Intracellular Localization of Some Key Enzymes of Crassulacean Acid Metabolism in *Sedum praealtum* 1

Received for publication September 25, 1978 and in revised form November 29, 1978

MARTIN H. SPALDING, MARK R. SCHMITT, S. B. KU, AND GERALD E. EDWARDS
Department of Horticulture, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT

The intracellular locations of six key enzymes of Crassulacean acid metabolism were determined using enzymically isolated mesophyll protoplasts of *Sedum praealtum* D.C. Data from isopycnic sucrose density gradient centrifugation established the chloroplastic location of pyruvate Pi dikinase, the mitochondrial location of NAD-linked malic enzyme, and exclusively nonparticulate (not associated with chloroplasts, peroxisomes, or mitochondria) locations of phosphoenolpyruvate carboxylase, NADP-linked malic enzyme, enolase, and phosphoglycerate mutase. The consequences of this enzyme distribution with respect to compartmentalization of the pathway and the transport of metabolites in Crassulacean acid metabolism are discussed.

CAM is one of the three major variations in the over-all pathway of photosynthetic CO₂ assimilation, along with the so-called C₃ and C₄ pathways. CAM is probably the least well understood of the three. The path of carbon is fairly well established over most of the pathway, but the compartmentation of the pathway has not been established (20). Most proposed schemes for flow-through compartments make use of information from a few inconclusive reports of intracellular locations of some enzymes (7, 18) combined with assumptions based on enzyme distribution within cells of C₄ plants. Well documented information on the intracellular distribution of enzymes in CAM is needed in order to investigate regulation at the enzyme level. For this reason, this investigation was undertaken.

Two major problems inherent in such efforts with CAM plants are the large amount of phenolic substances normally found in succulent leaves and the high concentrations of organic acids associated with CAM. We have utilized enzymically isolated protoplasts to be able to protect organelles and enzymes against these two problems more readily than would be possible when homogenizing pieces of leaf tissue containing many cells each. It has also been reported (22) that a larger proportion of organelles are maintained intact by the relatively gentle homogenization procedure of protoplast rupture as compared to mechanical tissue homogenization. Through the use of *Sedum praealtum* protoplasts, the intracellular distribution of some key enzymes in the pathway of CAM is demonstrated.

MATERIALS AND METHODS

**Chemicals.** Cellulysin was obtained from Calbiochem. The dextran used was 15,000 to 20,000 mol wt fraction from U. S. Biochemical Corp., Cleveland, Ohio. Methylcellulose was obtained from Fisher Scientific, Pittsburgh. PEP carboxylase was obtained from Worthington Biochemical Corp., Freehold, N. J. All other biochemicals were obtained from Sigma.

**Plant Material.** *S. praealtum* D.C. plants were grown under a 12-h photoperiod and a 30/15 C day/night temperature regime. The quantum flux density at soil level was about 600 μmol m⁻² s⁻¹. The plants were watered approximately every 3rd day and fertilized every 2 weeks. Young but fully expanded leaves were chosen for protoplast isolation (6th–9th leaf from apex). Isolations were begun in early morning just prior to the time lights would normally come on in the growth chamber. The temperature was kept at 30 C on the night preceding isolation to decrease both starch and acid content of the leaves.

**Protoplast Isolation.** The leaves were cut perpendicular to the leaf axis in slices about 1 mm thick with a sharp razor blade. The leaf slices were then washed once, vacuum-infiltrated, and again washed, all with 0.3 m sorbitol. The leaf slices were then incubated in an isolation medium containing 1.5% (w/v) Cellulysin and 0.3 m sorbitol at a pH of 5.0. After digestion at room temperature for 3 h, the mixture was swirled manually and passed through a nylon net with 210-μm apertures followed by another net with 155-μm apertures (Tetko, Inc., Elmsford, N. Y.) to remove undigested tissue, epidermal strips, and vascular strands. The protoplasts were allowed to settle out of the filtrate (about 15 min) over ice, and the loose pellet formed was used for protoplast purification.

Protoplast purification was accomplished by flotation on 10% (w/v) dextran. The loose protoplast pellet collected after settling was resuspended in a solution containing 20% (w/v) dextran, 0.3 m sucrose, 1% (w/v) PVP-40, 1 m CaCl₂, and 50 mm Tris-HCl (pH 8.4). Layered on top of this was about 1 ml of a solution of the same composition except that the dextran concentration was 10% (w/v). A third layer of about 1 ml of the sorbitol medium (same as the breaking medium described below) was then added. This gradient was centrifuged in a refrigerated clinical centrifuge at 240g for 5 min. The intact protoplasts, which floated up to the interface between the 10% dextran solution and the sorbitol medium, were collected with a Pasteur pipette and resuspended in the breaking medium.

**Differential Centrifugations.** The protoplasts in a breaking medium containing 0.15 m sorbitol, 1% (w/v) PVP-40, 0.2% (w/v) methylcellulose, 5 mm EDTA, 5 mm DTT, and 250 mm Tris-HCl (pH 8.4) were ruptured by three passes through a 44-μm nylon net which was attached to a 2.5-ml syringe. Ruptured protoplast preparations were centrifuged for 2 min at 240g in a refrigerated clinical centrifuge. The supernatant was separated from the pellet and the pellet resuspended in the sorbitol medium.

**Density Gradient Centrifugation.** Sucrose density gradient centrifugations were run on protoplast extracts prepared by the same

---

1 This work was supported by the College of Agriculture and Life Sciences, University of Wisconsin, Madison, by the University of Wisconsin Research Committee with funds from the Wisconsin Alumni Research Foundation, and by National Science Foundation Grant PCM 77-09384 to G. E. E.

2 Abbreviations: G3P: glyceraldehyde 3-phosphate; PEP: phosphoenolpyruvate; PGA: phosphoglycerate; RuBP: ribulose 1,5-bisphosphate.
procedure as for the differential centrifugations. The ruptured protoplast preparation (2.5 ml) was layered on top of a sucrose density gradient composed of a 4-ml cushion of 70% (w/w) sucrose, a 25-ml linear gradient from 60 to 40% (w/w) sucrose, and a 7-ml linear gradient from 40 to 10% (w/w) sucrose, all dissolved in 50 mM Tris-HCl, 1% (w/v) PVP-40, 5 mM DTT, and 5 mM EDTA (pH 8.0). The gradient was centrifuged at 25,000 rpm for 3.5 h at 4°C in a Beckman SW 27 rotor. At the end of the centrifugation, 1.2-ml fractions were collected using an ISCO model 640 density gradient fractionator while simultaneously monitoring the A$_{350}$.

Enzyme Assays. Pyruvate Pi dikinase (EC 2.7.9.1) was assayed spectrophotometrically as ATP-dependent activity essentially by the method of Hatch and Slack (10). Other enzymes assayed and reference to the method used were succinate dehydrogenase (EC 1.3.99.1) (4), RuBP carboxylase (EC 4.1.1.39) (16), NADP-triose-P dehydrogenase (EC 1.2.1.13) (15), PEP carboxylase (EC 4.1.1.31), and NADP-malic enzyme (EC 1.1.1.40) (24). NAD-malic enzyme (EC 1.1.1.38) (9), NAD-malate dehydrogenase (EC 1.1.1.37) (6), catalase (EC 1.11.1.6) (17), NAD-isocitrate dehydrogenase (EC 1.1.1.41) (3), hydroxypyruvate reductase (EC 1.1.1.29) (26), enolase (EC 4.2.1.11), and PGA mutase (EC 2.7.5.3) (14). Aliquots of fractions used for enzyme assays were routinely incubated for about 3 min in an equal volume of 0.1% (v/v) Triton X-100 to eliminate membrane integrity of organelles.

Other Analytical Methods. Chl was determined in 96% (v/v) ethanol by the method of Wintermans and De Mots (25). Sucrose concentration in each fraction of the gradient was determined by refractometry.

RESULTS

A typical preparation of purified mesophyll protoplasts from S. praealtum is illustrated in Figure 1. A similar preparation after three passes through a 44-μm nylon net is shown in Figure 2. If PVP was omitted from the breaking medium, chloroplasts at this stage appeared badly clumped and most of the activity of both mitochondrial and peroxisomal marker enzymes sedimented with the chloroplasts. Divalent cations (Mg$^{2+}$, Ca$^{2+}$), Pi, or a reduction in buffer concentration each also resulted in similar but less extreme co-sedimentation of marker enzymes for all three organelles. Distribution of some enzymes after a 240g centrifugation of extracts prepared in the optimal breaking medium is illustrated in Table I. In this experiment, Chl and NADP-triose-P dehydrogenase were used as chloroplast markers, hydroxypyruvate reductase and catalase were used as peroxisomal markers, and NADH-malate dehydrogenase was used as a marker for cytoplasm, mitochondria, and peroxisomes. NADP-triose-P dehydrogenase activity was found in the chloroplast pellet in approximately equal proportion to that of the Chl indicating sedimented chloroplasts are intact. Little activity of both PEP carboxylase and NADP-malic enzyme appeared in the chloroplast pellet which suggests that these enzymes are non-chloroplastic. The distribution of these two enzymes was consistent through all of several separations of chloroplasts by differential centrifugation.

Data from one of three consistent sucrose density gradient centrifugations are presented in Figure 3. The activity of peroxisomal marker enzymes (hydroxypyruvate reductase and catalase) which entered the gradient was primarily located in fractions 18 to 20 at a density of 1.24 to 1.26 g cm$^{-3}$ (Fig. 3b). The mitochondrial marker enzymes, succinate dehydrogenase, and NAD-isocitrate dehydrogenase had a sharp peak of activity in fraction 11 at a density of about 1.20 g cm$^{-3}$ (Fig. 3d). The chloroplast markers, RuBP carboxylase, NADP-triose-P dehydrogenase, and Chl had a peak of activity in fraction 28, which was the top of the cushion of 70% sucrose giving an equilibrium density between 1.31 and 1.36 g cm$^{-3}$ (Fig. 3, a and c). Chl was found in an additional peak in fraction 9 at a density of 1.17 g cm$^{-3}$ (Fig. 3a), but none of the assayed enzymes was associated to any extent with this peak. In addition, NAD-malate dehydrogenase activity was found to peak in the same fractions as the mitochondrial marker enzymes and, to a minor extent, with the peroxisomal and chloroplast markers. Among the enzymes of primary interest for their intracellular localization, PEP carboxylase, NADP-malic enzyme, enolase, and PGA mutase were found only at the top of the gradient (Fig. 3, e and f) and not associated with peroxisomal, mitochondrial, or chloroplast marker enzymes in the gradient. Pyruvate Pi dikinase activity in the gradient was found primarily in fraction 28 with
SPALDING ET AL.

TABLE 1. Distribution of enzyme activities after sedimenting chloroplasts by centrifugation of Sedum praealum protoplast extracts

| Measurement       | Total Activity$^1$ in Protoplast Extract | Activity in 240 g Pellet | Activity in 240 g Supernatant | distribution (%) |
|-------------------|------------------------------------------|--------------------------|-------------------------------|----------------
| chlorophyll       |                                          |                          |                               |                |
| NADP-triose-P     |                                          |                          |                               |                |
| dehydrogenase     | 591                                      | 56                       | 44                            |                |
| hydroxypruvate reductase | 450                                     | 20                       | 90                            |                |
| catalase          | 42,000                                   | 22                       | 78                            |                |
| NAD-malate        |                                          |                          |                               |                |
| dehydrogenase     | 17,150                                   | 7                        | 93                            |                |
| NADP-malate enzyme | 144                                      | 2                        | 98                            |                |
| PEP carboxylase   | 549                                      | 11                       | 89                            |                |

$^1$Total activity was calculated from the sum of the activities in the pellet and the supernatant.

The chloroplast markers (Fig. 3e). NAD-malic enzyme showed a peak of activity in fraction 11 with the mitochondrial marker enzymes (Fig. 3f).

DISCUSSION

A major limitation in research on the regulation of CAM at the cellular level has been the lack of good evidence as to the intracellular localization of enzymes involved in the pathway. We have prepared a sucrose density gradient which clearly separates all major organelles as demonstrated using marker enzymes. Although considerable breakage of organelles occurred, the proportion recovered (chloroplasts, 20–30%; mitochondria, 25–30%; peroxisomes, 15–20%) was sufficient to establish enzyme localization. The densities of all organelles except intact chloroplasts are consistent with established values in C₄ and C₃ plants (8, 21, 23). The very high density of intact chloroplasts probably reflects the large amount of starch which we observed by iodine staining (unpublished). In this report, data have been presented which demonstrate the distribution of six key enzymes in the CAM pathway.

Pyruvate Pi dikinase activity is clearly associated with intact chloroplasts, with most of the remaining activity at the top of the gradient. At a density of 1.17 g cm$^{-3}$ Chl was found without associated activities of chloroplast marker enzymes, indicative of broken chloroplasts (23), and considerable activity for both chloroplast marker enzymes was found at the top of the gradient. Therefore, the pyruvate Pi dikinase activity at the top of the gradient was very likely due to release of the enzyme from ruptured chloroplasts. Location of this enzyme in the chloroplast, although never reported for a CAM plant, is consistent with the reported location of this enzyme in C₄ plants (8).

NAD-malic enzyme activity is associated with the mitochondria. Activity at the top of the gradient follows a similar pattern of succinate dehydrogenase activity and probably reflects release of enzymes from broken mitochondria. The lack of activity of NAD-isocitrate dehydrogenase at the top of the gradient may be
attributed to the extreme lability of this enzyme, since no activity could be found in the protoplasm extract either under the assay conditions employed. Location of NAD-malic enzyme primarily in the mitochondria is consistent with data reported by Ditterich (5) on the distribution of this enzyme in Kalanchoë daigremontiana and its reported intracellular distribution in C₄ plants (21).

PEP carboxylase, NADP-malic enzyme, enolase, and PGA mutase activities were found only at the top of the gradient and not associated with any organelles. Extrachloroplastic locations of PEP carboxylase and NADP-malic enzyme are both contrary to the chloroplastic location of these two enzymes reported by Mukerji and Ting in Opuntia ficus-indica (18) and by Garnier-Dardart in Bryophyllum sp. (7). In both of these localization studies, nonaqueously isolated chloroplasts were used which have been demonstrated to contain cytoplasmic contamination often (1). This, combined with the facts that neither report indicates any determination of contamination by cytoplasm or other organelles or what proportion of the total NADP-malic enzyme or PEP carboxylase activity was found in the chloroplast fraction, makes interpretation of their results difficult. Inasmuch as in both studies all enzymes assayed were found to be particulate, this may reflect some nonspecific binding of enzymes similar to problems we encountered under certain conditions. Mukerji and Ting (19) have also reported the identification of three isoenzymes of NADP-malic enzyme in O. ficus-indica associated with chloroplasts, mitochondria, and the cytoplasm. It is possible that the reports mentioned above indicate that intracellular distribution of at least some enzymes of the CAM pathway is not the same in all CAM species. Location of PEP carboxylase outside the chloroplast, mitochondrion, and peroxisome is consistent with the intracellular distribution in C₄ plants (8), but an extrachloroplastic location of NADP-malic enzyme is at variance with the reported location of this enzyme in C₄ plants (21). This represents the first well documented demonstration of a difference in intracellular location of an enzyme associated with C₄ dicarboxylic acid metabolism in CAM versus C₄ plants.

With this demonstration of the compartmentalization of key enzymes in the CAM pathway, we are able to propose a tentative scheme for intracellular photosynthetic carbon flow in S. praeclum (Fig. 4). The pathway for nighttime acidification is illustrated in Figure 4A. Based on enzyme localization, the steps from 3-PGA to oxaloacetic acid are proposed to be located in the cytoplasm. The conversion of oxaloacetic acid to malate in the cytoplasm is also consistent with a major portion of NAD-malate dehydrogenase activity being apparently nonparticulate. The compartmentation of the pathway prior to the PGA mutase step is yet to be demonstrated, based on transport capabilities of chloroplasts from C₃ plants (11), the metabolites from starch breakdown most
likely to be transported out of the chloroplast would be either 3-PGA or G3P.

The proposed scheme for carbon flow during the decarboxylation and refixation phase is illustrated in Figure 4B. If pyruvate formed upon decarboxylation of malate by either NAD-malic enzyme or NAD-malic enzyme is to be incorporated into starch, the compartmentation of enzymes catalyzing steps from malate to 2-PGA requires that pyruvate, PEP, and probably either 3-PGA or G3P are transported across the chloroplast envelope. In addition, pyruvate formed from decarboxylation of malate via NAD-malic enzyme would need to be transported out of the mitochondrion.

The proposed transport is not unprecedented. Transport of pyruvate into and PEP out of NAD-malic enzyme-type C₄ mesophyll chloroplasts has been demonstrated (12, 13), and transport of pyruvate out of bundle sheath mitochondria is required for the currently accepted C₄ cycle of NAD-malic enzyme type C₄ plants (21). Verification of the pathway awaits isolation of organelles involved and demonstration of their ability to perform the proposed transport.

Demonstration of enzyme compartmentation in S. praelatum brings to light an important area of control, since both PEP carboxylase and NADP-malic enzyme appear to be located in the same compartment and operate in opposing directions. This becomes especially critical if the proposed scheme of carbon flow is correct, since substrates for both enzymes (PEP and malate) would be available in the cytoplasm during both the acidification and the decarboxylation and refixation phases. An important area of investigation in control of the CAM pathway will be regulation of PEP carboxylase and NADP-malic enzyme.

**LITERATURE CITED**

INTRACELLULAR ENZYME LOCALIZATION IN CAM


