CONVERSION OF CARBON 4 OF MALATE INTO PRODUCTS OF THE PENTOSE CYCLE BY ISOLATED BUNDLE SHEATH STRANDS OF *Digitaria sanguinalis* (L.) Scop. LEAVES

Peter Dittrich, Marvin L. Salin and Clanton C. Black

Department of Biochemistry, University of Georgia
Athens, Georgia 30602

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SUMMARY: Isolated bundle sheath strands of crabgrass, incubated with malate 4-^{14}C, were shown to take up malate and to incorporate ^{14}C into products of the pentose cycle. The conversion of ^{14}C into pentose cycle products was stimulated by light and by the addition of ribose-5-phosphate, ADP, and $NADP^+$. Malate was taken up near 0.25 μmoles/mg chlorophyll and this appeared to be the limiting factor for the conversion processes in isolated bundle sheath cells.

The C_4-dicarboxylic[1] pathway of leaf photosynthesis is based upon the interaction of mesophyll cells and bundle sheath cells. Several studies have shown a definite compartmentation of enzymes and different pathways of metabolism in each cell type (1). Initially, in intact leaves, most CO_2 is fixed in mesophyll cells via PEP carboxylase into C_4 acids such as oxaloacetic, malic, or aspartic. Specific organic acids subsequently are transported to the bundle sheath cells where decarboxylation occurs. The resulting CO_2 is refixed via RUDP carboxylase and thus enters the pentose cycle.

Supporting evidence for the theory of cell interaction in C_4 photosynthesis comes from studies showing specific enzyme compartmentalization in isolated mesophyll cells and bundle sheath cells or strands, leaf microscopy studies, and studies feeding various C_4 or pentose cycle intermediates to isolated leaf cell types (1). However, a crucial part of this theory, the refixation of CO_2 derived from the decarboxylation of a C_4 acid in bundle sheath cells, has not been demonstrated. We report in this paper that isolated bundle sheath strands from crabgrass take up malate and that label from C-4 of malate can be fixed

[1] Abbreviations used are: C_4, C_4-dicarboxylic; PEP, phosphoenolpyruvate; RUDP, ribulose-1,5-diphosphate; R-5-P, ribose-5-phosphate; pentose cycle, reductive pentose cycle; Chl, chlorophyll; PVP, polyvinylpyrrolidone-40; DTT, dithiothreitol.

into pentose cycle products.

METHODS

Mature leaves of *Digitaria sanguinalis* (L.) Scop. (crabgrass) were freshly harvested from greenhouse grown plants. Bundle sheath strands were isolated by the grinding and filtration methods developed previously (2). Leaves were ground in a buffer consisting of sorbitol, 330 mM; Tricine pH 7.8, 50 mM; $NaNO_3$, 2 mM; EDTA, 2 mM; $MnCl_2$, 1 mM; $MgCl_2$, 5 mM; PVP 2% (w/v); and DTT, 10 mM. The isolated bundle sheath strands were resuspended in the same medium except for the omission of PVP.

Malate uptake and metabolism was assayed in a 15 ml conical bottom tube placed in a water jacketed chamber maintained at 35°. The assay medium, total volume 10 ml, consisted of: cells (110-125 µg of Chl); R-5-P, 5 mM; ADP, 5 mM; malate 4-^{14}C, 2 mM (1 µC/µmole); and the same concentration of components used in the suspending medium. Cells were stirred with a magnetic stirrer constantly during the course of the experiment. At timed intervals a 1 ml aliquot was taken, passed through a 5 µ millipore filter, and washed with 10 ml of suspension buffer. The filter was placed in a scintillation vial and the radioactivity determined in a Packard Tri-Carb Liquid Scintillation Spectrophotometer (Model 2002). For the determination of the conversion of malate 4-^{14}C into other products, 300 µl of bundle sheath strands (16 µg of Chl) in suspension buffer were incubated with 100 µl (5 mM) malate 4-^{14}C (50 mC/mmole) under conditions described for the uptake of malate. Aliquots of 100 µl were taken at various times, extracted in hot 70% ethanol and chromatographed two dimensionally according to standard procedures (3). After radioautography labeled spots were cut out and counted by liquid scintillation counting.

Preparation of PEP carboxylase: Mesophyll cells of crabgrass were isolated as described previously (2). The cells were suspended in Tris buffer 0.1 M, pH 8.3 containing 10 mM DTT and 10 mM $MgCl_2$. The concentrated suspension was frozen in liquid nitrogen and ground till all cells were broken. The slurry

was centrifuged for 5 min at 25,000 x g and the supernatant was subjected to ammonium sulfate precipitation. The fraction between 40% and 55% saturation contained practically all of the PEP carboxylase.

Preparation of malic acid 4-^{14}C: The reaction mixture contained 50 units of crabgrass PEP-carboxylase, 50 units of malate dehydrogenase, 30 mM NADH, 30 mM PEP, 18 mM $NaH^{14}CO_3$ (50 mC/mmole), 0.1 M Tris buffer pH 8.3, 10 mM DTT and 10 mM $MgCl_2$ in a total volume of 1 ml. The reaction was run for 5 min at 35° and stopped with acetic acid and acetone. The suspension was centrifuged, the supernatant evaporated to dryness, and then chromatographed on Whatman No. 3 paper using a solvent of n-butanol-propionic acid-water 75-35-50 (v/v). After radioautography on Kodak X-ray film, the strip containing the labeled malate was cut out and stored overnight in a desiccator containing pellets of KOH to remove volatile acids. The malate was then eluted, counted, taken to dryness, and again resuspended in a small volume of suspension buffer.

Preparation of malic enzyme: Leaves of *Pennisetum purpureum* (L.) were washed, finely cut, and thoroughly ground with mortar and pestle in 0.1 M Tris buffer pH 8.3, containing 10 mM DTT, 5 mM $MgCl_2$, 5 mM $MnCl_2$, and 1% insoluble PVP. The extract was filtered through a 80 μ nylon filter and centrifuged for 10 min at 25,000 x g. Ammonium sulfate was added to the supernatant and most of the malic enzyme activity was detected in the fraction between 40% and 55% saturation. The crude enzyme was reprecipitated with ammonium sulfate before use.

Degradation of labeled malic acid: In order to establish the position of the label, malate was decarboxylated as follows. The assay contained 0.2 mM malate -^{14}C, 0.5 mM $NADP^+$, 0.5 mM NADH, 1 unit of lactic dehydrogenase, 0.1 unit of *Pennisetum purpureum* (L.) malic enzyme, 0.1 M Tris buffer pH 8.3, 10 mM DTT, 5 mM $MgCl_2$ and 5 mM $MnCl_2$ in a total volume of 0.5 ml. The temperature was 35°. Samples of 10 μl were taken at various times and the remaining radioactivity was determined. After an initial linear decrease, the loss of ^{14}C ceased after 20 min. The remaining radioactivity was 1.7% of the original

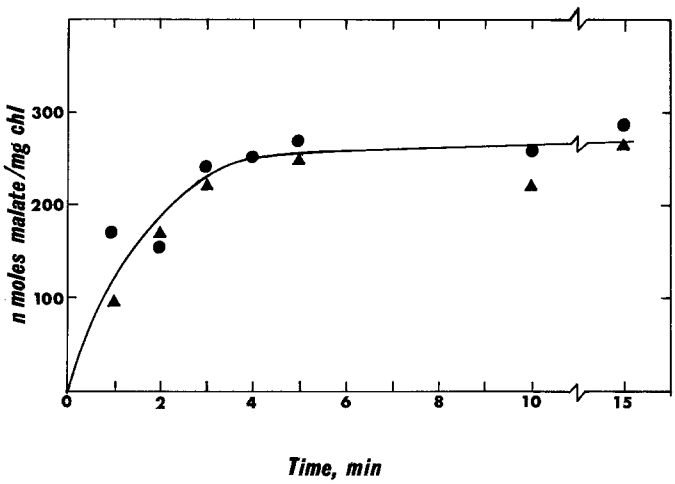

Figure 1. Time course of malate uptake by isolated crabgrass bundle sheath strands. Circles and triangles represent individual experiments, in the light, as described in METHODS.

content. Therefore 98.3% of the label was in the C-4 position of the synthesized malic acid.

Tubes containing the cells were illuminated by white light from a Sylvania sun gun focussed through a round bottom flask containing water, then passed through a flat bottle containing 2% (w/v) $CuSO_4$. The illumination intensity, as measured by a Yellow Springs Instrument Radiometer (Model 65A), was 1.5×10^5 ergs/cm^2/sec. Chlorophyll was determined in 96% ethanol using standard absorption coefficients (4).

RESULTS AND DISCUSSION

Previous radioautography directed at studying water soluble compounds in intact C_4 plant leaves after short time (2 to 10 sec) $^{14}CO_2$ fixation (5,6) demonstrated that label fixed initially in the mesophyll cells is rapidly transported to the bundle sheath compartment. Numerous plasmodesmata connecting the cell types presumably facilitate these intercellular movements (7,8). In isolated bundle sheath strands however, plasmodesmata are interrupted and possibly restricted. We questioned therefore, how readily compounds applied externally could be taken up by the isolated bundle sheath cell. According to

TABLE I. Compounds Formed by Bundle Sheath Strands of *Digitaria sanguinalis* (L.) Scop. Upon Incubation with Malate 4-^{14}C

Experiment	I			II			III	
Conditions	Light			Light + R-5-P, ADP, $NADP^+$			Dark + R-5-P, ADP, $NADP^+$	
Min of Incubation	2	8	15	2	8	15	5	10
Labeled Compounds Formed:								
Sugar diphosphates					3.8	3.8	1.1	1.9
Sugar monophosphates					2.6	3.8		
3-PGA	0.8*	1.5		2.6	63.7	97.5	11.2	26.2
Phosphoglycolate					6.4	3.4	3.7	3.7
UDP-glucose					15.0	18.7	7.5	11.2
Glycerate					12.8	7.5		
PEP	2.3	2.3	3		1.9	3.8	2.6	3.7
Aspartate	26.0	68.0	101	22.5	6.7	18.7	10.1	15.0
Citrate	7.5	19.0	15		3.8	11.2	8.2	11.2

*Values are given in 10^3 cpm/4 µg Chl. Experiments were performed as described in METHODS. 10^3 cpm = 1.3 x 10^{-5} µmoles.

the C_4 scheme of photosynthesis (1,2), crabgrass bundle sheath cells should utilize malate translocated from the mesophyll cells. Hence, as a preliminary approach it was necessary to investigate how permeable isolated bundle sheath strands were to malate.

Fig. 1 shows a time course of malate uptake in isolated bundle sheath strands of crabgrass. During the initial incubation phase malate was taken up maximally at a rate of 9 µmoles/mg Chl/hr. After 4 min, the cells reached a steady state concentration near 0.25 µmoles of malate/mg Chl. The rate of influx and the final concentration were independent of light, 20 µM DCMU, and a decrease in the concentration of external malate from 2 to 1 mM. Having shown that isolated bundle sheath cells take up malate, we proceeded to investigate its conversion into products of the pentose cycle. This conversion would involve decarboxylation of malate by malic enzyme followed by refixation of the libera-

ted CO_2 via RUDP carboxylase. Both enzymes are located predominantly (>98% of the total leaf activity) in bundle sheath cells (9,10).

Table I shows the results of three experiments in which malate 4-^{14}C was fed to isolated bundle sheath strands. In Experiment I, strands were incubated with labeled malate in the light. Though the conversion of malate into pentose cycle products was small, PGA was nevertheless detectable. After 15 min incubation, the label appeared in only a few compounds. During the procedure to isolate bundle sheath strands, which involves several washings and requires about one half hour of handling, compounds essential to CO_2 fixation may leach from the cells. Addition of photosynthetic intermediates is known to stimulate rates of CO_2 fixation by isolated cells (9,11). Therefore in Experiment II we added R-5-P, ADP, and $NADP^+$ to the incubation medium. PGA was a primary product under these conditions. The appearance of label in compounds such as sugar phosphates, phosphoglycolate, and UDP-glucose supports the contention that PGA was produced by refixation of $^{14}CO_2$ via the pentose cycle. The corresponding experiment carried out in the dark showed a similar formation of products as in the light. However, the rate of dark incorporation of $^{14}CO_2$ derived from malate into products of the pentose cycle was reduced, reflecting the influence of light during the fixation process. A similar relationship between light and dark fixation of added $NaH^{14}CO_3$ by bundle sheath cells supplemented with R-5-P and ADP was observed previously (9,11). The incorporation of ^{14}C in the dark (Expt. III) may be supported by NADPH formation from the oxidative decarboxylation reaction catalyzed by malic enzyme as well as ATP production from dark respiration.

The rate of conversion of malate 4-^{14}C into products of the pentose cycle varied between 2.4 and 3.6 μmoles/mg Chl/hr. This rate is low compared to leaf photosynthetic rates. Since malic enzyme and RUDP carboxylase activity in isolated bundle sheath strands exceed the rate of whole leaf photosynthesis (10), the incorporation of ^{14}C from malate appears to be substrate limited in isolated bundle sheath cells.

Our experiments show conclusively that carbon atom 4 of malic acid can be transferred into compounds of the pentose cycle by bundle sheath strands isolated from crabgrass leaves. The limiting factor in these *in vitro* experiments apparently was the low uptake of malate. However, in intact leaves the presence of numerous plasmodesmata (7,8) insure the direct contact of the cytoplasm of bundle sheath and mesophyll cells. In fact, recent radioautographic studies emphasize the speed with which ^{14}C fixed by the mesophyll cells (C_4 acids) is translocated into the bundle sheath cells (5,6). So translocation *in vivo* should not be a critical factor. Thus these studies support the currently accepted theory of C_4 photosynthesis (1) in which an organic acid produced by mesophyll cells is decarboxylated in the bundle sheath cells to furnish CO_2 for the pentose cycle of photosynthesis.

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