & \quad C_{b}^{*}
\end{align*}
\]

\(C_i\) is the external atmospheric concentration of \(^{14}CO_2\), \(C_i\) the internal \(^{14}CO_2\) concentration, and \(C_i^{*}\) and \(C_{b}^{*}\) are the respective radioactive \(^{14}CO_2\) concentrations. \(R\) is the rate of respiration, \(P\) is the rate of \(^{14}CO_2\) fixation, \(P^{*}\) the rate of \(^{14}CO_2\) fixation, and \(r\) the diffusive resistance to \(CO_2\) transport.

The following equations describe the model:

\[
\begin{align*}
(C_i - C_{i}^{*})/r + P = R \\
(C_i^{*} - C_{i})/r + P^{*} = 0
\end{align*}
\]

and \(P^{*} = PC_{i}^{*}/C_{i}\).

The rates of \(CO_2\) fixation in Figure 3 are calculated as \(P^{*}C_{i}/C_{i}^{*}\) which we represent by \(F\).

It can be shown:

\[
F = C_{i}P/(rR + C_{b})
\]

Equation 3 predicts the hyperbolic form shown by the data of Figure 3. Taking internal respiration, the sum of external respiration and \(CO_2\) fixation, to be 161 nmol hr\(^{-1}\) mg fresh wt\(^{-1}\) on days 10/11 and 72 nmol hr\(^{-1}\) mg fresh wt\(^{-1}\) on days 20/21, we calculate that on days 10/11, \(r\) was \(3.0 \times 10^{-3}\) (\%CO\(_2\) mg hr\(^{-1}\) nmol\(^{-1}\)), while on days 20/21 \(r\) was 15.3 \(\times 10^{-3}\).

Acknowledgments. — We wish to thank P. Mahoney for growing the plants and N. Lourie for excellent technical assistance.

**LITERATURE CITED**