MALATE CLEAVAGE REACTION IN PSEUDOMONAS SPECIES, (SHAW STRAIN MA).

Louis B. Hersh* and Edward Bellion

From the Department of Biochemistry, The University of Texas Southwestern Medical School, Dallas, Texas 75235 and the Department of Chemistry, The University of Texas at Arlington, Arlington, Texas 76010.

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SUMMARY

Extracts of a Pseudomonas species (Shaw strain MA) contain an inducible malate cleavage activity: Malate + ATP + CoA $\rightleftharpoons$ Glyoxylate + AcCoA + ADP + Pi. The stoichiometry of the forward reaction was shown to be one mole of glyoxylate produced per mole of ATP hydrolyzed, while in the reverse reaction all three products; malate, ATP, and CoA, were formed in equivalent amounts. The forward reaction shows a broad pH optima of 7-8, while the reverse reaction has a fairly sharp optima of pH 8.2. It is suggested that this enzyme plays a key role in the metabolism of reduced one-carbon compounds by Pseudomonas species-MA.

INTRODUCTION

Although a number of microorganisms are known to grow on reduced one-carbon compounds via a pathway involving serine as a key intermediate, the intimate steps of the metabolic pathway have yet to be elucidated (1). It is generally agreed that the reduced one-carbon compound is first oxidized to the level of formaldehyde, and then combines with glycine to yield serine (1). The key metabolic problem yet to be solved, is a mechanism for the regeneration of glycine required for serine synthesis.

We wish to report the finding of an inducible malate lyase (ATP cleaving-CoA acetylating) activity in extracts of a Pseudomonas species (Shaw strain MA) when the organism is grown on methylamine. The enzymatic cleavage of malate into acetyl CoA and glyoxylate has been characterized in only one

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other organism, Rhodospeudomonas spheroides (2-4), although Stern has reported finding this activity in Escherichia coli, Rhodospirillum rubrum, and rabbit liver (5). The metabolic function of the malate cleavage reaction, although unclear in Rhodospeudomonas spheroides, provides in Pseudomonas sp. (Shaw strain MA), a pathway for the net synthesis of acetyl CoA from two one-carbon units, and the regeneration of glycine (via glyoxylate).

RESULTS

Cell free extracts of Pseudomonas species, Shaw strain MA, (ATCC No. 23819) obtained from cells grown on methylamine contain an ATP - CoA dependent malate cleavage activity (Malate + ATP + CoA $\rightleftharpoons$ AcCoA + glyoxylate + ADP + Pi). Extracts of the same organism prepared from cells grown on acetate or succinate do not contain this activity.

The enzyme was purified approximately forty-fold as follows: Growth of the organism, cell extraction, 30 to 60% ammonium sulfate fractionation, and chromatography on Bio-Gel A 1.5 m were performed as described for the preparation of N-methyl glutamate synthetase (6) except that glutamate and FMN were deleted from the buffers. The enzyme obtained from the A 1.5 m column was concentrated using an Amicon ultrafiltration device and dialyzed against 20 mM potassium phosphate buffer, pH 7.4, containing 5 mM 2-mercaptoethanol. The dialyzed enzyme was applied to a DEAE-cellulose column (2.5 x 30 cm) previously equilibrated with the 20 mM potassium phosphate buffer. The enzyme was eluted with a linear gradient of 0 to 0.25 M potassium chloride (250 ml in the mixing chamber and 250 ml in the reservoir) and concentrated as described above. The Bio-Gel A 1.5 m and DEAE-cellulose steps were then repeated. A summary of the purification scheme is shown in Table I.

The stoichiometry of the reaction in both the forward and reverse directions is shown in Figure 1. In the forward reaction glyoxylate and ADP are formed at equal rates, while in the reverse reaction the rates of malate, ATP, and CoA formation are identical. Measuring either glyoxylate or ADP formation in the forward direction, no reaction was observed in the

TABLE I: Purification of Malate Cleavage Enzyme

Step	Total Protein (mg)	Total Activity (units)	S. A.	%
Extract	4432	164	0.037	(100)
30/60% $AmSO_4$	4018	162	0.040	99
Agarose A 1.5 m	979	139	0.141	85
DEAE-cellulose	480	101	0.210	62
Agarose A 1.5 m	239	76	0.32	38
DEAE-cellulose	52	43	0.80	26

Enzyme activity was determined by following glyoxylate formations according to the procedure of Dixon and Kornberg (7). Reaction mixtures contained 50 mM Tris hydrochloride buffer, pH 7.4, 3.6 mM phenylhydrazine, 10 mM L-malate, 3 mM ATP, 6 mM magnesium chloride, and 0.13 mM CoA in a final volume of 1.0 ml.

The formation of glyoxylate phenylhydrazine was followed at 324 nm. One unit of activity corresponds to the formation of 1 μmole of product per minute at 30°.

Protein was determined by the biuret method (8) in the crude extract and the 30/60% ammonium sulfate step, and thereafter by the method of Warburg and Christian (9).

absence of either malate, ATP, CoA, or Mg^{++}. The requirement for ADP and Pi in the reverse reaction serves to rule out malate synthetase as the enzyme responsible for malate formation.

The Michaelis constants for substrates of the forward and reverse reaction are summarized in Table II. In order to determine whether free ATP or a Mg^{++} - ATP complex is the reactive species, the effect of magnesium on the reactivity of ATP was investigated. As shown in Figure 2, a double reciprocal plot measuring the effect of Mg^{++} concentration on the reaction rate at a fixed level of ATP results in a triphasic curve. At concentrations of Mg^{++} equal to or less than the concentration of ATP, the reaction follows typical Michaelis-Menton kinetics. When the concentration of Mg^{++} exceeds that of ATP up to a ratio of Mg^{++}/ATP of about 50, the reaction is independent of Mg^{++}; at higher concentrations of Mg^{++} inhibition is observed.

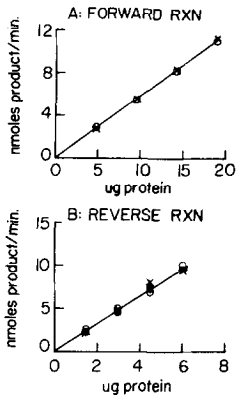


Fig. 1.

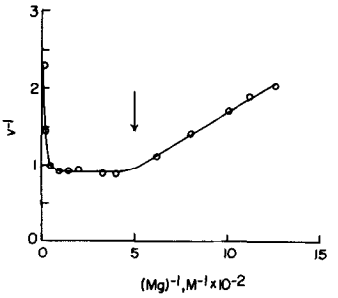


Fig. 2.

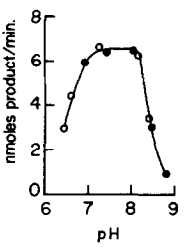


Fig. 3.

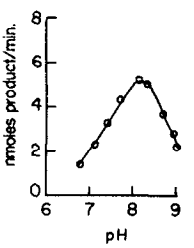


Fig. 4.

Figure 1: Stoichiometry of Malate Cleavage Reaction.

A. Forward Reaction: Reaction mixtures identical to those described in Table I were used to measure glyoxylate formation (0), except that potassium chloride was added to a final concentration of 50 mM. ADP formation (X) was measured by coupling the reaction to lactate dehydrogenase and pyruvate kinase. In place of phenylhydrazine, 0.125 μmoles DPNH, 5 units of lactate dehydrogenase, and 2 units pyruvate kinase were added.

B. Reverse Reaction: Reaction mixture contained 50 mM N-ethylmorpholine buffer, pH 7.5, 0.1 mM ADP, 1.0 mM glyoxylate, 10 mM potassium phosphate pH 7.5, 5 mM magnesium chloride, and 50 μM acetyl CoA. CoA release [0] was measured by carrying out the reaction in the presence of 0.2 mM DTNB. For measuring ATP formation (●), hexokinase (20 μg), glucose 6-phosphate dehydrogenase (2.5 units), glucose (10 mM), and TPN (0.125 mM) were added. For measuring malate formation (X) pig heart malic enzyme (5 units), and TPN (0.125 mM) were added.

Figure 2: Effect of Mg^{++} on the Reactivity of ATP.
Reaction mixtures identical to that of Table I, except that ATP was held constant at 2 mM and magnesium was varied as indicated. v^{-1} expressed as $(\mu moles/min/unit\ of\ enzyme)^{-1}$. The arrow denotes the equivalence point for magnesium and ATP.

Figure 3: Effect of pH on the Forward Malate Cleavage Reaction.
The forward reaction was measured by following ADP formation as described in Figure 1. 0 = Tris-hydrochloride buffer, ● = N-ethylmorpholine buffer.

Figure 4: Effect of pH on the Reverse Malate Cleavage Reaction.
The reverse reaction was measured by following ATP formation as described in Figure 1

This result is consistent with an active Mg^{++}-ATP complex formed at approximately equimolar concentrations. Above this 1-1 stoichiometry, no effect of Mg^{++} is observed until presumably inactive ATP complexes are formed at very high Mg^{++} concentrations.

Substrate Specificity: A number of compounds were tested as alternate substrates for the enzyme. L-malate could not be replaced by 20 mM D-malate, D,L α-hydroxyglutarate, D,L citramalate, or meso-tartrate. ATP was not replaced by 3 mM GTP, ITP, CTP, UTP, or TTP. Magnesium could be replaced by cobalt or manganese, 63% and 38% reactivity when compared to magnesium. Zn^{+2}, Ca^{+2}, and Ni^{+2} at concentrations equal to Mg^{+2} result in 62-78% inhibition of the reaction.

In the reverse reaction ADP could not be replaced by 0.01 mM GDP, UDP, or CDP, while glyoxylate could not be replaced by 1 mM acetaldehyde or 1 mM pyruvate. Phosphate could be replaced by arsenate, 67% activity at 10 mM arsenate compared to 10 mM phosphate.

pH-Rate Profiles: The effect of pH on the forward reaction is shown in Figure 3, while that of the reverse reaction is shown in Figure 4. It is interesting to note that the forward reaction exhibits a broad optima from pH 7 to 8, while the reverse reaction has a fairly sharp maxima at pH 8.2. The broad pH optima for the forward reaction differs from the fairly sharp optima of 7.8 found by Tuboi and Kikuchi (3).

Inhibition of the Reaction by Succinate: Tuboi and Kikuchi (3) noted that succinate was a potent inhibitor of partially purified malate cleavage enzyme. These workers proposed that this inhibition was due to a competition for coenzyme A by succinate thiokinase. A reinvestigation of this phenomena showed that succinate inhibition can be attributed to classical

TABLE II: Kinetic Constants for Malate Cleavage Enzyme

SUBSTRATE	Km (mM)
Forward Reaction	
Malate	0.30
ATP	0.36
CoA	0.037
Reverse Reaction	
Glyoxylate	1.2
Acetyl CoA	0.021
ADP	0.003
Pi	28

Kinetic constants for the forward reaction were determined by following glyoxylate formation as described in Table I. Reaction mixtures contained 50 mM Tris-hydrochloride buffer, pH 7.4, 3.6 mM phenylhydrazine, and when not varied 3 mM malate, 3 mM ATP, and 0.15 mM CoA. The ratio of Mg/ATP was held constant at 2.0.

Kinetic constants for the reverse reaction were determined by following CoA release with Ellman's reagent. Reaction mixtures contained 50 mM N-ethyl morpholine buffer, pH 8.1, 0.2 mM DTNB and when not varied 1.0 mM glyoxylate, 0.05 mM AcCoA, 0.01 mM ADP, and 10 mM Pi. When phosphate was varied the ratio of Mg^{++}/Pi was held constant at 0.5.

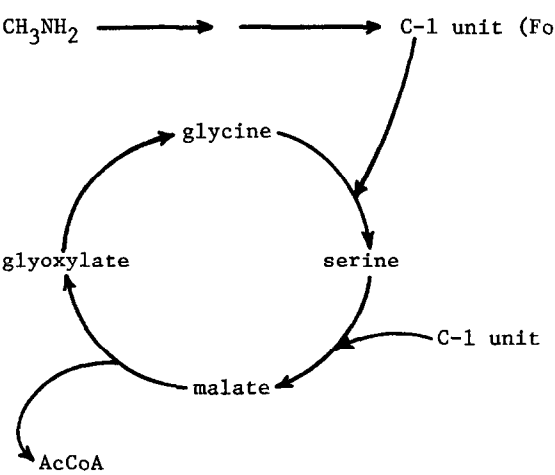
competitive inhibition with respect to malate. At the following substrate concentration: ATP = 3.0 mM, Malate = 0.3 mM, and CoA = 2 mM, a plot of $1/v$ vs the concentration of succinate gave an apparent KI of 0.24 mM. In addition to succinate, D-malate, glutarate, and D,L-isocitrate were also found to be competitive inhibitors with respect to malate. Under the above stated conditions the calculated KI values are 0.8 mM, 4.4 mM, and 5.2 mM respectively.

Molecular Weight: An estimation of the molecular weight of the malate

cleavage enzyme was obtained by the method of Andrews (10) using Bio-Gel A 1.5 m. Using urease, pyruvate kinase, fumarase, and alcohol dehydrogenase as standards, a molecular weight of 275,000 was estimated.

DISCUSSION

The data presented in this communication clearly establish the presence of an ATP-CoA dependent malate cleavage reaction in Pseudomonas sp. MA. The involvement of the malate cleavage reaction in one-carbon metabolism is suggested by the specific induction of the enzyme by methylamine. Previous work in this laboratory has provided evidence that methylamine is oxidized to formaldehyde via the intermediate formation of N-methylglutamate (11). Unpublished experiments (E. Bellion and L. Hersh, manuscript in preparation) suggest that formaldehyde can condense with glycine to yield serine, and that serine reacts with a one-carbon unit to eventually yield malate. We thus propose that the function of the malate cleavage reaction is to regenerate the glycine (via glyoxylate) needed for serine synthesis, and yield a net conversion of two one-carbon units into acetyl CoA:



We have also found this cleavage enzyme, malate lyase (CoA acetylating), in extracts of Hyphomicrobium WC after growth on methylamine, (J. C. Spain and E. Bellion, unpublished results), lending further support to the suggestion of its involvement in one-carbon metabolism.

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