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CO_2 Metabolism in Corn Roots. II. Intracellular Distribution of Enzymes[1]

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Summary. Three enzymes assumed to mediate CO_2 metabolism in corn root tips, P-enolpyruvate carboxylase, malic dehydrogenase, and the malic enzyme, were extracted to determine their relative specific activities and their partitioning between soluble and particulate fractions. The data indicated that the intracellular location of these 3 enzymes is nonparticulate and thus these enzymatic reactions of CO_2 metabolism are apparently nonparticulate. The soluble malic dehydrogenase fraction differed from the particulate fraction in several kinetic properties, viz., response to the thionicotinamide analog of nicotinamide-adenine dinucleotide, oxaloacetate substrate inhibition at pH 8.3, and Km's for nicotinamide-adenine dinucleotide and L-malate. It was concluded that the soluble-malic dehydrogenase differed from the particulate forms in both structure and function. The soluble malic dehydrogenase is apparently involved in CO_2 metabolism.

In the previous paper of this series, kinetic data were presented in support of a metabolic sequence involving the carboxylation of P-enolpyruvate via a PEP[3] carboxylating enzyme (presumed to be PEP carboxylase) to form oxaloacetate, subsequent reduction by malic dehydrogenase to form malic acid, and decarboxylation of malic acid via the malic enzyme to form CO_2 and pyruvic acid (19). Although these data did indicate that PEP carboxylase activity was sufficient to account for the in vivo uptake of CO_2, other carboxylating proteins are known to occur in plant tissue. For example, the nucleotide dependent carboxylation of PEP carboxykinase has been reported by several workers (20). Furthermore, since the metabolism seems to be nonmitochondrial in corn roots (9, 10), it was of importance to determine the intracellular location of the above enzymes. In this regard, malic dehydrogenase is of especial interest since in corn root tips 3 electrophoretically different bands have been reported (18), one of which is apparently located in the soluble fraction of the cell and 2 in the particulate fraction. Thus it was also of interest to determine whether or not the 2 fractions were kinetically different.

In this report, we present data indicating that the 3 enzymes of interest are located in the soluble fraction and that the soluble and particulate malic dehydrogenase fractions differ kinetically.

Materials and Methods

Plant Material. Burpee's Golden Bantam Cross seeds were used to obtain root tissue for all procedures. The method of growth was the same as previously described (19).

Extraction and Preparation of Enzymes. Fresh root tips were ground in a mortar with acid washed sand (3:1 w/w) for 2 minutes in 25 mM tris- 0.5 M sucrose buffer at pH 7.5 (5°). The ratio of roots to buffer was 1:1 (w/v). All operations were completed in the cold room. The homogenate was centrifuged at 1000 × *g* for 5 minutes to remove cellular debris and strained through 4 layers of cheesecloth. The filtrate was centrifuged at 10,000 to 12,500 × *g* for 15 minutes to obtain a supernatant (soluble) and pellet (particulate) fraction. Electron micrographs indicated that the pellet fraction contained intact mitochondria as well as membrane fragments. The pellet was washed once with a small amount of the sucrose buffer and recentrifuged; the supernatant solution was discarded. Solubilization of the pellet was conducted by either freeze-thawing or sonication for 60 seconds at 60 watts. The latter was found to be more efficient. After solubilization, the fraction was recentrifuged to obtain a solubilized particulate fraction. Both fractions were dialyzed against 2 changes of 25

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[3] The following abbreviations are used: PEP, P-enolpyruvate; GDP, 5'-pyrophosphate of guanosine; IDP, 5'-pyrophosphate of inosine; DNP, 2,4-dinitrophenol.

mM tris pH 7.5, for 15 to 20 hours. [Dialysis tubing was treated by boiling in 1 mM EDTA (disodium salt) for 5–10 mins, rinsing, and storing in distilled water.] After the dialysis procedure, the non-diffusible material was centrifuged at 10,000 to 12,500 × *g* and the supernatant was used as the solubilized particulate fraction.

Protein Determination. Protein content was estimated by the 280 mμ to 260 mμ ratio method described by Layne (8). All samples used had a 280 to 260 ratio equal to or greater than 0.7.

P-Enolpyruvate Carboxylase Assay. The reaction mixture for the assay of P-enolpyruvate carboxylase [Orthophosphate:oxaloacetate carboxylyase (phosphorylating) EC 4.1.1.31] consisted of 0.5 ml of 30 mM P-enolpyruvate trisodium salt), 0.3 ml of 20 mM $NaHCO_3$, 0.3 ml of 20 mM $MgCl_2$, and 50 mM tris, pH 7.4, plus enzyme homogenate to make a total of 2 ml. All materials were prepared in the buffer. Two methods of assay were used. Firstly, oxaloacetate was determined as the 2,4-dinitrophenylhydrazone derivative according to Friedemann and Haugen (5). Reagent volumes were modified to detect 50 to 200 μmoles of oxaloacetate. Under these conditions, the PEP carboxylase assay was linear up to an OD_{450} of 0.400 and was linear with time for 7 minutes.

Secondly, PEP carboxylase was assayed spectrophotometrically by coupling the reaction with endogenous malic dehydrogenase and measuring the change in optical density at 340 mμ. For this assay, 0.1 ml of 1.2 mM NADH was substituted for 0.1 ml of buffer.

Malic Dehydrogenase Assay. Malic dehydrogenase (L-malate:NAD oxidoreductase-EC 1.1.1.37) was assayed spectrophotometrically at 340 mμ in the direction of oxaloacetate reduction with 0.2 ml of 6 mM oxaloacetate (neutralized with NaOH), 0.2 ml of 1.2 mM NADH, and buffer plus extract to make a total of 3 ml. The buffer was either pH 7.4 or 8.3, 50 mM tris. The reaction was started with the addition of extract, diluted 50 to 200 times. The assay was linear with enzyme concentration up to a $\triangle OD_{340}$ mμ / $\triangle$ t (min) = 0.055. Activity is defined as μmoles of NADH oxidized per minute and specific activity as the activity per mg protein. The reverse direction was assayed with 0.3 ml of 1.0 M L-malic acid (neutralized with NaOH), 0.2 ml of 41 mM NAD, and 2.5 ml of extract plus 50 mM tris, pH 7.4 or 8.3. The extract was usually diluted 5 to 20 times. The assay was linear with enzyme concentration up to a $\triangle OD_{340}$ / $\triangle$ t (min) = 0.055. Activity is defined as μmoles of NAD reduced per minute and specific activity as the activity per mg protein.

NAD analog ratio experiments were conducted according to Kaplan and Ciotti (7). The assay procedure was the same as that for the assay of malic dehydrogenase in the direction of malate oxidation except that 0.1 ml of 14 mM NAD was used.

Michaelis constants were determined in the usual manner and analyzed statistically according to the method of Elmore et al. (4).

Malic Enzyme Assay. The malic enzyme (L-malate:NADP oxidoreductase (decarboxylating) EC 1.1.1.40) was assayed spectrophotometrically at 340 mμ with 0.2 ml of 0.1 M L-malic acid, 0.2 ml of 20 mM $MnCl_2$, 0.1 ml of 50 mM AMP, 0.2 ml of 1.2 mM NADP, and extract plus 50 mM tris, pH 7.4 to make a total volume of 3.0 ml.

The OD_{340} was read at 1 minute intervals for 4 minutes. The assay was linear with time for 10 minutes and with enzyme concentration up to $\triangle OD_{340}$ / $\triangle$ t (min) = 0.020.

Results

PEP Carboxylase. Maximum PEP carboxylase activity was present when PEP, HCO_3^-, Mg^{2+}, and NADH were added to the homogenate. The initial linear rate in the absence of HCO_3^- was 56 % of the complete reaction. Bandurski (1) previously made the same observation with extracts from spinach leaves, and under stringent conditions in which dissolved CO_2 was removed, the rate of oxaloacetate formation was reduced to 18 %. The necessity for CO_2 was demonstrated with $^{14}CO_2$ (2). In the absence of Mg^{2+}, the initial linear rate was 10 % of the complete reaction while the absence of PEP gave a rate of 7 % of the complete. The latter rate was due to the presence of an NADH oxidase. The amount of extract required for the PEP carboxylase assay, however, was such that NADH oxidase did not interfere significantly, and the oxidase activity was essentially negligible under all conditions.

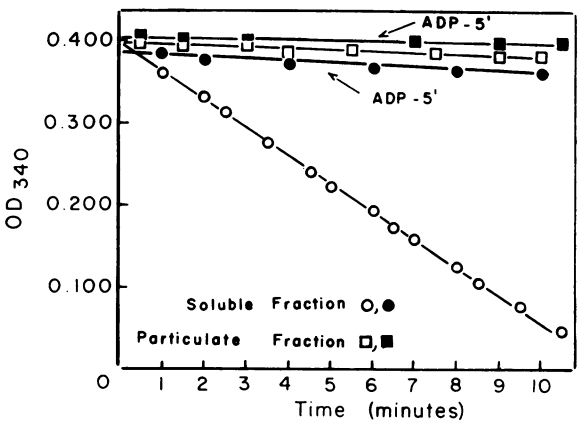

FIG. 1. Assay for PEP carboxylase and PEP carboxykinase in the supernatant and particulate fractions. No PEP carboxykinase was detectable and PEP carboxylase was apparently present only in the soluble fraction. The reaction mixture contained 0.5 ml of 30 mM PEP, 0.3 ml of 20 mM $NaHCO_3$, 0.3 ml of 20 mM $MgCl_2$, 0.2 ml of 1.2 mM NADH, and 50 mM tris (pH 7.4) plus extract to a total of 2 ml, with or without ADP.

Supernatant and particulate fractions were assayed for PEP carboxylase activity by coupling with endogenous malic dehydrogenase. As previously shown, both fractions contain malic dehydrogenase (18 and see next section). The results of these studies indicated that there was no detectable PEP carboxylase activity in the particulate fraction (fig 1). When a nucleotide (ADP, GDP, or IDP) was added to the extract as a phosphate acceptor, i.e., an assay for PEP carboxykinase, not only was there no kinase activity, but the carboxylase activity in the supernatant fluid was completely inhibited. The inhibition may have been due to activation of pyruvate kinase activity which we have measured in our preparations (our unpublished data). Thus these data indicate that in corn root tips PEP carboxylase activity is confined to the soluble fraction of the cell, and that under these conditions, no PEP carboxykinase activity could be detected. In spinach leaf tissue, PEP carboxylase activity was detected in particulate rather than soluble fractions, however, in cauliflower buds and pea seedlings most of the activity was localized in the cytoplasm (11). In barley roots, both fractions give positive assays for PEP carboxylase (6).

Malic Dehydrogenase. As previously indicated, malic dehydrogenase represents a special case since isozymes exist in both soluble and particulate fractions. The following series of experiments was designed to determine whether or not the soluble and particulate fractions differ kinetically in order to gain some insight as to their possible catalytic roles. Our existing hypothesis is that the soluble malic dehydrogenase, and not the particulate, functions in dark CO_2 metabolism in this tissue (18).

Table I. *Oxaloacetate Substrate Inhibition of Malic Dehydrogenase in Soluble and Particulate Fractions*

The data are ratios of specific activities at 8 mM to 0.4 mM oxaloacetate. Maximum rates were obtained with 0.4 mM. The spectrophotometric assay was conducted with 0.2 ml of 1.2 mM NADH, oxaloacetate, and 50 mM tris buffer plus extract to a total of 3 ml.

Prep	Soluble	Particulate	pH
1	0.85	0.56	8.3
2	0.96	0.61	8.3
2	0.56	0.61	7.4

Table II. *Ratio of Malic Dehydrogenase Specific Activity at pH 8.3 to pH 7.4*

Assay was spectrophotometric with 0.2 ml of 6 mM oxaloacetate, 0.2 ml of 1.2 mM NADH or 0.3 ml of 1.0 M L-malate, 0.2 ml of 41 mM NAD and 50 mM tris buffer plus extract. Total volume for the assays was 3 ml.

	Oxaloacetate → malate	Malate → oxaloacetate
Soluble	0.85	2.2
Particulate	0.72	2.6

Raval and Wolfe (13) reported oxaloacetate substrate inhibition with pig heart malic dehydrogenase. The optimum substrate concentration for the soluble and particulate malic dehydrogenases in corn root tips was 0.4 mM oxaloacetate. An oxaloacetate concentration of 8 mM resulted in little or no inhibition of the supernatant malic dehydrogenase, yet the particulate fraction was inhibited about 40 % at a pH of 8.3. At a pH of 7.4, both fractions were inhibited about equally (table I). Thus with respect to substrate inhibition, there appears to be kinetic differences between the 2 fractions.

As shown by Raval and Wolfe (12), the forward and reverse directions of malic dehydrogenase activity have different pH optima. They reported an optimum for oxaloacetate to malate of pH 7.5 and malate to oxaloacetate of pH 8.9. Our results with partially purified preparations were essentially the same with no apparent differences between the 2 fractions (table II).

A sensitive method for differentiating between malic dehydrogenase isozymes is their reaction in the presence of nucleotide analogs (7). When measured at a pH of 8.3, the thionicotinamide (TN-NAD) analog of NAD resulted in an increased rate of soluble malic dehydrogenase activity (malate to oxaloacetate), and a decreased rate of the particulate (table III). Similar results were obtained at pH 7.4. The deamino analog was not as active but the 3-acetylpyridine derivative was more active in both fractions. The fraction differences of the latter 2 analogs were slight, while the differences between the rates in the presence of TN-NAD indicate significant differences between the 2 malic dehydrogenase fractions.

Michaelis constants for the 2 main substrates and nucleotides were determined at pH 8.3 for both fractions. There were no apparent statistical differences between the fractions with respect to oxaloacetate and NADH, however, the Km's for malic acid and NAD were statistically different at the 1 % confidence level (table IV). Thus these data also indicate significant protein differences and further suggest different functional roles for the malic dehydrogenase fractions.

Malic Enzyme. Maximum malic enzyme activity required the presence of a metal ion (Mn^{2+}), NADP, and L-malic acid. Magnesium ions can replace Mn^{2+}; however, only about 70 % of the rate was obtained. Because of metal ion protein precipitation, AMP was found to be necessary when Mn^{2+} was used, but not when Mg^{2+} was used. NAD would not replace NADP. Thus the enzyme is similar to previously reported malic enzymes (3).

A comparison between soluble and particulate fractions indicated that malic enzyme activity was not present in the latter (fig 2), nor was any activity present in either fraction when NAD was used in place of NADP. Since in the presence of NAD and malic acid, the proper substrates are

Table III. *Malic Dehydrogenase Activity as a Ratio of NAD to an NAD Analog*

Assay was spectrophotometric in the direction of malate oxidation with 0.3 ml of 1.0 M L-malate, and 0.1 ml of 14 mM NAD or analog plus 50 mM tris buffer and extract to a total of 3 ml.

Prep	Analog	pH	Soluble	Particulate
1	TN-NAD*	7.4	0.48	2.4
2	TN-NAD*	7.4	0.48	3.5
3	TN-NAD*	8.3	0.62	3.8
3	De-NAD**	8.3	3.2	5.3
3	AP-NAD***	8.3	0.68	0.48

* TN-NAD = Thionicotinamide analog.
** De-NAD = Deamino analog.
*** AP-NAD = Acetylpyridine analog.

Table IV. *Apparent Km's Measured at pH 8.3 for Soluble and Particulate Malic Dehydrogenases*

Assay was spectrophotometric.

Substrate	Soluble Km (M)	Particulate Km (M)	Significant difference
OAA	$2.46 \times 10^{-5} \pm 0.176 \times 10^{-5}$	$2.22 \times 10^{-5} \pm 0.199 \times 10^{-5}$	N.S.
NADH	$2.78 \times 10^{-5} \pm 0.395 \times 10^{-5}$	$3.54 \times 10^{-5} \pm 0.657 \times 10^{-5}$	N.S.
MAL	$9.32 \times 10^{-4} \pm 1.00 \times 10^{-4}$	$16.98 \times 10^{-4} \pm 1.51 \times 10^{-4}$	**
NAD	$1.74 \times 10^{-4} \pm 0.214 \times 10^{-4}$	$5.94 \times 10^{-4} \pm 1.75 \times 10^{-4}$	**

N.S. = Not significant.
** 1 % Confidence level.

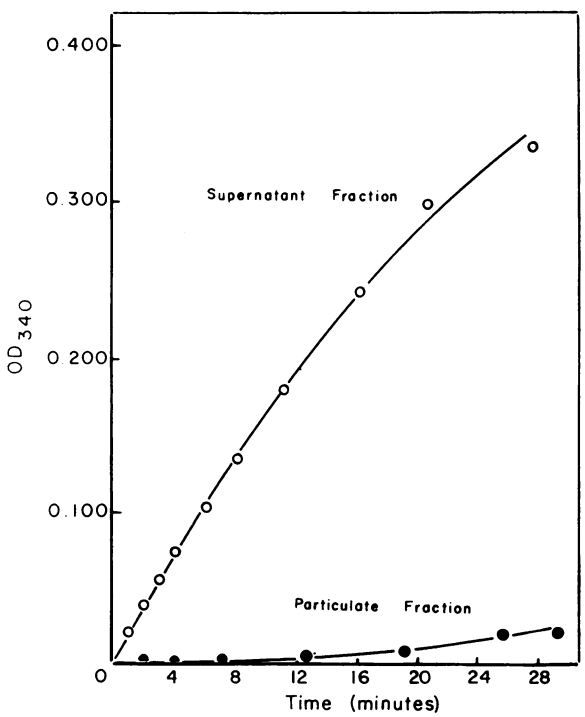

FIG. 2. Assay of malic enzyme in soluble and particulate fractions from corn root tips. Essentially, no activity was detectable in the particulate fraction. The reaction mixture contained 0.1 ml of 50 mM L-malate, 0.2 ml of 2.5 mM NADP, 0.5 ml of 20 mM $MnCl_2$, 0.2 ml of 50 mM AMP and 50 mM tris, pH 7.4 plus extract to a volume of 3.0 ml.

present for malic dehydrogenase activity, some reaction might be expected when measured spectrophotometrically. The Km's for these substrates, however, are sufficiently high so that the reaction is not measurable (see table IV).

Discussion

Nonphotosynthetic uptake of CO_2 by corn root tips and other tissues is a well known and repeatedly verified observation. The biological significance, however, is not by any means completely understood for all tissues (20). The presence of CO_2 is apparently necessary for optimum root growth (15) and this has been shown for corn root tips (14). Splitstoesser (14) recently demonstrated a correlation between protein synthesis and CO_2 metabolism in corn root tissue. Furthermore, more CO_2 metabolism takes place at the root tip (14, 17). Thus CO_2 is intimately linked with growth and development. Lips and Beevers (9) have shown a strict compartmentalization of the products of CO_2 fixation and acetate metabolism. Under certain conditions of stress, such as DNP inhibition, there seems to be a transfer of CO_2 fixation products, i.e., malic acid, to the mitochondria (10). Therefore, under certain conditions CO_2 fixation is anaplerotic in corn roots. Under most conditions, however, the 2 pools of malic acid are separated intracellularly.

Previously it was reported that $^{14}CO_2$ taken up by corn root tips was rapidly utilized as measured

by $^{14}CO_2$ release (16). The same observation was made for *Opuntia* root tips (17). Further, Ting and Dugger (16) postulated a turnover of carbon after CO_2 fixation via the malic enzyme and suggested that CO_2 metabolism in corn root tips would regulate reduced and oxidized nucleotide levels in the soluble portion of the cell.

In the previous paper of this series (19), the kinetics of carboxylation and decarboxylation were shown to be compatible with the sequence PEP carboxylase → malic dehydrogenase → malic enzyme. The latter was based on the assumption that the metabolism was extra-mitochondrial and that the turnover of malic acid was not due to respiratory activity.

In this paper we have shown that the carboxylating enzyme is probably PEP carboxylase, and that it is a nonparticulate enzyme in corn root tips.

That the soluble malic dehydrogenase activity differs kinetically from the particulate in several properties, viz., oxaloacetate substrate inhibition, activity with the NAD analog, TN-NAD, and Km's for malate and NAD, they would appear to function differently intracellularly. Since these properties are for the most part pH dependent and probably dependent on other intracellular conditions as well, little can be said for their exact meaning. It is interesting, however, that the supernatant malic dehydrogenase is less affected by oxaloacetate substrate inhibition at pH 8.3 than the particulate forms. Based on the Km's, both the soluble and particulate forms seem to favor oxaloacetate → malic acid rather than malic acid → oxaloacetate. The latter seems to be true for malic dehydrogenase in general (12). The data in this paper also indicate that the malic enzyme in corn root tips is not a particulate enzyme. We conclude, therefore, that these 3 enzymes (PEP carboxylase, soluble malic dehydrogenase, and the malic enzyme) are nonparticulate in corn root tips and that the CO_2 metabolism mediated by these enzymes is evidently not directly related to mitochondrial metabolism.

With regard to the differences noted between the soluble fraction and particulate fraction malic dehydrogenases, it must be emphasized that our fractions are not by any means pure. Electrophoretically, the soluble fraction appears to have predominantly the soluble malic dehydrogenase, but is contaminated with the 2 bands which seem to be associated with the particulate fraction (18). The particulate fraction, contained 2 bands and was apparently free from the soluble form (18). It is clear that the 2 fractions are dominated by their respective proteins, however, the specific data which we obtained must be considered in light of the cross interactions.

The $^{14}CO_2$ data reported in the previous paper (19) indicated that PEP carboxylase activity was sufficient to account for the uptake of CO_2 by corn root tips. Earlier, evidence was presented to suggest that the decarboxylation of malic acid via the malic enzyme could account for the observed rate of $^{14}CO_2$ turnover (16). The ratio of the rate of malate decarboxylation via the malic enzyme to the rate of malate formation via PEP carboxylase and soluble malic dehydrogenase as calculated from our data was 0.455. Thus these data indicate that the breakdown of malic acid by the malic enzyme could take place at a rate of 45 % of its formation. The rate of ^{14}C turnover (measured as $^{14}CO_2$ release), after a period of $^{14}CO_2$ uptake, seems to be on the order of 50 % or more of the uptake rate (16, 17). These data, however, reflect the synthesis and subsequent turnover of all products. The rate of turnover of malic ^{14}C from $^{14}CO_2$ fixation seems to be much the same (see fig 8 of 19). Therefore, these data also suggest that the enzymic sequence PEP carboxylase → soluble malic dehydrogenase → malic enzyme could account for much of the CO_2 fixation and turnover observations. The distinct possibility that other enzymes contribute to the turnover of malic acid is, of course, not eliminated.

In sum, the data in this paper are consistent with the hypothesis that CO_2 metabolism to form malic acid in corn root tips is mediated in part by PEP carboxylase, soluble malic dehydrogenase, and the malic enzyme, and that the major portion of the metabolism mediated by these enzymes is probably nonparticulate.

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