Anapleurotic CO₂ Fixation by Phosphoenolpyruvate Carboxylase in C₃ Plants

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ABSTRACT
The role of phosphoenolpyruvate carboxylase in photosynthesis in the C₃ plant Nicotiana tabacum has been probed by measurement of the ¹³C content of various materials. Whole leaf and purified ribulose bisphosphate carboxylase are within the range expected for C₃ plants. Aspartic acid purified following acid hydrolysis of this ribulose bisphosphate carboxylase is enriched in ¹³C compared to whole protein. Carbons 1–3 of this aspartic acid are in the normal C₄ range, but carbon-4 (obtained by treatment of the aspartic acid with aspartate β-decarboxylase) has an isotopic composition in the range expected for products of C₄ photosynthesis (<5‰), and it appears that more than half of the aspartic acid is synthesized by phosphoenolpyruvate carboxylase using atmospheric CO₂/HCO₃⁻. Thus, a primary role of phosphoenolpyruvate carboxylase in C₃ plants appears to be the anapleurotic synthesis of four-carbon acids.

Measurements of the ¹³C contents of natural materials have been important in understanding the dynamics of CO₂ fixation in plants (12, 22, 23). Initial studies focused on the distinction between C₃ and C₄ plants, but subsequent studies have revealed a wealth of detail with regard to relative rates of carboxylation, stomatal diffusion, CO₂ hydration, and other steps (2, 7, 13).

For isotopic analysis, materials are converted to CO₂ by combustion or chemical or enzymatic degradation prior to measurement of the isotope ratio R, defined by

\[ R = \frac{^{12}CO_2}{^{13}CO_2} \]  

For convenience, R values are ordinarily converted to values of δ¹³C, defined by

\[ \delta^{13}C (‰) = \left( \frac{R[sample]}{R[standard]} - 1 \right) \times 1000 \]  

The standard (δ¹³C = 0) is CO₂ obtained from PDB limestone (1). Analysis of whole leaf carbon gives a clear distinction between C₃ plants (δ¹³C near −28‰) and C₄ plants (δ¹³C near −14‰), with virtually no overlap of the two distributions. Theories are now available for explaining why these distributions are different (5, 12, 16). The principal factors are the isotopic fractionations associated with stomatal diffusion (4.4‰; Ref. 12), RuBP carboxylase (29‰; Ref. 18), and PEP carboxylase (2‰; Ref. 14).

Combustion-based studies give only an integrated, overall view of carbon metabolism and provide only limited information with regard to specific metabolic pathways. Recently, studies of metabolites have begun to provide more detailed insights into certain pathways. Lipids are unique among metabolites in being significantly depleted in ¹³C compared to whole leaf carbon (3, 12) and an explanation in terms of the mechanism of fatty acid synthesis has been given (3). The isotopic composition of malate in CAM plants has been used to provide details of environmental effects on nocturnal CO₂ uptake (2, 7, 13).

All plants contain both enzymes of CO₂ fixation, RuBP carboxylase and PEP carboxylase, but the proportions differ with different photosynthetic pathways. In C₄ plants, primary CO₂ uptake is catalyzed by PEP carboxylase, which concentrations CO₂ to improve the efficiency of RuBP carboxylase (4, 8, 17). In this case, the ratio of the two enzymes is near 1:1 (9). C₃ plants, on the other hand, contain a large excess (typically 15:1) of RuBP carboxylase over PEP carboxylase. The role of the latter enzyme in C₃ plants is uncertain. Suggestions have included a role in recapturing respiratory carbon, in synthesizing four-carbon acids (principally malate and aspartate), as well as other possible functions (9).

An attractive possibility for the role of PEP carboxylase in C₃ plants is that it provides four-carbon skeletons for synthesis of aspartate, malate, and perhaps other compounds, as follows.

\[ CO_2 \xrightarrow{\text{carbonic anhydrase}} \text{HCO}_3^- \xrightarrow{\text{PEP carboxylase}} \text{oxalacetate} \]  
\[ \text{Oxalacetate} \xrightarrow{\text{malate dehydrogenase}} \text{malate} \]  
\[ \text{Oxalacetate} \xrightarrow{\text{aspartate aminotransferase}} \text{aspartate} \]  

In such a scheme, PEP would presumably be synthesized from products of the C₃ photosynthetic pathway, and only carbon-4 of malate or aspartate would come from the atmosphere via the PEP carboxylase pathway. CO₂ for carbon 4 would be derived from the same CO₂/HCO₃⁻ pool as is used by RuBP carboxylase (although RuBP carboxylase is in the chloroplast and PEP carboxylase is at least partially in the cytoplasm, there is rapid transport of CO₂ back and forth between the two locations, so it is adequate to consider all CO₂ to be in the same pool).

Even if this scheme is qualitatively correct, we do not know at present what fraction of four-carbon acids might be so derived, nor do we know the extent to which acids derived from this pathway would be in chemical or isotopic equilibrium with products derived from the C₃ pathway. However, insofar as this pathway functions as a source of aspartate or malate, carbon-4 of these materials should be isotopically different from carbons 1–3 because of the difference in isotope fractionations between

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2 Abbreviations: RuBP, ribulose bisphosphate, PEP, phosphoenolpyruvate.
the C3 and C4 photosynthetic pathways. In the limit where all carbon-4 of aspartate is derived via this route and isotope exchange with other metabolites is slow, we would expect carbons 1-3 of aspartate (presumably derived from glycolysis) to be in the usual range for metabolites from C3 plants (near -28‰; [12, 22, 23]), whereas carbon-4 would be expected to be in the range for metabolites from C4 plants (near -14‰; [12, 22, 23]), or even more positive (vide infra).

Because of the many roles of malic acid in metabolism, and because of the likely randomization of carbon-1 and carbon-4 of malate by fumarase, it is unlikely that an unambiguous isotopic signal can be obtained by studying malate. Instead, we chose to look at aspartic acid, which is kinetically more remote from metabolism (it communicates with malate via aspartate aminotransferase and malate dehydrogenase) and thus has a better chance of preserving an isotopic signal from any contribution of PEP carboxylase. Rather than trying to look at the small and probably dynamic pool of free aspartic acid, we chose to look at a stable form of aspartate, namely aspartate in proteins. To provide a reproducible source of material and to facilitate comparison among species, we focused our attention on the aspartate (and asparagine) in RuBP carboxylase. This enzyme constitutes 25 to 50% of soluble protein in the leaves of C3 plants and can easily be obtained pure in large quantities (10). We report here that the isotopic content of aspartic acid indicates a significant contribution to aspartic acid synthesis by anapleurotic CO2 fixation by PEP carboxylase in the C3 plant, tabacco.

MATERIALS AND METHODS

L-Aspartic acid β-carboxylase (specific activity 31 units/mg) was from Alcaligenes faecalis (19). RuBP carboxylase, purified from leaves of Nicotiana tabacum, was provided by Dr. Richard Jensen. Glutamic-oxalacetic transaminase and malate dehydrogenase were obtained from Sigma Chemical Co.

The concentration of aspartic acid was determined by a coupled enzyme method using glutamic-oxalacetic transaminase and malate dehydrogenase (19). Isozyme ratio measurements were made on a Finnigan Delta-E isotope-ratio mass spectrometer and are given relative to the usual PDB standard (1). Combustions were conducted in sealed tubes (2). Hydrolysis of protein (40-60 mg per experiment) was carried out with 6 N HCl for 40 min at 155°C. Samples were dried on a rotary evaporator. Yields of aspartic acid were quantitative.

Aspartic acid was separated from acid hydrolysates by ion-exchange chromatography. The concentrated protein hydrolysate was redissolved in 1 ml H2O and freed from chloride ions by a small column of Dowex 50-X8 (1.1 × 9 cm, 200-400 mesh, H+ form). After washing with 40 ml of H2O, the amino acids were eluted with 1 M NH3 and concentrated to 2 ml. This solution was loaded on an anion exchange column (Bio-Rad AG-1-X8, 1.6 × 30 cm, 100-200 mesh, formate form) and the column was eluted with 250 ml of a gradient from 0.05 to 1 N HCOOH at a flow rate of 50 ml/h. Amino acids were detected with ninhydrin. Aspartic acid eluted as the last amino acid after about 130 ml in a volume of 28 to 32 ml. The combined aspartic acid-containing fractions were brought to dryness on a rotary evaporator. The aspartic acid was redissolved and dried several times to remove traces of HCOOH. Control experiments demonstrated that the aspartic acid so obtained is pure and that aspartic acid subjected to this procedure maintains its isotopic integrity.

Aspartic acid was decarboxylated with aspartate β-carboxylase (19). To eliminate any isotope fractionation in the decarboxylation, all reactions were carried to 100% decarboxylation. The isotopic fidelity of this procedure has previously been demonstrated (19), and this was confirmed in the present study.

RESULTS

We have obtained pure RuBP carboxylase from Nicotiana tabacum and have measured the following isotopic compositions: (a) whole leaf (measured by combustion); (b) pure RuBP carboxylase protein (measured by combustion); (c) pure aspartic acid (i.e. carbons 1-4 measured by combustion following acid hydrolysis of the carboxylase and ion-exchange chromatography); (d) carbon-4 of aspartic acid (measured by action of aspartate β-decarboxylase on the aspartate [c] above); and (e) carbons 1-3 of aspartic acid (by calculation from [c] and [d] above). All measurement techniques have been thoroughly checked. The results of three to five analyses of each type are shown in Figure 1.

The isotopic composition of the whole leaf (δ13C = -27.4‰) is in the range usually observed for C3 plants (12, 22, 23). The value for RuBP carboxylase protein (-23.0 ± 0.2‰) is slightly more positive. Aspartic acid from RuBP carboxylase (-17.6 ± 0.3‰) is significantly more positive than whole protein, but carbons 1-3 (-21.8 ± 0.4‰) are not significantly different from whole protein. Carbon-4 of aspartic acid (-5.1 ± 0.3‰) is very positive.

DISCUSSION

The isotopic compositions of leaves and of various fractions and metabolites from C3 plants have isotopic contents averaging near -28‰, with most values falling within ±5‰ of that value. Leaves and various other materials in C4 plants average near -14‰. Carbon-4 of aspartic acid is at the positive end of the range expected if aspartate is synthesized principally by the action of PEP carboxylase. However, as we will discuss below, because of diffusional and other factors, the isotopic content of C4 plants does not provide an adequate basis for predicting the isotopic content of carbon-4 of aspartic acid if this material is synthesized by PEP carboxylase in C3 plants.

Before we conclude that PEP carboxylase is responsible for the abnormal isotopic content of aspartic acid, several other possibilities must be eliminated: (a) There is a large equilibrium isotope effect between aspartate and other metabolites. This is not possible because the equilibrium isotope effect required (more than 15‰) is larger than is reasonable. (b) Aspartate is synthesized via the C3 pathway with a large kinetic isotope effect. This is not possible because such an isotope effect would tend to deplete aspartate in 13C, rather than enrich it. (c) Aspartate and malate are in isotopic equilibrium and carbon-4 of these materials is becoming enriched in 13C because of the action of malic enzyme. A large isotope effect can be observed for malic enzyme provided that CO2 is removed as rapidly as it is formed (6). However, in the present case, we would expect the malic enzyme reaction to operate near equilibrium, and the isotope fractionation should be small. Furthermore, if this explanation were correct, then the isotopic composition of carbon-4 of malic acid should be about the same as that of carbon-4 of aspartic acid, and this is not the case (E Melzer, MH O'Leary, unpublished data). Other reactions of malate are not expected to show a large

![FIG. 1. δ13C values of various fractions from Nicotiana tabacum.](image-url)
isotope fractionation at carbon-4.

What $\delta^{13}C$ is expected for carbon-4 of aspartate synthesized via PEP carboxylase in a C4 plant? In C3 plants, the answer is near $-11\%$, but in that case stomatal diffusion is principally limiting and the $\delta^{13}C$ value of the internal CO2 pool is near $-11\%$. This is not so in C4 plants. Instead, carboxylation and diffusion are jointly limiting and PEP carboxylase is drawing off only a minor fraction of the same CO2/HCO3- pool used by RuBP carboxylase. The $\delta^{13}C$ value for the internal CO2 pool in a C4 plant can be estimated from previous models1 to be about $+1\%$. This material is more positive than air because of the isotope fractionation associated with RuBP carboxylase. Synthesis of aspartate involves three steps (a) Conversion of CO2 to HCO3-, which will shift $\delta^{13}C$ to a more positive value4 by $9\%$ (11); (b) absorption of HCO3- by PEP carboxylase, which shows an isotope fractionation of $2\%$ (14); (c) transamination to aspartate, which should involve no further isotope fractionation.

Thus, we estimate that carboxyl carbons so introduced will be about $7\%$ more positive than internal CO2 or near $+8\%$. The observed value, $-5\%$, thus indicates that a major source of aspartic acid in this plant is anaplerotic CO2 fixation by PEP carboxylase. If the above estimates are correct, then slightly more than half of aspartate is synthesized by this pathway. The remaining aspartic acid is presumably synthesized from products of the C3 photosynthetic pathway without participation of PEP carboxylase. It has been suggested (9) that PEP carboxylase functions to recapture respirated carbon in C3 plants. Respirated CO2 should have a $\delta^{13}C$ value near that of the leaf and should lead to aspartate having a $\delta^{13}C$ value for carbon-4 of $-15$ to $-20\%$. Thus, we cannot eliminate the possibility that some of the remaining aspartic acid is derived via this route, but our data clearly indicate that the primary role of PEP carboxylase is anaplerotic, rather than recapture.

Schmidt and Winkler (21) have discussed the possibility that PEP carboxylase activity has an influence on $\delta^{13}C$ of C3 plants. Our data suggest that one carbon of one amino acid comes via this route, but for proteins this is probably less than 1% of total carbon. A contribution of this magnitude is consistent with 14C labeling studies of Scheibe and Beck (20). PEP carboxylase might also contribute to the synthesis of other four-carbon acids, but it seems unlikely that this total contribution would exceed 5% of total carbon fixed. The latter value would cause a shift of only 1% in the $\delta^{13}C$ value of whole leaf and thus would be a minor contributor to leaf isotopes.

3 This is obtained by writing equation 12 of Ref. 12 twice—one for $^{13}C$ and once for $^{14}C$—and taking the ratio of the two equations. The known values of the individual isotope fractions, the $\delta^{13}C$ value of air ($-8\%$), and the estimated value for k$_2$/k$_1$ (0.6) can then be used to calculate the isotopic composition of internal CO2.

4 However, if CO2 and HCO3- fail to reach isotopic equilibrium, this fractionation will be smaller and the resulting oxaloacetate will contain more $^{13}C$ (15). In the limiting case in which HCO3- formation is rate limiting, the predicted $\delta^{13}C$ value for carbon-4 of aspartate is the same as that of the internal CO2 pool, or $+1\%$. Because of the presence of significant carbonic anhydrase in C4 plants, the latter case is unlikely.

Similar experiments with spinach have revealed a pattern of isotopic labeling consistent with that shown for tobacco above. Experiments are in progress to see whether this same phenomenon occurs in other C3 plants.

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LITERATURE CITED

6. HERMES JD, CA ROSEKE, MH O'LEARY, WW CLELAND 1982 The use of multiple isotope effects to determine enzyme mechanisms and intrinsic isotope effects. Malic enzyme and glucose-6-phosphate dehydrogenase. Biochemistry 21: 5106-5114
8. KLUGE M 1983 The role of phosphoenolpyruvate carboxylase in C4-photosynthesis and Crassulacean acid metabolism. Physiol Veg 21: 817-825
22. TROUGHTON J 1979 $\delta^{13}C$ as an indicator of carboxylation reactions. Encycl Plant Physiol, New Ser 6: 140-149