Two Forms of 'Malic' Enzyme with Different Regulatory Properties in *Trypanosoma cruzi*

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1. Cell-free extracts from culture epimastigotes of *Trypanosoma cruzi* contained two forms of NADP⁺-linked 'malic' enzyme (EC 1.1.1.40), I and II, with the same molecular weight but different electrophoretic mobilities and kinetic and regulatory properties. 2. The apparent K_m for L-malate was lower for 'malic' enzyme I, with hyperbolic kinetics, whereas the kinetic pattern for 'malic' enzyme II was slightly sigmoidal (h 1.4). The kinetics for NADPH were hyperbolic for 'malic' enzyme I, and very complex for 'malic' enzyme II, suggesting both positive and negative co-operativity. 3. 'Malic' enzyme II was markedly inhibited by adenine nucleotides; AMP was the most effective, at least in the presence of an excess of MnCl₂. 'Malic' enzyme I was much less affected by the nucleotides. Both enzyme forms were inhibited by oxaloacetate, competitively towards L-malate, but the apparent K_i for 'malic' enzyme I (9 μ M) was 10-fold lower than the value for 'malic' enzyme II. 'Malic' enzyme II, but not 'malic' enzyme I, was activated by L-aspartate and succinate (apparent K_a of 0.12 and 0.5 mM respectively); the activators caused a decrease in the apparent K_m for L-malate and, to a lesser extent, in the apparent K_m for NADP⁺. L-Aspartate, but not succinate, increased the apparent V_{max} . 4. The inhibition by AMP suggests regulation by energy charge, with the L-malate-decarboxylation reaction catalysed by 'malic' enzyme II fulfilling a biosynthetic role. The inhibition by oxaloacetate and the activation by succinate are probably involved in the regulation of the 'partial aerobic fermentation' of glucose which yields succinate as final product. The activation by L-aspartate would facilitate the catabolism of this amino acid, when present in excess in the growth medium.

Trypanosoma cruzi, the causative agent of South American trypanosomiasis (Chagas' disease) aerobically catabolizes glucose only partially to CO₂, with a substantial amount of glucose carbon being excreted into the medium as succinate and acetate; the percentage values are respectively 55, 12 and 17% for the bloodstream form, and 32, 34 and 20% for the culture epimastigote form (Bowman, 1974). Because of this incomplete glucose oxidation, *T. cruzi* has been considered to be a 'partial aerobic fermenter' (von Brand, 1967). The synthesis of succinate, which is accumulated to maintain the redox balance of the nicotinamide nucleotide coenzymes (Bowman, 1974), necessarily requires fixation of CO₂ on a C₃ acid (Bowman *et al.*, 1963). Cell-free extracts from epimastigotes of *T. cruzi* contain NADP⁺-linked 'malic' enzyme (L-malate-NADP⁺ oxidoreductase, decarboxylating; EC

1.1.1.40), which catalyses the decarboxylation of L-malate to pyruvate and CO₂ with the concomitant reduction of NADP⁺ to NADPH (Raw, 1959); we have partially purified the enzyme, and studied some of its properties (Cazzulo *et al.*, 1977). Raw (1959) suggested that 'malic' enzyme was responsible for the CO₂ fixation that occurs concomitantly with glucose catabolism and succinate production by *T. cruzi*; our studies suggest, however, that this role is fulfilled instead by an ADP-linked phosphoenolpyruvate carboxykinase (EC 4.1.1.49) (Cataldi de Flombaum *et al.*, 1977), and 'malic' enzyme decarboxylates L-malate, as in most organisms studied (Kornberg, 1966; Sanwal & Smando, 1969). C₄ dicarboxylic acids, in order to be degraded through the tricarboxylic acid cycle, must first be converted to L-malate or oxaloacetate and decarboxylated to pyruvate, this then being taken into the cycle as acetyl-CoA. Activation of 'malic' enzyme by some C₄ dicarboxylic acids would therefore be useful when these acids are present in substantial amounts in the

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culture medium; such a situation arises during the last stages of growth of epimastigotes in culture, when, after the glucose in the medium is exhausted, the succinate initially excreted into the medium is incorporated again and catabolized (Cáceres & Fernandes, 1976). On the other hand, during 'partial aerobic fermentation' of glucose with production of succinate, the decarboxylating activity of 'malic' enzyme should be decreased, in order to prevent wasteful recycling of C_4 dicarboxylic acids to C_3 monocarboxylic acids. Inhibition of the enzyme by oxaloacetate (Cazzulo *et al.*, 1977) is probably important in this context. A rather complex regulation of 'malic' enzyme activity would be essential for the control of glucose catabolism in the parasite.

During the course of kinetic studies some strikingly atypical results made us suspect the presence of more than one enzyme form, with different kinetic behaviour. We describe here the isolation of two forms of $NADP^+$ -linked 'malic' enzyme from epimastigotes of *T. cruzi*, and some of their kinetic and regulatory properties.

Materials and Methods

Organism and culture

T. cruzi (Tulahuén strain) was cultured, the cells were disrupted and the cell-free extract was obtained as previously described (Cataldi de Flombaum *et al.*, 1977).

Enzyme purification

The cell-free extract (in a typical preparation, 64.1 ml was obtained from 6 g wet wt. of cells) was fractionated with $(NH_4)_2SO_4$: 18.7 g of solid $(NH_4)_2SO_4$ was added, with stirring, at $0^\circ C$; after centrifugation at 32000 g for 20 min at $4^\circ C$, the precipitate was discarded, and to the supernatant (70 ml) was added 11.1 g of solid $(NH_4)_2SO_4$. The precipitate was collected by centrifugation as above, dissolved in 1.0 ml of 50 mM-Tris/HCl/1 mM-EDTA/0.4 M-KCl buffer, pH 7.6, and percolated through a Sephadex G-200 column (2.5 cm $\times$ 40 cm); fractions (4 ml) were collected at a rate of 15 ml/h at $4^\circ C$. The active fractions were pooled (19.5 ml), precipitated with 58.5 ml of satd. $(NH_4)_2SO_4$ solution at $0^\circ C$, and the precipitate was collected by centrifugation as above, dissolved in 1.1 ml of 22.5 mM-Tris/12.5 mM-boric acid/0.4 mM-EDTA buffer, pH 8.6, and dialysed against 500 vol. of the same buffer for 5 h at $0^\circ C$; the volume after dialysis was 2.3 ml. The solution was subjected to starch-gel electrophoresis by the method of Smithies (1959). Gels (22 cm $\times$ 1.2 cm $\times$ 0.6 cm) were made up of 10% (w/v) starch in 22.5 mM-Tris/12.5 mM-boric acid/0.4 mM-EDTA buffer, pH 8.6. The enzyme solution was pipetted into small slots in the gel. Each

gel contained eight slots which enabled the insertion of up to 1 ml of sample (approx. 0.12 ml per slot). The gel was connected by filter paper wicks to electrode tanks that contained 90 mM-Tris/50 mM-boric acid/1.6 mM-EDTA buffer, pH 8.6, the upper one (cathode) and 64.3 mM-Tris/35.7 mM-boric acid/1.14 mM-EDTA buffer, pH 8.6, the lower one (anode). Electrophoresis was carried out at $2^\circ C$, a voltage gradient of 7 V/cm (6 mA/gel) being applied for 18 h. After electrophoresis, a narrow strip from one of the edges comprising one slot was sliced horizontally and the two 'malic' enzyme forms were identified by overlaying the gel with a solution containing 25 mM-L-malate, 0.15 mM- $NADP^+$, 2 mM- $MnCl_2$, phenazine methosulphate (0.05 mg/ml) and Blue Tetrazolium (0.08 mg/ml) in 50 mM-Tris/HCl buffer, pH 7.6. After incubation at $30^\circ C$ in the dark for 1–3 h, the areas containing enzyme activity were made visible by the formation of the deep-blue precipitate of formazan. With the aid of the stained strip, zones of the unstained block corresponding to both molecular forms of the 'malic' enzyme were cut out. Elution of the enzyme was performed as described by Smithies (1955). The excised pieces of starch gel were frozen at $-30^\circ C$; then they were thawed in the cold-room at $4^\circ C$, and eluates were obtained by squeezing the sponge-like pieces with the aid of a nylon cloth.

For the reasons stated in the Results section, it was advantageous to carry out the electrophoresis in the presence of 20% (v/v) glycerol added to the gel mixture described above. In this case a voltage gradient of 10 V/cm (9 mA/gel) was used and the run lasted about 20 h. In spite of the fact that glycerol was not an inhibitor of the enzyme, staining of the gels for activity under these conditions was unsuccessful. For this reason, after electrophoresis, horizontal strips of the gels, approx. 10 mm wide, were cut out, eluted as described above, and the two forms of the 'malic' enzyme localized by measuring activity in the eluates.

The soluble starch used in the present work was prepared from potato starch, supplied by BDH, Poole, Dorset, U.K., by the method of Smithies (1955), except that the acetone/HCl treatment was carried out at $30^\circ C$ for 5.5 h.

Mobilities were not calculated for the starch-gel electrophoresis experiments, since they were essentially preparative and therefore the run was continued long after the tracking dye had left the gel in order to obtain better resolution.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis was performed by the method of Davis (1964), with 7.5% (v/v) acrylamide. Enzyme activity was detected on the gels by immersing them in about 2 ml of a reaction mixture containing 50 mM-Tris/HCl buffer, pH 8.0, 37.4 mM-

L-malate, 1.2 mM-NADP⁺, 2 mM-MnCl₂, phenazine methosulphate (0.1 mg/ml) and Blue Tetrazolium (1.0 mg/ml). The gels were incubated in the dark at 30°C until the bands of activity appeared (1–3 h). Gels run in parallel were stained for protein with Coomassie Blue. The amount of protein in the bands corresponding to enzyme activity was determined, as a percentage of the total protein in the gel, with a Chromoscan MK II densitometer.

Assay methods

'Malic' enzyme was assayed in the direction of L-malate decarboxylation in a Gilford 2000 recording spectrophotometer fitted with a temperature-controlled cuvette holder at 30°C, by measuring the increase in A_{340} concomitant with the reduction of NADP⁺. The reaction mixtures, unless stated otherwise, contained, in a final volume of 1 ml, 50 mM-Tris/HCl buffer, pH 7.6, 9 mM-L-malate, 0.15 mM-NADP⁺, 2 mM-MnCl₂ and enzyme preparation, which was added last to start the reaction.

The CO₂-fixing activity of 'malic' enzyme was assayed radiochemically. The reaction mixtures contained, in a final volume of 0.5 ml, 50 mM-Tris/acetate buffer, pH 5.4, 8 mM-NaH¹⁴CO₃ (59.2 μ Ci/ μ mol), sodium pyruvate, NADPH and MnCl₂, as stated in the legends to the Figures, and enzyme, which was added last to start the reaction. After incubation for 5 min at 30°C, the reactions were stopped and the samples processed and counted for radioactivity as previously described (Cataldi de Flombaum *et al.*, 1977).

Enzyme activities are expressed as nmol of NADP⁺ reduced, or ¹⁴CO₂ fixed, per min.

Protein was determined in crude extracts and (NH₄)₂SO₄ fractions by the method of Lowry *et al.* (1951), with bovine serum albumin as standard; in the Sephadex G-200 fractions it was determined by the spectrophotometric method of Christian & Warburg (1941), and in the electrophoresis eluates by the method of Bensadoun & Weinstein (1976), with the same standard.

The concentrations of nucleotide solutions were determined spectrophotometrically at their respective absorbance maxima. Oxaloacetate was assayed with malate dehydrogenase (Hohorst & Reim, 1963), pyruvate with lactate dehydrogenase (Bücher *et al.*, 1963) and L-malate with malate dehydrogenase (Hohorst, 1963).

Chemicals

L-Malic acid, oxaloacetic acid, L-aspartic acid, succinic acid, sodium pyruvate, NADP⁺, NADPH, all nucleotides, bovine serum albumin, malate dehydrogenase (EC 1.1.1.37) and lactate dehydrogenase (EC 1.1.1.27) were obtained from Sigma Chemical

Co., St. Louis, MO, U.S.A.; phenazine methosulphate was from Calbiochem, Los Angeles, CA, U.S.A.; Blue Tetrazolium was from BDH. NaH¹⁴CO₃ was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were analytical reagents.

Results

Presence of two forms of 'malic' enzyme in cell-free extracts of T. cruzi

When cell-free extracts from *T. cruzi* were subjected to polyacrylamide-gel electrophoresis and stained for enzyme activity two distinct enzyme activity bands were observed (Fig. 1a). The faster enzyme band will be referred to as 'malic' enzyme I and the slower one as 'malic' enzyme II. The same result was obtained with partially purified preparations obtained after gel filtration on Sephadex G-200. When a crude extract was subjected to starch-gel electrophoresis, a similar pattern was obtained (Fig. 1b). The latter procedure was applied on a preparative scale for further purification and separation of both enzyme forms from the Sephadex G-200 eluate. The two forms were eluted from the gel, and glycerol was added to a final concentration of 30% (v/v); this was then used for the experiments described below. When glycerol was not present during electrophoresis, the recovery was very poor, being at most 10% of the activity in the Sephadex G-200 eluate; moreover, when these separated enzyme forms were kept frozen for 24 h at -30°C in the absence of glycerol, over 90% of the activity was lost. Glycerol afforded considerable protection: the presence of 20% (v/v) glycerol during electrophoresis increased the yield to about 20%, and the eluted enzyme forms, in the presence of 30% (v/v) glycerol, were completely stable for at least 1 month at -30°C. The two enzyme forms were still not pure after electrophoresis, as shown in Figs. 1(c) to 1(f). The protein bands with 'malic' enzymes I (Fig. 1c) and II (Fig. 1e) activities were estimated by densitometry to account for about 25 and 20% of the total protein present in the respective gels (Figs. 1d and 1f). The electrophoretic mobilities in polyacrylamide-gel electrophoresis of 'malic' enzymes I (Fig. 1c) and II (Fig. 1e), relative to Bromophenol Blue, were 0.47 and 0.25 respectively. In a typical preparation, the specific activities of partially purified 'malic' enzyme II and 'malic' enzyme I were 3.1 and 1.1 μ mol/min per mg of protein respectively, when assayed in the direction of L-malate decarboxylation.

Effects of adenine nucleotides

As shown in Fig. 2(a), the L-malate-decarboxylating activity of 'malic' enzyme II was inhibited by adenine nucleotides at concentrations up to 2 mM, AMP being

Fig. 1. Electrophoretic patterns of 'malic' enzymes from *T. cruzi*

(a), (c), (d), (e) and (f) Polyacrylamide-gel electrophoresis; (b) starch-gel electrophoresis; (a) 0.2 mg of protein from a cell-free extract; (b) 0.6 mg of protein from a cell-free extract; (c) and (d) 32 μ g of partially purified 'malic' enzyme I; (e) and (f) 30 μ g of partially purified 'malic' enzyme II. Gels (a), (b), (c) and (e) were stained for enzyme activity, and gels (d) and (f) for protein, as described in the Materials and Methods section. The photograph of gel (a) has been magnified 1.5-fold.

the most effective and ATP the least active. 'Malic' enzyme I, on the other hand, was only little affected by the nucleotides. When the effect of the adenylates on the carboxylation reaction was studied (Fig. 2b), both enzyme forms were inhibited, although again 'malic' enzyme II was more affected, and AMP was the more effective inhibitor.

The specificity of the inhibition with respect to the base was studied for both enzyme forms. Table 1 shows that the L-malate-decarboxylation reaction catalysed by 'malic' enzyme II was preferentially inhibited by AMP (2 mM), the other nucleoside monophosphates being considerably less effective. A similar pattern was observed for the nucleoside

Fig. 2. Inhibition of 'malic' enzymes I and II by adenine nucleotides

(a) L-Malate-decarboxylating activity was measured with 2.7 mM-L-malate, 0.03 mM-NADP⁺, 2.5 mM-MnCl₂ and the indicated adenine nucleotides. ○, ●, AMP; □, ■, ADP; △, ▲, ATP. ○, □, △, 7.9 μ g of 'malic' enzyme I/ml; ●, ■, ▲, 7.5 μ g of 'malic' enzyme II/ml. Other experimental conditions were as described in the Materials and Methods section. (b) CO₂-fixing activity was measured with 5 mM-pyruvate, 0.03 mM-NADPH, 2.5 mM-MnCl₂ and the indicated adenine nucleotides. The symbols are the same as for (a); 32 μ g of 'malic' enzyme I/ml and 15 μ g of 'malic' enzyme II/ml were used. The experiment was performed three times, only one of which is shown. The lines were drawn by eye. Other experimental conditions were as described in the Materials and Methods section.

Table 1. *Base-specificity of the inhibition of 'malic' enzymes I and II by nucleotides*
L-Malate-decarboxylating activity was measured as indicated in the legend to Fig. 2(a). Nucleotide concentrations were 2mm.

Base	Inhibition (%)					
	Monophosphate		Diphosphate		Triphosphate	
	'Malic' enzyme I	'Malic' enzyme II	'Malic' enzyme I	'Malic' enzyme II	'Malic' enzyme I	'Malic' enzyme II
A	16	74	2	43	0	20
G	0	24	0	23	15	43
I	3	16	4	1	7	35
C	4	15	6	14	8	38
U	4	16	9	10	8	37
T	—	—	0	27	8	29

diphosphates; in the case of the triphosphates, on the other hand, all the nucleotides tested were more effective than ATP, although none of them was as effective as AMP. In the case of 'malic' enzyme I the inhibition was much less; the only nucleotides effective to some extent were AMP and GTP, both of which inhibited by about 15%. ADP and ATP did not inhibit at all.

Since 'malic' enzyme requires a bivalent cation activator, and nucleotides such as ATP and ADP are well-known chelating agents, it was necessary to discard the possibility that the observed inhibition was merely due to chelation. Table 2 shows that, when the nucleotide concentration (2mm) was higher than the MnCl₂ concentration (1 mm), ATP and ADP were the most effective inhibitors, as expected from the known dissociation constants of their complexes with Mn²⁺ (Burton, 1961). When the concentration of MnCl₂ was increased to 10mm, the inhibition by ATP and ADP disappeared, whereas that by AMP remained only slightly diminished. A similar, although not so clear-cut, result was obtained when a mixture of 1mm-MnCl₂ and 9mm-MgCl₂ was used. This suggests that the free uncomplexed nucleotides are responsible for the inhibition observed.

Table 2. *Effect of bivalent cation concentration on the inhibition of 'malic' enzyme II by adenine nucleotides*
L-Malate-decarboxylating activity was measured as indicated in the legend to Fig. 2(a), except for the bivalent cation concentrations, which were as indicated, and the enzyme concentration, which was 3.6µg of 'malic' enzyme II/ml. Nucleotide concentrations were 2mm.

Cation	Inhibition (%)		
	AMP	ADP	ATP
1mm-MnCl ₂	76	94	100
1mm-MnCl ₂ +9mm-MgCl ₂	65	23	30
10mm-MnCl ₂	56	0	7

Fig. 3. *Inhibition of 'malic' enzyme II by adenine nucleotides with L-malate as the variable substrate*
Reaction mixtures contained 0.03mm-NADP⁺, 2.5mm-MnCl₂ and 2mm-adenine nucleotide where indicated. ○, No inhibitor; △, AMP; □, ADP; ●, ATP. Concentration of 'malic' enzyme II: 10.5 µg/ml. The experiment was performed three times, only one of which is shown. The lines were drawn by eye. Other experimental conditions were as described in the Materials and Methods section.

The experiments on base-specificity and effect of bivalent cations on the inhibition were also performed in the direction of CO₂ fixation on pyruvate, with essentially similar results,

Fig. 3 shows double-reciprocal plots for L-malate as variable substrate, at a fixed concentration of NADP⁺ (0.03 mm), for 'malic' enzyme II. This enzyme form was inhibited competitively by AMP, ADP and ATP, which increased the apparent K_m value from 2.74mm to 7.4, 4.1 or 4.55mm respectively. 'Malic' enzyme I, on the other hand, was only little inhibited by AMP, which seemed to be uncompetitive, decreasing the apparent K_m value from 1 to 0.86mm.

Fig. 4 shows double-reciprocal plots for NADP⁺ as the variable substrate, at a fixed concentration of L-malate (2.7 mm), for 'malic' enzyme II. This enzyme

Fig. 4. Inhibition of 'malic' enzyme II by adenine nucleotides with NADP^+ as the variable substrate

Experimental conditions were as described in the legend to Fig. 3, except for the fixed concentration of L-malate, which was 2.7 mM.

form was again inhibited by the nucleotides in a competitive manner, AMP being the most effective inhibitor. The apparent K_m value for NADP^+ was increased from 28 μM in absence of nucleotides, to 91, 50 or 46 μM , in the presence of 2 μM -AMP, -ADP or -ATP respectively. 'Malic' enzyme I was again little inhibited by the three nucleotides, which increased the apparent K_m for NADP^+ from 28 to 36 μM .

Figs. 5(a) and 5(b) show double-reciprocal plots for pyruvate as the variable substrate in the CO_2 -fixation reaction. Both enzyme forms were inhibited by the nucleotides, although again 'malic' enzyme II was the more sensitive. Inhibition of 'malic' enzyme II by AMP was competitive with respect to pyruvate, the apparent K_m value being increased from 4.4 to 13.3 mM in the presence of 2 mM-AMP; ADP and

ATP, on the other hand, inhibited non-competitively towards pyruvate (Fig. 5a). Fig. 5(b) shows that 'malic' enzyme I was inhibited competitively by the three adenine nucleotides, AMP being again the most effective. The apparent K_m for pyruvate was increased from 4.4 mM in the absence of the nucleotides to 9.1 mM in the presence of AMP, or 6.7 mM in the presence of ADP or ATP.

Fig. 6 shows the plot of 'malic' enzyme II activity as a function of the concentration of NADPH for the

Fig. 6. Plot of 'malic' enzyme II activity as a function of the concentration of NADPH in the absence and presence of adenine nucleotides

The experimental conditions were as described in the legend to Fig. 2(b), except for the varying concentration of NADPH and the enzyme addition, which was 21.6 μg of 'malic' enzyme II/ml. Nucleotides were added at a final concentration of 2 mM, where indicated. $\circ$, No inhibitor; Δ , AMP; $\square$, ADP; $\bullet$, ATP.

Fig. 5. Inhibition of 'malic' enzymes I and II by adenine nucleotides with pyruvate as the variable substrate

Experimental conditions were as described in the legend to Fig. 2(b) except for the varying concentration of pyruvate and the adenine nucleotides, which were added at a final concentration of 2 mM where indicated. $\circ$, No inhibitor; Δ , AMP; $\square$, ADP; $\bullet$, ATP. (a) 13.4 μg of 'malic' enzyme II/ml; (b) 24.6 μg of 'malic' enzyme I/ml. The experiment was performed three times, only one of which is shown. The lines were drawn by eye.

CO₂-fixation reaction, both in the absence and presence of the inhibitors. The control curve shows three different plateau regions, with zones of apparently sigmoidal behaviour between them, suggesting both positive and negative co-operativity. Although no kinetic analysis was done, because of the complexity of the pattern, it is apparent that AMP again was the most effective inhibitor, and that the peculiar kinetic pattern was conserved in the presence of the nucleotides. The linearity of the reaction with time in all zones of the curves was checked, thus discarding the possibility of an experimental artifact. When the experiment was repeated several times, the general pattern was conserved, but with a tendency to lose the positive co-operativity observed at the lower NADPH concentrations as the preparation aged, suggesting some desensitization.

Fig. 7 shows that, in contrast, the kinetic behaviour for NADPH presented by 'malic' enzyme I was hyperbolic, as indicated by the straight double-reciprocal plots, both in the absence and presence of the nucleotides.

Effect of oxaloacetate on the L-malate-decarboxylation reaction

Figs. 8(a) and 8(b) show double-reciprocal plots for L-malate as the variable substrate, in the presence of a saturating concentration of NADP⁺ (0.15 mM), in the absence or presence of oxaloacetate. Both enzyme forms were inhibited by oxaloacetate in an apparently competitive manner, but whereas 'malic' enzyme II followed sigmoidal kinetics, as shown by the fact that the double-reciprocal plots were concave upwards (Fig. 8a), 'malic' enzyme I presented

Fig. 7. Inhibition of 'malic' enzyme I by adenine nucleotides with NADPH as the variable substrate

The experimental conditions were as described in the legend to Fig. 6, except for the fixed concentration of pyruvate, which was 2.6 mM, and the enzyme addition, which was 24.6 µg of 'malic' enzyme I/ml. ○, No inhibitor; △, AMP; □, ADP; ●, ATP.

Michaelian kinetics (Fig. 8b). The apparent K_m for L-malate of 'malic' enzyme II, obtained by extrapolation of the linear segments of the curves, was increased from 2.1 mM in the absence of oxaloacetate to 5.0, 7.7 or 12.5 mM in the presence of 0.125 mM- or 0.50 mM-oxaloacetate respectively; an apparent K_i value of 94 µM was calculated from these results. The apparent h values, calculated from Hill plots (not shown), were about 1.4 both in the absence and presence of the inhibitor. In the case of 'malic' enzyme I, the apparent K_m value for L-malate was increased from 0.2 mM to 1.3, 2.8 or 5.9 mM, in the presence of 0.05 mM, 0.125 mM or 0.25 mM-oxaloacetate respectively; the apparent K_i value calculated

Fig. 8. Inhibition of 'malic' enzymes I and II by oxaloacetate with L-malate as the variable substrate

Reaction mixtures contained 0.15 mM-NADP⁺, 2 mM-MnCl₂ and oxaloacetate at the following concentrations: ○, none; ▲, 0.05 mM; △, 0.125 mM; □, 0.25 mM; ●, 0.5 mM. Other experimental conditions were as described in the Materials and Methods section. The experiment was performed three times, only one of which is shown. The lines were drawn by eye. (a) 3.75 µg of 'malic' enzyme II/ml; (b) 7.9 µg of 'malic' enzyme I/ml.

Fig. 9. Activation of 'malic' enzyme II by L-aspartate and succinate

L-Malate-decarboxylating activity was measured with 0.45 mM-L-malate, 0.15 mM-NADP⁺, 2 mM-MnCl₂, 1.5 µg of 'malic' enzyme II/ml and L-aspartate (a) or succinate (b) as indicated on the abscissa. Other experimental conditions were as described in the Materials and Methods section. A double-reciprocal plot of these data in which v_0 and v refer to the activity in the absence and presence of L-aspartate or succinate is shown in the inset. The experiment was performed three times, only one of which is shown. The lines were drawn by eye.

from these results was 9 µM. The apparent h value was about 1 in all cases. The differences in the apparent K_m values for L-malate obtained from the plots shown in Figs. 3(a) and 3(b) and 8(a) and 8(b) can be accounted for by the different fixed NADP⁺ concentration used in each case. Also, the sigmoidicity observed for 'malic' enzyme II was readily apparent in the presence of the higher NADP⁺ concentration.

Effects of L-aspartate and succinate on the decarboxylation of L-malate

'Malic' enzyme II was strongly activated by L-aspartate (Fig. 9a) and succinate (Fig. 9b) in the presence of a low concentration of L-malate (0.45 mM). The activation by both dicarboxylic acids seemed to be hyperbolic, as shown by the linearity of the double-reciprocal plots presented as insets in Figs. 9(a) and 9(b). The maximal activation by 0.25 mM-L-aspartate was about 6-fold, with an apparent K_a (concentration of activator for half-maximal activation) of 0.12 mM, and that by 1.75 mM-succinate was about 4-fold, with an apparent K_a of 0.5 mM. The activity of 'malic' enzyme I was very little affected by L-aspartate or succinate, at concentrations up to 1 mM. Under conditions where 'malic' enzyme II was activated by L-aspartate by 2.4-fold, the maximal activation of 'malic' enzyme I was about 9%; the corresponding

values for succinate were 1.54-fold and 4% respectively.

Fig. 10(a) shows double-reciprocal plots of 'malic' enzyme II for L-malate as the variable substrate, at a fixed concentration of 0.15 mM-NADP⁺, in the absence or presence of dicarboxylic acid activators. The apparent K_m for L-malate was decreased from 2.17 mM in the absence of activators to 0.42 and 0.12 mM, in the presence of 1 mM-succinate and 0.5 mM-L-aspartate respectively. The apparent V_{max} was not significantly changed by succinate, and was increased by about 23% by L-aspartate. The sigmoidal kinetics of 'malic' enzyme II for L-malate (see above) were not changed by the activators.

Fig. 10(b) shows double-reciprocal plots of 'malic' enzyme II for NADP⁺ as the variable substrate, in the presence of a fixed concentration of 9 mM-L-malate; the apparent K_m for NADP⁺ was decreased from 18.5 µM in the absence of activators, to 14.3 and 10.2 µM, in the presence of 1 mM-succinate and 0.5 mM-L-aspartate respectively. Again succinate did not change the apparent V_{max} , whereas L-aspartate increased it by about 21%.

Action of other possible effectors

Acetyl-CoA, at concentrations up to 0.5 mM, did not inhibit either of the 'malic' enzymes. Glyoxylate

Fig. 10. Activation of 'malic' enzyme II by L-aspartate and succinate with L-malate or NADP⁺ as the variable substrate. Reaction mixtures contained 2 mM-MnCl₂, 1.5 µg of 'malic' enzyme II/ml and the activators as follows: ○, none; △, 0.5 mM-L-aspartate; □, 1 mM-succinate. Other experimental conditions were as described in the Materials and Methods section. (a) L-Malate variable in the presence of 0.15 mM-NADP⁺. (b) NADP⁺ variable in the presence of 9 mM-L-malate. Three experiments were performed, only one of which is shown. The lines were drawn by eye.

was not very effective, inhibiting 'malic' enzyme II by 13% and 'malic' enzyme I by 32% at 2 mM. NADH (0.3 mM) did not inhibit 'malic' enzyme II, and inhibited 'malic' enzyme I by 11%.

Discussion

Culture epimastigotes of the Tulahuén strain of *T. cruzi* contain two different forms of NADP⁺-linked 'malic' enzyme, which have the same or very similar molecular weight, since they were eluted from Sephadex G-200 as a single symmetric peak (not shown), but they have different electrophoretic mobilities and kinetic and regulatory properties. The two isoenzymes were partially purified; purification to homogeneity was not attempted, because of the low yields and considerable lability of both 'malic' enzyme forms after starch-gel electrophoresis.

'Malic' enzyme I and 'malic' enzyme II had, under similar experimental conditions, the same apparent K_m values for NADP⁺ and pyruvate (28 µM and 4.4 mM respectively); the apparent K_m value for L-malate, on the other hand, was between 3- and 10-fold lower for 'malic' enzyme I, depending on the concentration of the co-substrate, NADP⁺. Moreover, the kinetics for L-malate shown by 'malic' enzyme II were slightly sigmoidal (apparent h of 1.4), whereas 'malic' enzyme I showed hyperbolic behaviour.

The most striking kinetic difference between the two enzyme forms was in the effects of varying the concentration of NADPH on the velocity of the CO₂-fixation reaction. Thus, whereas 'malic' enzyme I had hyperbolic kinetics, 'malic' enzyme II presented a very complex kinetic pattern, which suggests the

presence of both positive and negative co-operativity. We have not yet analysed this behaviour, which seems to be even more complex than that of the anthranilate synthetase from *Salmonella typhimurium* (Henderson *et al.*, 1970), which also showed three plateau regions, but with apparently hyperbolic behaviour in the zones between them. It is noteworthy that 'malic' enzyme II had normal hyperbolic kinetics for NADP⁺ in the decarboxylation reaction; the anomaly therefore only affects one direction of the reaction. It is possible that NADPH may act in the CO₂-fixation reaction both as a substrate and as an allosteric effector, as reported, although with a much simpler kinetic pattern, for the NADP⁺-linked 'malic' enzyme from *Escherichia coli* (Sanwal & Smando, 1969).

Both enzyme forms were also different in regulatory properties. Thus 'malic' enzyme II was markedly inhibited by adenine nucleotides, whereas 'malic' enzyme I was very little affected, at least for the decarboxylation reaction, which is probably the physiologically important one (Cazzulo *et al.*, 1977; Cataldi de Flombaum *et al.*, 1977). The results shown in Table 2 suggest that only free nucleotides and not bivalent-cation-complexed nucleotides are inhibitory. In fact, ATP and ADP were very effective inhibitors when they were in excess or at nearly equimolar concentration with respect to the bivalent cation, when an appreciable concentration of free nucleotide must exist, but lost their effectiveness when assayed in the presence of a great excess of bivalent cation, when nearly all the nucleotide must be present as the complex ion. Table 2 shows that this happened not only in the presence of 10 mM-MnCl₂, a concentration clearly non-physiological, but also in

the presence of a mixture of 1 mM-MnCl₂ and 9 mM-MgCl₂, which probably is more similar to the situation inside some eukaryotic cells (Cazzulo & Stoppani, 1969). It is noteworthy that MgCl₂ in this case must be acting only by forming complexes with ATP or ADP, since it is not itself a very effective activator of the 'malic' enzymes from *T. cruzi* (Cazzulo *et al.*, 1977). When tested at concentrations higher than that of the bivalent cation, ATP and ADP could also be inhibiting merely by chelating the bivalent cation activator strictly required for activity (Cazzulo *et al.*, 1977). The inhibition by AMP, which is a poor chelating agent (Burton, 1961), was only slightly decreased by a considerable excess of MnCl₂. These results, together with the base specificity of the inhibition, suggest that the physiologically significant inhibitor of 'malic' enzyme II is AMP. This suggests that the enzyme activity is regulated by the energy charge of the cell (Atkinson, 1966), 'malic' enzyme II being maximally active when the energy charge is high, and therefore the AMP concentration is low. This, in its turn, suggests that the role of 'malic' enzyme II is biosynthetic, probably as a source of NADPH. It is noteworthy that the reactions going from phosphoenolpyruvate to oxaloacetate via phosphoenolpyruvate carboxykinase, from oxaloacetate to L-malate via malate dehydrogenase, and from L-malate to pyruvate via 'malic' enzyme, would act as a transhydrogenase, transferring reduction equivalents from NADH to NADP⁺, and causing at the same time the net conversion of phosphoenolpyruvate to pyruvate and the production of ATP, as if pyruvate kinase had acted. The latter enzyme is also present, although its activity is rather low (Juan *et al.*, 1976).

To the best of our knowledge, this is the first report of inhibition of a 'malic' enzyme by AMP. Kleber (1975) has reported inhibition of the 'malic' enzyme from *Acinetobacter calcoaceticus* by ATP and ADP, but AMP was completely ineffective. The NAD⁺-linked 'malic' enzyme from *E. coli* was also inhibited by ATP and ADP, but AMP was much less effective (Sanwal, 1970).

Both forms of 'malic' enzyme were inhibited by oxaloacetate, although the *K_i* for this inhibitor was 10-fold lower in the case of 'malic' enzyme I. It is noteworthy that this enzyme form was little affected by AMP. The inhibition of 'malic' enzyme from *T. cruzi* by oxaloacetate (Cazzulo *et al.*, 1977), like that of 'malic' enzyme from *Crithidia fasciculata* (Marr, 1973), is probably related to the regulation of 'partial aerobic fermentation' of glucose with production of succinate. This production of succinate would participate in the reoxidation of glycolytic NADH, through the reactions catalysed by malate dehydrogenase and a hypothetical fumarate reductase, demonstrated by Klein *et al.* (1975) in *Trypanosoma brucei brucei* and *Trypanosoma mega*. If

'malic' enzyme was fully active during this process, it would decarboxylate the L-malate arising from the oxaloacetate generated by phosphoenolpyruvate carboxykinase, avoiding the second reductive reaction and reversing the first, by acting as a transhydrogenase as suggested above. The inhibition of 'malic' enzyme I by low concentrations of oxaloacetate would avoid this useless draining of C₄ dicarboxylic acid. The presence of a second enzyme form, 'malic' enzyme II, with higher apparent *K_m* for L-malate and much less sensitive to this inhibition, would allow some transhydrogenation to take place to provide NADPH for biosynthesis when L-malate accumulates, even in the presence of oxaloacetate.

'Malic' enzyme II, but not 'malic' enzyme I, was activated by L-aspartate and succinate. The activation by the amino acid would increase its degradation when present in excess in the growth medium. The activation by succinate might be physiologically significant as a control point of the metabolic flow via 'aerobic fermentation' or tricarboxylic acid cycle; in fact, transient accumulation of succinate inside the cell would activate 'malic' enzyme II, which would then drain part of the excess of C₄ dicarboxylic acid produced towards the tricarboxylic acid cycle. This activation could also be useful when the epimastigotes incorporate and catabolize the succinate initially excreted into the medium, after glucose has been exhausted (Cáceres & Fernandes, 1976). Finally, activation by succinate might be a simple reflection of structural similarity with respect to L-aspartate; the effects of both activators on 'malic' enzyme II were not additive, which suggests that they are acting at the same site.

It is noteworthy that the enzyme form activated by L-aspartate and succinate, 'malic' enzyme II, was less inhibited by oxaloacetate, and therefore the more likely to be directly involved in the catabolism of C₄ dicarboxylic acids. So far as we know, there are two other examples of effects of L-aspartate on 'malic' enzymes: the NAD⁺-linked enzyme of *E. coli* was also activated by the amino acid (Sanwal, 1970), and one of the two NADP⁺-linked 'malic' enzymes from *Neurospora crassa* was inhibited by L-aspartate (Zink, 1972).

It is likely that the two molecular forms of 'malic' enzyme are placed in different subcellular compartments; we have not been able yet to investigate this possibility, since the peculiar structure of the kinetoplast-mitochondrion complex present in these haemoflagellates (Paulin, 1975) makes it extremely difficult to obtain intact mitochondria and clean subcellular fractions.

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