CO_2 FIXATION BY MALIC ENZYME IN A SPECIES OF MICROCOCCUS

T.I. Matula, I.J. McDonald and S.M. Martin

Division of Biology, National Research Council, OTTAWA, Canada

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Micrococcus sp. (A.T.C.C. No. 407) grown in a synthetic medium produced large amounts of malic enzyme. Growth of the organism and the malic enzyme production by the cells was enhanced by the addition of $NaHCO_3$ to the medium. CO_2 fixation was catalysed by malic enzyme in cell free extracts. This enzymatic reaction required pyruvate, NADPH and Mg^{++}. The radioactive product was identified by paper chromatography as malic acid. This evidence suggests that the physiological role of malic enzyme is in the maintenance of the tricarboxylic acid-cycle.

A number of CO_2 fixation reactions are responsible for replenishing C4 intermediates of the tricarboxylic acid-cycle as they are removed for synthesis of amino acids in the living cell. Phosphoenolpyruvate (PEP) carboxylase (EC 4.1.1.31) and phosphoenolypyruvate carboxykinase (EC 4.1.1.32) are believed to be the major enzymes involved in CO_2 assimilation in heterotrophic bacteria (1,2). Malic enzyme (L-malate: $NADP^+$ oxidoreductase decarboxylating, EC 1.1.1.40), which is widely distributed in a variety of cells, could also serve in this function (3). However, no direct evidence of CO_2 fixation by malic enzyme has been obtained in bacterial systems (4) and indeed recent reports on CO_2 fixation in certain species of Enterobacteriaceae excluded the possibility of malic enzyme functioning in the direction of CO_2 fixation (1,5,6). It has been suggested that malic enzyme in bacteria merely plays an anabolic role or, that it serves as a NADPH generating system (7,8). There is, therefore, uncertainty concerning the metabolic function of this enzyme. Results presented in this communication show evidence of CO_2 fixation by

malic enzyme in a Micrococcus species and suggest an alternative metabolic role for this enzyme.

MATERIALS AND METHODS

Organism and cell free extracts:

Micrococcus sp. (M. freudeureichii A.T.C.C. No. 407) was grown for 24 hrs on a rotary shaker at 25°C in synthetic medium (9) containing 0.5% glutamate and 0.4% maltose. Cells were harvested by centrifugation, washed in distilled water, recentrifuged and suspended in 0.05 M phosphate buffer (pH 7.4) containing 0.1 mM dithiothreitol. Cells were treated for 12 min in the presence of glass beads with a Branson sonifier. Cell debris was removed by centrifugation at 25,000 x g for 20 min. Cell-free extracts could be stored for 3-4 days at -10°C without loss of activity.

Enzyme assays:

Malic enzyme was assayed by following the reduction of $NADP^+$ at 340 mμ in a recording spectrophotometer at 23-24°C. The reaction, started by the addition of substrate, was followed for 2-3 minutes. One enzyme unit is defined as the amount of enzyme which brought about an increase in optical density of 0.001 per minute. Specific activity is expressed as units of enzyme per mg of protein.

CO_2-fixation by cell-free extracts was determined in a reaction mixture containing $NaH^{14}CO_3$ (see Table II). Perchloric acid (0.1 ml of a 45% solution) was added and the mixture was centrifuged. Aliquots (0.1 ml) of the supernatant were spotted on Whatman 2 MM filter paper (2 x 4 cm) and dried. The filter paper was then placed in 10 ml of toluene containing 0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis-2(5-phenyloxozolyl-benzene) and the radioactivity was measured in a Beckman liquid

TABLE I: Activity of Malic Enzyme in Cell-free Extract Micrococcus No. 407

Reaction system*	Activity**
Complete	0.180
$MgCl_2$ omitted	0.050
$MgCl_2$ omitted, $MnCl_2$ added	0.196
$NADP^+$ omitted, NAD^+ added	0.00
Malate omitted	0.00

*Complete reaction system contained in 3.0 ml volume: tris Cl buffer (pH 8.5), 150 µmoles; $MgCl_2$, 10 µmoles; $NADP^+$, 1 µmole; l-malate, 30 µmoles and cell-free extract, 0.1 ml.

**Δ in O.D. at 340 mµ/min

TABLE II: CO_2 fixation by cell-free extracts of Micrococcus No. 407

Additional components*	Total CO_2 fixed (counts/min)	
	Exp. 1	Exp. 2
pyruvate, NADPH	6,272	8,352
pyruvate, ATP	112	0
PEP**	1,296	672
PEP, ADP	1,872	944
pyruvate, $NADP^+$, malate	25,824	-

The reaction system contained: 0.5 ml cell-free extract, tris Cl buffer (pH 7.5), 100 µmoles; $MgCl_2$, 5 µmoles; $NaH^{14}CO_3$, 1 µcurie; $NaHCO_3$, 10 µmoles; cysteine, 2.5 µmoles and water to 1.5 ml. Incubated for 20 min at 25°C.

*Components added: pyruvate, malate, PEP, ATP and ADP, 10 µmoles each and $NADP^+$ or NADPH, 1 µmoles each.

**PEP: phosphoenolpyruvate.

Scintillation System (LS 200).

Chromatographic procedures:

The products of the reaction were separated by paper chromatography using Whatman No. 1 paper in two different solvent systems: n-butanol-formic acid-water, (10-2-10) and phenol-formic acid-water (75-25-1). Organic acids were detected by spraying with ethanolic bromcresol green (0.04%). Perchloric acid was precipitated with KOH and cations were absorbed on Dowex 50 (H^+ form). Distribution of radioactivity was determined by cutting duplicate portions of chromatograms into 2 x 4 cm segments and counting.

Keto acids were isolated from reaction mixtures as 2,4-dinitrophenylhydrazone derivatives. Five mg of oxaloacetate was added to 0.5 ml of supernatant, this mixture was treated with 2 ml of 0.5% 2,4-dinitrophenylhydrazine in 2N HCl for 2-3 hrs at room temperature. The precipitated hydrazones were collected on filter paper, washed with 5 ml of 2N HCl, air dried and counted. The reaction product of malic enzyme assay was also isolated and characterized as the phenylhydrazone by the method of Cavallini and Frontali (10).

RESULTS AND DISCUSSION

Cells of *Micrococcus* sp. produced large amounts of malic enzyme when grown on synthetic medium containing glutamate and maltose. The enzyme was found in cell-free extracts and showed specificity for $NADP^+$ and was activated by Mg^{++} or Mn^{++} ions (Table 1). The product of enzyme action on malate was pyruvate, indicating a reductive decarboxylation of malate. The addition of $NaHCO_3$ to the growth medium resulted in stimulation of growth and in an increase of malic enzyme level in the cells. Upon addition of $NaHCO_3$ the differential rate of enzyme synthesis

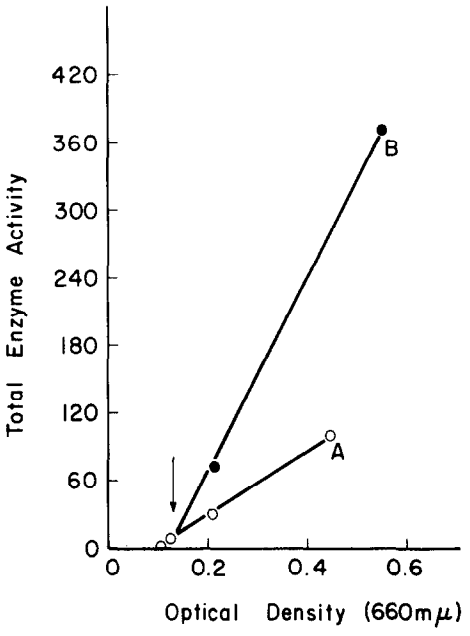

Figure 1. Effect of $NaHCO_3$ on the formation of malic enzyme in Micrococcus No. 407. Grown on synthetic medium, A-without $NaHCO_3$, B- with $NaHCO_3$ (0.1%). $NaHCO_3$ added at the point indicated by the arrow. Total enzyme activity is expressed as specific activity x cell density.

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increased immediately, leading to a much higher malic enzyme level than found in cells growing without $NaHCO_3$ (Fig. 1). This inducer-like effect of $NaHCO_3$ on the malic enzyme production prompted an investigation of CO_2 fixation as a possible function of the enzyme in this organism.

Cell-free extracts of Micrococcus No. 407 assimilated CO_2 in the presence of pyruvate, NADPH and Mg^{++} ions suggesting the involvement of malic enzyme (Table II). The incorporation of radioactivity when pyruvate, $^{14}CO_2$ and malate were added to the reaction system indicated reversibility of the reaction. Limited CO_2 fixation was also observed in reaction mixtures containing PEP or PEP and ADP. These results, therefore, do not exclude the possibility of some PEP-carboxylase and PEP-carboxykinase activity

in the cell extracts. However, the observation that no CO_2 was fixed in the presence of pyruvate and ATP indicated that pyruvate carboxylase was not involved in this reaction.

Pyruvate and NADPH were specifically required for incorporation of radioactive CO_2 (Table III). The relatively high labelling in mixtures without added $MgCl_2$ may be due to divalent cations in the cell extracts.

Activity of PEP-carboxylase, the major CO_2-fixing enzyme in cell extracts of Escherichia coli, is under control by intermediates of the tricarboxylic acid-cycle and related compounds (6). No significant effect on CO_2-fixation by Micrococcus enzyme was found when malate, oxaloacetate, succinate or aspartate (9,792, 7,040, 7,248 counts per min. respectively, control, 7,022) was added to the reaction mixture. The slightly higher incorporation with malate added indicated reversibility of the reaction. These results, and the fact that hydrazones were not radioactive in any of the reaction mixtures, indicate that oxaloacetate was not formed by carboxylation of pyruvate.

TABLE III: Component requirement for CO_2 fixation by the cell-free extracts of Micrococcus No. 407

Reaction system*	Total CO_2 fixed (counts/min)
Complete	8,096
with boiled extracts	16
pyruvate omitted	336
$MgCl_2$ omitted	4,288
NADPH omitted, NADH added	688

*Reaction system contained: 0.5 ml cell-free extracts; $NaH^{14}CO_3$, 1 µcurie; $NaHCO_3$, 10 µmoles; tris Cl buffer (pH 7.5), 100 µmoles; $MgCl_2$, 5 µmoles; pyruvate, 10 µmoles; cysteine, 2.5 µmoles; NADPH or NADH, 1 µmoles and water to 1.5 ml.

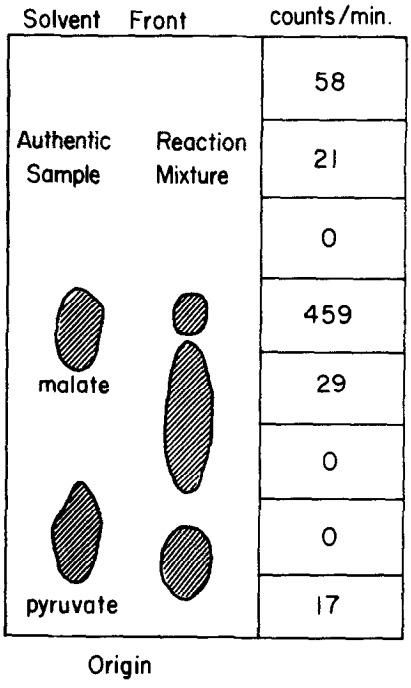

Figure 2. Demonstration of malate formation by malic enzyme. Reaction mixture as described for complete system in Table III. Chromatogram developed in n-butanol, formic acid, water (10, 2, 8) solvent system. Counts/min shown indicate C^{14} activity in 50 µl sample.

The reaction product of CO_2 fixation was identified as malic acid by paper chromatography (Fig. 2). All C^{14} activity in a sample of reaction system migrated the same distance as the authentic malic acid in two solvent systems. The identification of enzymically formed malic acid was confirmed by eluting the radioactive spot and rechromatographing the eluant with authentic malic acid.

Malic enzyme, which catalyses the reductive decarboxylation of malate, was one of the first enzymes shown to fix CO_2 in animal tissues (3). Although malic enzyme has the potential for synthesis of C4-dicarboxylic acids from CO_2 and pyruvate its action in this manner has not previously been shown in bacteria.

Jacobson et al. (8) concluded that the metabolic role of malic enzyme of Pseudomonas putida was decarboxylation under conditions when C4 compounds were available to the cells. Studies on CO_2 fixation in Enterobacteriaceae indicated that C4-acids were formed from C3-acids by the catalytic action of PEP-carboxylase and that malic enzyme was not involved in this reaction (2,1). However, present findings that malic enzyme may be involved in CO_2 fixation and that its production is influenced by bicarbonate, suggest that the physiological role of this enzyme in Micrococcus No. 407 is in an anaplerotic sequence primarily to supply C4-dicarboxylic acids. Studies on regulation of the formation and activity of malic enzyme are now in progress.

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