The Malic Enzyme from *Trypanosoma cruzi*

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INTRODUCTION

Cell-free extracts from the culture form of *Trypanosoma cruzi*, the causative agent of American trypanosomiasis, Chagas' disease, contain a NADP-linked malic enzyme (EC. 1.1.1.40; Raw, 1959) which catalyses the decarboxylation of L-malate to pyruvate and CO₂, with the concomitant reduction of NADP to NADPH. Raw (1959) suggested this enzyme was responsible for the CO₂ fixation which occurs during glucose oxidation by intact epimastigotes, and which yields succinate as a final product (Bowman, Tobie & von Brand, 1963). However, in most organisms studied, the malic enzyme is thought to decarboxylate L-malate (Kornberg, 1966; Sanwal & Smando, 1969).

In this paper, we describe some properties of the partially purified malic enzyme from *T. cruzi*.

METHODS

Partial purification of the enzyme. *Trypanosoma cruzi*, Tulahuén strain, was grown and the cells were harvested and washed as described previously (Gerez de Burgos *et al.*, 1976). The strain was maintained at 30 °C in a biphasic medium supplemented with haemin (20 mg l⁻¹); this was omitted from the pre-harvest cultures. The parasites (mostly epimastigotes with about 2 % trypomastigotes) were suspended (100 mg wet wt ml⁻¹) in 50 mM-Tris/HCl buffer, pH 7.6, containing 1 mM-EDTA, 10 mM-MgCl₂ and 0.2 M-KCl, and broken in a Sorvall Omnimixer at 6000 rev. min⁻¹ for 4 min in an ice bath. The homogenate was centrifuged at 12000 g for 15 min at 4 °C, and the stirred supernatant was brought to 50 % saturation at 0 °C with saturated ammonium sulphate solution (adjusted to pH 7 with NH₄OH containing 1 mM-EDTA). The suspension was then centrifuged at 37000 g for 20 min at 4 °C, the pellet was discarded, and the supernatant was brought to 75 % saturation with saturated ammonium sulphate solution and centrifuged as before. The pellet was dissolved in 0.8 ml 50 mM-Tris/HCl buffer, pH 7.6, containing 1 mM-EDTA and 0.4 M-KCl, and loaded on to a column (31 × 1.2 cm) of Sephadex G-200 and eluted with the same buffer at 6.1 ml h⁻¹ at 6 °C. Fractions (1 ml) were collected and assayed for protein and malic enzyme activity. The most active fractions were pooled; in a representative preparation, malic enzyme was purified about sevenfold [sp. act., 0.61 µmol NADPH formed min⁻¹ (mg protein)⁻¹] with a yield of 63 %. Little parasite material was available (about 1 g batches) which prevented further purification of the enzyme. The partially purified preparation contained malate dehydro-

genase (EC. 1.1.1.37) which, being strictly NAD specific, did not interfere with the enzyme assays even in the presence of oxaloacetate.

Assay methods. Protein was estimated in crude extracts and ammonium sulphate fractions by the method of Lowry *et al.* (1951) using bovine serum albumin as standard; it was estimated in the Sephadex G-200 fractions by the spectrophotometric method of Warburg & Christian (1941).

Malic enzyme was assayed using a Pye Unicam SP1800B recording spectrophotometer by measuring the increase in extinction at 340 nm concomitant with the reduction of NADP at 30 °C. Unless stated otherwise, the final reaction mixtures contained ($\mu\text{mol ml}^{-1}$): Tris/HCl buffer (pH 7.6), 50; L-malate, 5; NADP, 0.12; MnCl_2 , 1; and 0.015 ml enzyme preparation. The reaction was started by adding the enzyme and activity was measured after 1 min.

The radiochemical assay for $^{14}\text{CO}_2$ fixation on pyruvate used a standard reaction mixture containing (μmol) in a final volume of 0.4 ml: Tris/HCl buffer (pH 7.6), 20; MnCl_2 , 0.1; sodium pyruvate, 4; NADPH, 0.12; $\text{NaH}^{14}\text{CO}_3$ (2.2 μCi), 4; and 0.03 ml enzyme preparation. The reactions were started by adding the enzyme and, after 15 min incubation at 30 °C, were stopped by adding 1.2 ml absolute ethanol. The samples were processed and counted, and the enzyme activity was expressed as previously described (Vidal & Cazzulo, 1972).

Determination of molecular weight. The approximate molecular weight of the malic enzyme was determined by gel filtration through Sephadex G-200, according to Andrews (1965). The experimental conditions were similar to those used for the gel filtration step of the purification method. The proteins used as markers were bovine serum albumin (Sigma; 5 mg; mol. wt 68000), citrate synthase (EC. 4.1.3.7; Boehringer; 0.02 mg; mol. wt 100000), lactate dehydrogenase (EC. 1.1.1.27; Boehringer; 0.2 mg; mol. wt 140000), and pyruvate kinase (EC. 2.7.1.40; Sigma; 0.02 mg; mol. wt 240000). The corresponding elution volumes were 25.8, 23.1, 20.7 and 17 ml, respectively. The elution volume of malic enzyme (4 mg of the 50 to 75 % ammonium sulphate fraction) was 21 ml. The void volume of the column was 12.7 ml as determined with Blue Dextran (Pharmacia). Bovine serum albumin was assayed in a separate experiment by measuring the extinction at 280 nm; lactate dehydrogenase was assayed as described by Andrews (1965), citrate synthase as described by Srere (1969) and pyruvate kinase as described by Bücher & Pfeleiderer (1955).

RESULTS AND DISCUSSION

The partially purified malic enzyme from *T. cruzi* catalysed both the decarboxylation of L-malate in the presence of NADP, and the reductive carboxylation of pyruvate (Table 1). The reaction velocity was about 40-fold greater in the direction of decarboxylation. This result agrees well with the role generally ascribed to malic enzyme, i.e. the production of pyruvate, and of NADPH for lipid biosynthesis (Sanwal & Smando, 1969). A role for the enzyme in the anaplerotic (Kornberg, 1966) fixation of CO_2 has not been suggested except in a *Micrococcus* species (Matula, McDonald & Martin, 1969). Our results, together with those reported by Marr (1973) for the enzyme from *Crithidia fasciculata*, suggest that malic enzyme in trypanosomatids is not responsible for CO_2 fixation. In *C. fasciculata*, there is evidence for the presence of pyruvate carboxylase (EC. 6.4.1.1) and phosphoenolpyruvate carboxykinase (EC. 4.1.1.32) (Bacchi *et al.*, 1970); the latter enzyme is also present in *T. cruzi* (Cannata, Cataldi de Flombaum, Segura & Cazzulo, unpublished results).

The malic enzyme from *T. cruzi* was NADP-linked; the low activity with NAD in the decarboxylation reaction (Table 1) was probably due to the action of malate dehydrogenase. The optimum pH for the decarboxylation reaction, determined in the presence of 0.1 M-Tris/acetate buffers, was about 8.5.

Table 1. *Requirements of the reaction catalysed by the malic enzyme from Trypanosoma cruzi*

The basal reaction mixtures were those described in Methods, with the omissions or additions indicated. The enzyme preparation was exhaustively dialysed against 50 mM-Tris/HCl buffer, pH 7.6, containing 1 mM-EDTA immediately before use.

Additions or omissions	Reaction velocity [nmol min ⁻¹ (mg protein) ⁻¹]
Decarboxylation of L-malate	
None	468
Plus 35 mM-NH ₄ Cl	205
Minus L-malate	0
Minus NADP	0
Minus NADP, plus 0.12 mM-NAD	45
Minus MnCl ₂	0
Carboxylation of pyruvate	
None	11.8
Plus 26 mM-NH ₄ Cl	3.1
Minus Na pyruvate	0.8
Minus NADPH	0
Minus NADPH, plus 0.3 mM-NADH	0.4
Minus MnCl ₂	0.8

The divalent cation requirement was satisfied by very low concentrations of MnCl₂ (Table 1) or CoCl₂; both cations at 1 μ M gave a reaction velocity which was about 70 % of that in the presence of 1 mM-MnCl₂. Concentrations of CoCl₂ higher than 0.2 mM caused a relative inhibition, which was not observed with MnCl₂ up to 1 mM. MgCl₂ was a very poor activator, even at 10 mM. This low efficiency of MgCl₂ as an activator, together with the lack of activation and, indeed, inhibition by monovalent cation salts, such as NH₄Cl (Table 1), differentiates the malic enzyme of *T. cruzi* from that of some bacteria (Massarini & Cazzulo, 1975; Vidal & Cazzulo, 1976).

The malic enzyme from *T. cruzi* differed from that of both eukaryotic and prokaryotic organisms (Hsu & Lardy, 1969; Massarini & Cazzulo, 1975) in that the apparent K_m for L-malate was considerably higher with the flagellate enzyme. However, like the malic enzyme from a marine pseudomonad (Massarini & Cazzulo, 1975), the value of the apparent K_m for L-malate depended on the nature of the divalent cation activator, with 0.12 mM-NADP the values were 0.76 and 12.5 mM in the presence of 1 mM-CoCl₂ or MnCl₂, respectively. The apparent V_{max} was about threefold higher with MnCl₂. At a fixed concentration of 7.5 mM-L-malate, the apparent K_m for NADP was 30 μ M in the presence of either CoCl₂ or MnCl₂. The apparent K_m values for L-malate and NADP in the presence of 10 mM-MgCl₂ were very high, 74.3 mM and 0.33 mM, respectively. The apparent kinetic constants were obtained from double-reciprocal plots (Lineweaver & Burk, 1934) which were linear in all cases.

The molecular weight of the malic enzyme from *T. cruzi*, determined by gel filtration through Sephadex G-200 (Andrews, 1965), was about 130000 daltons. This value is about half of that of the pigeon liver enzyme, which is 280000 daltons (Hsu & Lardy, 1969), and about a third of the molecular weight of the *Escherichia coli* enzyme, which has been reported to be 345000 $\pm$ 15000 daltons (Sanwal & Smando, 1969). Since the pigeon liver enzyme is a tetramer (Hsu & Lardy, 1969), it is tempting to speculate that the *T. cruzi* enzyme might be a dimer, the basic subunit being of similar weight in both cases.

The malic enzyme from *T. cruzi* was inhibited by oxaloacetate, which caused the saturation curve for the substrate L-malate to become sigmoid; the apparent h values, obtained from Hill plots (Monod, Wyman & Changeux, 1965), were 1.1 in the absence of oxaloacetate and about 1.5 in the presence of 0.7 mM or 1.4 mM-oxaloacetate. The $V_{\max}$ was apparently not affected by the inhibitor, but the $[S]_{0.5}$ values (concentration of substrate for half-maximum velocity) obtained from the Hill plots were 12, 17.6 and 25.5 mM-L-malate in the absence of oxaloacetate, and in the presence of 0.7 mM and 1.4 mM-oxaloacetate respectively. The inhibition by oxaloacetate was of the mixed type towards NADP; the apparent K_m for NADP increased from 30 μ M without the inhibitor to 50 μ M with 1.5 mM-oxaloacetate, with a concomitant threefold decrease in the $V_{\max}$. The linearity of the double-reciprocal plots for NADP was not affected by the inhibitor. These results suggest that the inhibition of the malic enzyme from *T. cruzi* by oxaloacetate, as in the case of the enzyme from *E. coli* (Sanwal & Smando, 1969) and one of the NADP-linked malic enzymes from *Neurospora crassa* (Zink, 1972), is allosteric, L-malate being the target substrate (Sanwal, 1970) for the inhibitor in all cases.

The K_i for oxaloacetate was 0.21 mM as calculated from Dixon plots (Dixon & Webb, 1964) of the reciprocal reaction velocity as a function of the concentration of oxaloacetate in the presence of three fixed concentrations of L-malate (2, 5 and 10 mM). This value is considerably higher than that reported for the *E. coli* enzyme (Sanwal & Smando, 1969) and raises some doubts about the physiological value of this inhibition. The possibility that the high K_i value was due to decarboxylation of the inhibitor in the reaction mixtures seems unlikely; at pH 7.6 the production of pyruvate from 1 mM-oxaloacetate, measured with lactate dehydrogenase and NADH in reaction mixtures for malic enzyme lacking NADP and containing the same amount of enzyme preparation used in the kinetic experiments, was only about 6 nmol min⁻¹. Marr (1973) determined an even higher K_i value for oxaloacetate (0.7 mM) for the malic enzyme of *C. fasciculata*; however, he proposed that the inhibition might be physiological, because compartmentation might allow a relatively high local concentration of the inhibitor.

Glucose catabolism in *T. cruzi* leads to the excretion of considerable amounts of succinate into the medium, even in aerobiosis (Bowman, 1974). The inhibition of the malic enzyme by oxaloacetate [probably produced, as in some invertebrates (Hochachka & Mustafa, 1972), by CO₂ fixation via phosphoenolpyruvate carboxykinase], might be involved in the regulation of this aerobic fermentation, in order to avoid a recycling of C₄ dicarboxylic acids to pyruvate. A similar suggestion has been proposed by Marr (1973) for *C. fasciculata*. If this were so, the production of NADPH for biosynthesis would not be seriously impaired by the inhibition of the malic enzyme, since epimastigotes of *T. cruzi* contain high activities of isocitrate dehydrogenase (Agosin & Weinbach, 1956) and glutamate dehydrogenase (unpublished results), both of which are NADP-linked.

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