Plant Physiol. (1984) 75, 873–875
0032-0889/84/75/0873/03/$01.00/0

Short Communication

Sulfate Assimilation in C_4 Plants

INTERCELLULAR AND INTRACELLULAR LOCATION OF ATP SULFURYLASE, CYSTEINE SYNTHASE, AND CYSTATHIONINE β-LYASE IN MAIZE LEAVES

Received for publication March 7, 1984 and in revised form May 9, 1984

JAMES N. BURNELL*
Division of Plant Industry, CSIRO, GPO Box 1600, Canberra, ACT 2601, Australia

ABSTRACT

The activity of ATP sulfurylase, cysteine synthase, and cystathionine β-lyase was measured in crude leaf extracts, bundle sheath strands, and mesophyll and bundle sheath chloroplasts to determine the location of sulfate assimilation of C_4 plant leaves. Almost all the ATP sulfurylase activity was located in the bundle sheath chloroplasts while cysteine synthase and cystathionine β-lyase activity was located, in different proportions, in both chloroplast types.

A new spectrophotometric assay for measuring ATP sulfurylase activity is also described.

The C_4 pathway of CO_2 assimilation is compartmentalized into specific cell types with atmospheric CO_2 initially being fixed into organic acids in the mesophyll cells of C_4 plants and then transported to the bundle sheath cells. Within the two types of cells the reactions are compartmentalized into chloroplasts, cytoplasm and mitochondria. The intercellular compartmentation of sulfur metabolism is less well defined.

The intercellular compartmentation of ATP sulfurylase (EC 2.7.7.4) has been reported in a wide variety of C_4 plants (8, 9). An hypothesis for the pathway of sulfur assimilation in leaves of C_4 plants has been described based upon the location of ATP sulfurylase and, to a lesser extent, thiosulfonate reductase/sulfite reductase (8). Since ATP sulfurylase and sulfite reductase are involved in only the initial stages of assimilatory sulfate pathway in plants, the location of enzymes involved in the synthesis of cysteine and methionine remains unresolved.

Although it is generally agreed that in C_3 plants, chloroplasts are the main site of SO_4^{2-} assimilation (activation and reduction to S^{2-}) (1) the subcellular distribution of cysteine synthase (EC 4.2.99.8) appears to differ between plant species. In wheat and rape leaves and kidney bean seedlings, the enzyme is apparently cytoplasmic (3, 14, 16) whereas in spinach, white clover, and pea leaves the enzyme is associated with chloroplasts (7, 15). A recent report suggests that cystathionine β-lyase (EC 4.4.1.8) in barley leaves is at least partially chloroplastic (17).

This paper reports on the inter- and intracellular location of ATP sulfurylase, cysteine synthase and cystathionine β-lyase in *Zea mays*. A new spectrophotometric assay for ATP sulfurylase is also described.

MATERIALS AND METHODS

Maize (*Zea mays*, cv Dekalb XL81) was grown in sterile soil in a naturally illuminated glasshouse between 22 to 30°C and was used 18 to 20 d after germination. All biochemicals were of analytical reagent grade and were purchased from either Sigma Chemical Co. or Boehringer-Mannheim, Australia. PDRP[1] was partially purified as described previously (4).

Mesophyll chloroplasts were isolated and purified as described previously (13) except that 2 mM DTT was included in the extraction, washing and Percoll density buffers. Bundle sheath strands and chloroplasts were prepared as described by Jenkins and Boag (12). Crude extracts were prepared as described previously (13). Chl was determined as described previously (2).

Pyruvate,Pi dikinase was activated as described previously (13) except that 0.3 units partially purified PDRP was added. Pyruvate,Pi dikinase (EC 2.7.9.1) and PEP carboxylase (EC 4.1.1.31) activities were measured as described previously (6, 11), and NADP-malic enzyme (EC 1.1.1.40) activity was measured as described previously (12).

ATP sulfurylase activity was measured spectrophotometrically by coupling the reverse reaction catalyzed by ATP sulfurylase with hexokinase and glucose-6-P dehydrogenase and measuring the APS- or PPi-dependent reduction of NADP. Reaction mixtures contained in a volume of 1 ml: 1 μmol APS, 1 μmol PPi, 5 μmol $MgCl_2$, 5 μmol glucose, 0.3 μmol NADP, 5 units hexokinase, 5 units glucose-6-P dehydrogenase, and 50 μmol Tris-HCl, pH 8.0. Cysteine synthase and cystathionine β-lyase were assayed by the methods described previously (5).

All enzyme assays were run at 25°C in the presence of 0.1% (v/v) Triton X-100 added to ensure organelle breakage.

RESULTS

NADP-malic enzyme was assayed as a marker for bundle sheath chloroplasts, pyruvate,Pi dikinase as a marker for mesophyll chloroplasts and PEP carboxylase to determine the level of contamination by mesophyll cell cytoplasm. The activities of the marker enzymes indicated that there was little cross-contamination of the two types of chloroplast extracts (Table I). In addition, the low level of PEP carboxylase activity in either chloroplast preparation indicated little mesophyll cell cytoplasm contamination of either chloroplast preparation.

When initially measured, the pyruvate,Pi dikinase activity in both crude extracts and mesophyll chloroplasts was low and extended incubation at 25°C in the presence of Pi did not activate the enzyme. However, the enzyme was activated when mesophyll chloroplast and crude extracts were incubated with both PDRP and Pi (Table II). This result suggests that either the PDRP in both crude and mesophyll extracts was inactivated during prep-

[1] Abbreviations: PDRP, pyruvate,Pi dikinase regulatory protein; PEP, phosphoenolpyruvate; APS, adenosine 5′-phosphosulfate.

Table I. *Localization of Enzymes in Chloroplasts Isolated from Maize Leaves*

Enzyme	Crude Extract	Mesophyll Chloroplasts	Bundle Sheath Chloroplasts
		μmol/min·mg Chl	
NADP-malic enzyme	12.2	3.05 (2.01)[a]	34.1 (11.6)
Pyruvate,Pi dikinase	6.3	10.9 (7.19)	0.32 (0.11)
PEP carboxylase	22.2	0.07 (0.05)	0.35 (0.12)
ATP sulfurylase	0.69	0.06 (0.04)	1.64 (0.56)
Cysteine synthase	1.58	1.45 (0.96)	1.72 (0.58)
Cystathionine β-lyase	0.15	0.18 (0.12)	0.06 (0.02)

[a] Values in parentheses are the specific activities of the enzymes after correcting for the relative distribution of Chl in the mesophyll (66%) and bundle sheath (34%) chloroplasts.

Table II. *Effect of Pyruvate,Pi Dikinase Regulatory Protein and Pi upon Pyruvate,Pi Dikinase Activity in Crude and Chloroplast Extracts of Maize Leaves*

	Pyruvate,Pi Dikinase Activity		
	Crude	Mesophyll Chloroplasts	Bundle Sheath Chloroplasts
		μmol/min·mg Chl	
No addition	4.9	3.8	0.3
Plus PDRP[a]	5.9	4.0	0.3
Plus Pi	4.8	3.9	0.3
Plus PDRP and Pi	6.3	10.9	0.3

[a] Extracts were incubated in the presence or absence of partially purified pyruvate,Pi dikinase regulatory protein (PDRP) (0.7 mg) and Pi (5 mM) at 25°C. Pyruvate,Pi dikinase was measured after 30 and 60 min, no increase in activity as detected after 30 min.

aration of the fractions, or that the PDRP was converted to a form which does not catalyze Pi-dependent activation during extraction.

ATP sulfurylase activity was measured in the reverse direction using a spectrophotometric assay to follow the formation of ATP. The reaction was completely dependent upon the addition of APS and PPi and all components of the coupled assay system. The background rate of NADP reduction was determined in the absence of PPi and the initial rate of NADP reduction on addition of PPi was used as a measure of ATP sulfurylase activity; endogenous inorganic pyrophosphatase did not affect these initial rates since the addition of purified pyrophosphatase had no effect.

Using the spectrophotometric assay, more than 95% of the ATP sulfurylase activity was located in the bundle sheath strands. The specific activity of ATP sulfurylase in bundle sheath strands ranged from 1.62 to 1.67 μmol/min·mg Chl, close to that observed in the bundle sheath chloroplasts indicating that ATP sulfurylase is located in the chloroplasts of the bundle sheath strands.

Only a very low level of ATP sulfurylase activity was detected in the mesophyll chloroplasts and on a percentage distribution basis it was lower than the level expected by contamination. Following PDRP-dependent activation, a high activity of pyruvate,Pi dikinase was detected in the same preparation, however, which indicated that soluble proteins were not lost during isolation and purification of the mesophyll chloroplasts.

Both cysteine synthase and cystathionine β-lyase activities were detected in crude extracts and in both types of chloroplasts, with a greater percentage of the total cysteine synthase activity in the mesophyll chloroplasts (Table I). Similarly, a greater percentage of the cystathionine β-lyase activity was located in the mesophyll chloroplasts. In the bundle sheath strands the activities of cysteine synthase and cystathionine β-lyase ranged from 1.67 to 1.73 and from 0.05 to 0.06 μmol/min·mg Chl, respectively, close to those values observed in the bundle sheath chloroplasts (Table I) indicating that both enzymes appear to be located in the chloroplasts of the bundle sheath strands.

DISCUSSION

The location of ATP sulfurylase in bundle sheath chloroplasts is in agreement with earlier findings (9). However, the activity of ATP sulfurylase expressed on a Chl basis was about five times higher than that previously reported for whole leaf and bundle sheath cell extracts from maize leaves (9). Since the values previously reported for ATP sulfurylase activity in both whole leaf and bundle sheath extracts of maize were about 20% of the average level of activity of the enzyme found in other C_4 plants examined, the reported values may be an underestimation of the enzyme activity in maize leaf tissue.

Expressed on a Chl basis, the cysteine synthase activity in the mesophyll and bundle sheath chloroplasts are similar to those reported for pea and clover chloroplasts (15).

Significant levels of both cysteine synthase and cystathionine β-lyase were detected in both mesophyll and bundle sheath extracts. That the maize leaf cysteine synthase activity appears to be associated with chloroplasts in both mesophyll and bundle sheath cells is similar to the enzyme from spinach, white clover, and pea leaves (7, 15). When these results are combined with the finding that most, if not all, of the ATP sulfurylase activity is located in the bundle sheath cells, they indicate that sulfur must be transferred from bundle sheath to mesophyll cells following sulfate activation. The previous finding, that thiosulfonate reductase/sulphite reductase activity has been detected in both mesophyll and bundle sheath cell extracts (8), suggests that sulfite must be transferred from the bundle sheath to the mesophyll cells prior to reduction to sulfide. One sulfite has been transferred from the bundle sheath chloroplasts, the mesophyll cells contain the enzymes to reduce sulfite to sulfide and to incorporate it into cysteine.

Although homocysteine may be synthesized by either transsulfuration or direct sulfhydration in plants, it appears that transsulfuration accounts for essentially all homocysteine biosynthesis in the plant kingdom (10). Therefore, the site of cystathionine β-lyase activity in plants can be used as an indicator of the site of homocysteine synthesis. With almost 85% of the cystathionine β-lyase activity located in the mesophyll chloroplasts, the major site of homocysteine synthesis appears to be the mesophyll chloroplast. Since it was recently reported that methionine synthase (EC 2.1.1.13) is located in the cytoplasm of pea and barley leaves (17) no conclusions can be made regarding the location of methionine biosynthesis in maize.

The results presented in this paper together with previously published data (8, 9), indicate that SO_4^{2-} is activated in the bundle sheath chloroplasts of maize leaves and it is then transferred to the mesophyll cell chloroplasts in the form of APS or SO_3^{2-} (in a free or bound form—see Ref. 1).

The cellular compartmentation of sulfur assimilation in leaves of C_4 plants is analogous to that of carbon assimilation. Carbon assimilation is initiated in the mesophyll cells close to the source of gaseous carbon, CO_2, while sulfur assimilation is initiated in the bundle sheath cells close to the source of dissolved sulfur, SO_4^{2-}.

Acknowledgments—The expert technical assistance of Ms. Helen Franklin is gratefully acknowledged.

LITERATURE CITED

1. ANDERSON JW 1980 Assimilation of inorganic sulphate into cysteine. *In* BJ Miflin, ed, The Biochemistry of Plants, Vol 5. Academic Press, New York, pp 203–223

2. ARNON DI 1949 Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1–15
3. ASCANO A, DJD NICHOLAS 1977 Purification and properties of O-acetyl-L-serine sulphydrylase from wheat leaves. Phytochemistry 16: 889–893
4. BURNELL JN, MD HATCH 1984 Regulation of C_4 photosynthesis: Catalytic phosphorylation as a prerequisiste for ADP-mediated inactivation of pyruvate, P_i dikinase. Biochem Biophys Res Commun 118: 65–72
5. BURNELL JN, FR WHATLEY 1977 Sulphur metabolism in *Paracoccus denitrificans* Purification, properties and regulation of serine transacetylase, O-acetylserine sulphydrylase and β-cystathionase. Biochim Biophys Acta 481: 246–265
6. CHAPMAN KSR, MD HATCH 1981 Regulation of C_4 photosynthesis: Mechanism of activation and inactivation of extracted pyruvate, inorganic phosphate dikinase in relation to dark/light regulation. Arch Biochem Biophys 210: 82–89
7. FANKHAUSER H, C BRUNOLD, KH ERISMANN 1976 Subcellular localization of O-acetylserine sulphydrylase in spinach leaves. Experientia 32: 1494–1496
8. GERWICK BC, CC BLACK 1979 Sulfur assimilation in C_4 plants. Plant Physiol 64: 590–593
9. GERWICK BC, SB KU, CC BLACK 1980 Initiation of sulfate activation: A variation in C_4 photosynthesis plants. Science 209: 513–515
10. GIOVANELLI J, SH MUDD, AH DATKO 1980 Sulphur amino acids in plants. *In* BJ Miflin, ed, The Biochemistry of Plants, Vol 5. Academic Press, New York, pp 453–505
11. HATCH MD, IR OLIVER 1978 Activation and inactivation of phosphoenolpyruvate carboxylase in leaf extracts from C_4 species. Aust J Plant Physiol 5: 571–580
12. JENKINS CLD, TS BOAG 1984 Isolation and characterization of intact, functional bundle sheath chloroplasts from *Zea mays*, an NADP-ME type C_4 plant. Aust J Plant Physiol. In press
13. JENKINS CLD, VJ RUSS 1984 Large scale, rapid preparation of functional mesophyll chloroplasts from *Zea mays* and other C_4 species. Plant Sci Lett. In press
14. MASADA M, K FUKUSHIMA, G TAMURA 1975 Cysteine synthase from rape leaves. J Biochem 77: 1107–1115
15. NG BH, JW ANDERSON 1978 Chloroplast cysteine synthases of *Trifolium repens* and *Pisum sativum*. Phytochemistry 17: 879–885
16. SMITH IK 1972 Studies of L-cysteine biosynthetic enzymes in *Phaseolus vulgaris* L. Plant Physiol 50: 477–479
17. WALLSGROVE RM, PJ LEA, BJ MIFLIN 1983 Intracellular localization of aspartate kinase and the enzymes of threonine and methionine biosynthesis in green leaves. Plant Physiol 71: 780–784