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pH DEPENDENCE OF THE KINETIC PARAMETERS FOR THE
OXALACETATE DECARBOXYLATION AND PYRUVATE
REDUCTION REACTIONS CATALYZED
BY MALIC ENZYME

THESIS

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Ascaris suum NAD-malic enzyme catalyzes the decarboxylation of oxalacetate and reduction of pyruvate. Thus, the present classification (E.C. 1.1.1.39) for this enzyme should be changed to E.C. 1.1.1.38. In the absence of nucleotide, both the chicken liver NADP-malic enzyme and Ascaris suum NAD-malic enzymes catalyze the decarboxylation of oxalacetate. A study of the pH dependence of kinetic parameters for oxalacetate decarboxylation and pyruvate reduction was carried out for the NAD(P)-malic enzyme with Mg^{2+} and Mn^{2+} in the presence and absence of nucleotide. In all cases, an enzyme residue is required in its protonated form for reaction while for oxalacetate decarboxylation the β -carboxyl of oxalacetate is required unprotonated. Of a number of inhibitory binding analogs of malate tested, oxalate is the tightest binding inhibitor for Ascaris suum enzyme.

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LIST OF ABBREVIATIONS

DTT		1,4-Dithiothreitol
EDTA		Ethylenediaminetetraacetic acid
Hepes	. . .	N-(2-hydroxyethyl)piperazine-N'-2-ethane-sulfonic acid
LDH		Lactate Dehydrogenase
Mes		2-(N-morpholino)ethanesulfonic acid
NAD		β -Nicotinamide Adenine Dinucleotide
NADH		β -Nicotinamide Adenine Dinucleotide, reduced
NADP		β -Nicotinamide Adenine Dinucleotide Phosphate
OAA		Oxalacetate
SDS		Sodium Dodecyl Sulfate

CHAPTER I

INTRODUCTION

Malic enzyme (E.C. 1.1.1.38-40) catalyzes the reaction depicted in Fig. 1 except that E.C. 1.1.1.39 does not catalyze the decarboxylation of oxalacetate (reaction 2). Malic enzyme has been purified from various sources such as animals, plants, insects and microorganisms and its characteristics and possible physiological role(s) have been reviewed (Ochoa, 1952; Fernandez et al., 1967; Scrutton, 1971; Frenkel, 1975). Possible roles for malic enzyme include the production of reducing equivalents for fatty acid synthesis and the production of pyruvate for gluconeogenesis in all organisms investigated. A divalent cation is needed as an activator in all of the malic enzyme reactions (1-3) and also appears to be required for binding and polarization of the keto group of keto acid substrates.

All of the above reactions are catalyzed by pigeon liver malic enzyme (Hsu, 1970; Tang and Hsu, 1973) which has been purified in crystalline form by Hsu and Lardy (1967). Reaction 1 is catalyzed at neutral and high pH (the most favorable pH is 7.5, Veiga Salles and Ochoa, 1950). In addition to oxidative decarboxylation of L-malate, the pigeon liver malic enzyme catalyzes the decarboxylation of oxalacetate at a similar rate. Therefore, Hsu (1970) suggested that oxalacetate is an intermediate in the oxidative

Figure 1

Reactions catalyzed by malic enzyme.

1. Oxidative decarboxylation of malate
2. Decarboxylation of oxalacetate
3. Reduction of pyruvate

decarboxylation of malate. Reaction 2 is catalyzed by pigeon liver malic enzyme at low pH and requires a divalent metal and NADP (Schimerlik and Cleland, 1977b).

For reaction 3, oxalacetate is the best substrate for the NADP-malic enzyme from pigeon liver. The maximum velocity is about 10% of reductive carboxylation of pyruvate. Pyruvate, ketomalonate and other keto acids are slower substrates than oxalacetate by an order of magnitude. The pH optimum for reaction 3 has been shown to be 6.5 (Tang and Hsu, 1973).

Each of the malic enzymes studied thus far is distinguished by its nucleotide specificity and oxalacetate decarboxylase ability. Three different kinds of malic enzyme have been suggested. The first classification is E.C. 1.1.1.40 (L-malate:NADP oxidoreductase(oxalacetate decarboxylating)) which includes crystalline pigeon liver malic enzyme and chicken liver malic enzyme. The second classification is E.C. 1.1.1.39 (L-malate:NAD oxidoreductase(oxalacetate non-decarboxylating)) which includes malic enzyme from the group D streptococcus (London and Meyer, 1969), cauliflower (MacRae, 1971), potato tuber (Grover et al., 1981) and Ascaris mitochondria (Saz and Hubbard, 1957; Fodge et al., 1972). Finally, the third type is E.C. 1.1.1.38 (L-malate:NAD oxidoreductase(oxalacetate decarboxylating)) which includes malic enzyme from Lactobacillus arabinosus (Korkes et al., 1950) and the enzyme from dung beetle catharsius (Imbuga and Pearson,

1982). E.C. 1.1.1.38 exists only at low levels in vertebrates (Sauer, 1973a,b; Lin and Davis, 1974), but more activity is found in certain invertebrate tissues.

The Ascaris suum NAD-malic enzyme has a steady-state random kinetic mechanism for reaction 1 (Park et al., 1984).

In addition, the location of rate limiting step along the reaction path has been determined (Kiick, 1985). Ascaris suum NAD-malic enzyme was classified in E.C. 1.1.1.39.

Attempts have been made to detect whether or not this enzyme decarboxylates oxalacetate. The present study shows that this enzyme does catalyze the decarboxylation of oxalacetate. Thus, the classification for this enzyme should be changed to E.C. 1.1.1.38. In addition, it has been shown that the nucleotide (NAD) is not required for the decarboxylation reaction at all pH values tested. The present study also shows that the chicken liver NADP-malic enzyme does not require NADP for oxalacetate decarboxylation at all pH values tested, but the presence of the nucleotide does increase the affinity of enzyme for oxalacetate. In this thesis, data are presented for the pH dependence of the steady-state kinetic parameters of reaction 2 (by chicken liver and Ascaris suum malic enzyme) and 3 (by Ascaris suum malic enzyme).

A chemical mechanism is proposed for these reactions. Included are preliminary studies with inhibitors which may serve to define the specificity of the malate binding site on the Ascaris suum enzyme. All inhibitors are competitive vs. malate except oxalate which is noncompetitive.

In addition to the work presented in the body of this thesis, I have also carried out studies toward a determination of the kinetic mechanism of the NAD-malic enzyme from Ascaris suum. This work resulted in a full paper in Biochemistry (Park et al., 1984) for which I collected all the data except the data for initial velocity studies using a narrow range of Mg^{2+} concentrations (Table I in the paper) and the pH 7.3 data in Table II. The latter were collected by Dennis M. Kiick in this laboratory. Since this study is already published, all of the work will be excluded from the thesis, but the abstract is reproduced below.

Park, Sang-Hoon, Kiick, Dennis, M., Harris, B. G. and Cook, Paul F.* Kinetic Mechanism in the Direction of Oxidative Decarboxylation for NAD-Malic Enzyme from Ascaris suum.

Measurement of the initial rate of the malic enzyme reaction varying the concentration of NAD at several different fixed levels of Mg^{2+} (0.25-1.0 mM) and a single malate concentration gave a pattern which intersects to the left of the ordinate. Repetition of this initial velocity pattern at several additional malate concentrations and treatment in terms of a terreactant mechanism suggests an ordered mechanism in which NAD adds prior to Mg^{2+} which must add prior to malate. On the other hand, when a broader concentration range of Mg^{2+} (0.25-50 mM) is used, data are consistent with a random mechanism in which Mg^{2+} must add prior to malate. By use of product inhibition studies, pyruvate is competitive vs. malate and noncompetitive vs. NAD, while NADH is competitive vs. NAD and noncompetitive vs. malate. These results are consistent with the random addition of substrates and further suggest rapid equilibrium random release of products. Tartro-nate, a dead-end analogue of malate, is competitive vs. malate and noncompetitive vs. NAD. Thio-NAD is a slow substrate which is used at 2.4% the maximum rate of NAD. When used as a dead-end analogue of NAD, thio-NAD is competitive vs. NAD and

gives a complex inhibition pattern vs. malate in which competitive inhibition is apparent at low concentrations of malate (<12.5 mM), and this changes to uncompetitive inhibition at high concentrations of malate (>12.5 mM). These data are consistent with a steady-state random mechanism in the direction of oxidative decarboxylation in which Mg^{2+} adds in rapid equilibrium prior to malate. However, calculations based on intracellular levels of substrates suggest that the ordered mechanism discussed above probably operates under physiologic conditions.

CHAPTER II

MATERIALS AND METHODS

Chemicals

The nucleotides, NAD and NADP, were from Boehringer-Mannheim. L-malate, NADH, oxalacetate (OAA) and dithiothreitol (DTT) were from Sigma. Pyruvate was from United States Biochemical Corporation. An oxalacetate solution was made fresh daily in ice-cooled water and titrated to pH 5.0 with KOH and allowed to establish tautomeric equilibrium for one hour (Dolin, 1968; Pogson and Wolfe, 1972). It was then stored in an ice bath. Only the keto form of oxalacetate is enzymatically active (Schimerlik and Cleland, 1977b).

The concentration of oxalacetate was determined enzymatically using 15 units of malate dehydrogenase and 0.3 mM NADH. A pyruvate solution was prepared using the same procedure as for the oxalacetate solution discussed above, but the potassium salt was used eliminating the need to titrate with KOH. The concentration of pyruvate was determined enzymatically with 30 units of lactate dehydrogenase in the presence of excess NADH (0.4 mM). The concentration of NAD, NADP and NADH were determined spectrophotometrically using an extinction coefficient of $17,800 \text{ M}^{-1} \text{ cm}^{-1}$ at 259 nm for the oxidized nucleotides and $6,220 \text{ M}^{-1} \text{ cm}^{-1}$ for the reduced nucleotide at 340 nm. All other chemicals and reagents were obtained from commercial sources and were of the highest purity available.

Enzyme Assay

Mitochondrial NAD-malic enzyme from Ascaris suum was purified by B. G. Harris at the Texas College of Osteopathic Medicine according to the procedure of Allen and Harris (1981). Enzyme was homogeneous by the criterion of SDS-polyacrylamide gel electrophoresis run according to the method of O'Farrell (1975) as modified by Atkins et al. (1975). Enzyme had a final specific activity of 35 u/mg and enzyme concentration was assayed in the direction of oxidative decarboxylation using 100 mM Hepes, pH 7.5; 1.0 mM DTT, 310 mM malate (24 mM when corrected for Mg:malate); 32.8 mM NAD (2 mM when corrected for Mg:NAD) and 618 mM MgSO_4 (300 mM when corrected for Mg:malate and Mg:NAD). Purified Ascaris suum malic enzyme was stored at -20°C in a storage buffer containing 15 mM triethanolamine-maleate, pH 7.5, 1 mM EDTA, 10 mM DTT, and 5% glycerol. The enzyme was stable for several months at -20°C . Freeze-thawing had no apparent effect on activity. Since in later studies (for example, those for reduction of pyruvate to give lactate) large concentrations of enzyme were used, assay mixtures were pooled, reconcentrated by Amicon PM-10 and dialyzed extensively for use in further studies.

Chicken liver NADP-malic enzyme (Sigma) was dialyzed twice against 2 l of buffer which contained 10 mM potassium phosphate, pH 6.0, including 40 mM β -mercaptoethanol, 3 mM EDTA, and 10% glycerol.

The resulting enzyme was stored using conditions identical to those given above for Ascaris suum malic enzyme and assayed in the direction of oxidative decarboxylation using a mixed buffer (see pH studies). The assay for enzyme activity at pH 7.0 contained 1.0 mM DTT; 10.8 mM L-malate (6 mM when corrected for Mg:Malate); 0.2 mM NADP (0.1 mM when corrected for Mg:NADP) and 24.9 mM MgSO_4 (20 mM when corrected for Mg:-Malate and Mg:NADP). Throughout this thesis enzyme concentration (catalytic site) is expressed in units of nM using the following equation:

$$\text{Enzyme (nM)} = \text{Enzyme} \left(\frac{\text{U}}{\text{ml}} \right) \div \text{specific activity} \left(\frac{\text{U}}{\text{mg}} \right) \div \text{M.W.} \left(\frac{\text{mg}}{\text{mmole}} \right) \quad (1)$$

All assays were carried out at 25° C using a Gilford 2600 spectrophotometer and Hewlett-Packard 7225B Graphics Plotter and Gilford Thermal Printer II. The temperature was maintained with a circulating water bath with the capacity to heat and cool the thermospacers of the Gilford 2600. Reaction cuvettes were 1 cm in path length and 1 ml in volume. All cuvettes were incubated for at least 10 min in the waterbath. All assays described below reflected initial velocity conditions with less than 10% of the limiting reactant utilized over the time course of the reactions.

Initial velocity studies of malate oxidative decarboxylation in the presence of malate analogs were run in 100 mM MES adjusted to pH 6.5 by addition of KOH. A typical assay contained 100 mM buffer, 1 mM DTT, variable concentrations of malate, metal, saturating NAD and malate analog and 2.2 to

6.6 nM Ascaris suum malic enzyme. The reaction was initiated by the addition of enzyme, and the production of NADH was monitored at 340 nm.

The decarboxylation of oxalacetate was monitored using the disappearance of the enolic oxalacetate absorbance at 282.5 nm in the presence of Mn^{2+} and 263.5 nm in the presence of Mg^{2+} . In the case of the chicken liver enzyme, velocity was linearly dependent on enzyme concentration over the range 90-360 nM with Mn^{2+} and 480-2560 nM with Mg^{2+} as the metal ion (Fig. 2). For the Ascaris suum enzyme, velocity was also linearly dependent on enzyme over the range 550-1360 nM concentration with Mg^{2+} as the divalent metal ion (Fig. 3).

Oxalacetate was simultaneously added to 4 cuvettes 30 sec before enzyme and a background rate was obtained for each point using same cuvette before adding enzyme. The dilution effect resulting from the addition of enzyme to a blank cuvette was negligible unless the added volume was more than 40 μ l. The reported velocities are those plus enzyme minus those of the background. The background rate results from nonenzymatic oxalacetate decarboxylation catalyzed by the divalent metal. Dissolved CO_2 does not affect the enzymatic velocities even at low pH (4.0). A typical assay of the chicken liver malic enzyme contained mixed buffer (see pH studies) pH 4.0-6.5, 20 mM Mg^{2+} or Mn^{2+} , 1 mM DTT, in the presence and absence of NADP with 178 nM (using Mn^{2+}) and 355 nM (using Mg^{2+}) enzyme and oxalacetate varied from 0.1-3 mM. Although the apparent K_m for oxalacetate (using Mn^{2+}) is about 0.2 mM in the presence

Figure 2

Velocity vs. enzyme concentration for oxalacetate decarboxylation by chicken liver NADP-malic enzyme.

- A. Reaction mixtures contained 100 mM mixed buffer, pH 5, 1 mM oxalacetate, 20 mM MnSO_4 .
- B. Reaction mixtures contained 100 mM mixed buffer, pH 4.0, 1 mM oxalacetate, 20 mM MgSO_4 .

Figure 3

Velocity vs. enzyme concentration for oxalacetate decarboxylation by Ascaris suum NAD-malic enzyme. Reaction mixtures contained 100 mM mixed buffer.
pH = 4.5, 2.25 mM oxalacetate, 20 mM MgSO_4 .

of NADP, it is 3 mM in the absence of NADP (1 mM and 3 mM, respectively, using Mg^{2+}). The highest oxalacetate concentration used was 3 mM since absorbance was too high at higher oxalacetate concentrations. As a result, there is some error in the V_{max} values obtained in the absence of NADP. A typical assay for the Ascaris suum malic enzyme contained mixed buffer (see pH studies) from pH 4.0-6.5, 20 mM Mg^{2+} or Mn^{2+} , 1 mM DTT, in the absence and presence of NAD with 264 nM (using Mn^{2+}) and 1056 nM (using Mg^{2+}) enzyme and oxalacetate varied from 0.25-2.5 mM.

The reduction of pyruvate was monitored using the disappearance of NADH at 340 nm. The velocity of the Ascaris suum malic enzyme was linearly dependent on enzyme concentration over the range 440 nM-1760 nM with Mn^{2+} (Fig. 4). One cuvette was used for both blank and experimental as for oxalacetate decarboxylation. The presence of CO_2 had no effect on the reaction at pH 5.0. Since large volumes of enzyme were added, there was a possibility of inhibition by maleate present in the enzyme storage buffers. However, no appreciable rate decrease was found up to 1 mM maleate. The reported velocities are the pyruvate reduction by Ascaris suum malic enzyme in the presence of saturating metal minus pyruvate reduction by contaminating LDH activities in the absence of metal, and the presence of 1 mM EDTA. A typical assay contained 100 mM Mes buffer, pH 4.7-6.7, 1758 nM enzyme, 1 mM DTT, pyruvate 1-4 mM, NADH 0.35 mM, Mn^{2+} (80 mM) or Mg^{2+} (100 mM).

Figure 4

Velocity vs. enzyme concentration for metal dependent pyruvate reduction by Ascaris suum NAD-malic enzyme.

Reaction mixtures contained 100 mM Mes buffer, pH = 6.7, 1 mM pyruvate, 0.4 mM NADH, 80 mM MnSO_4 .

Metal Chelate Correction

The concentration of the reactants malate and NAD added to the reaction mixture were corrected for the concentration of the metal-ligand chelate complex. The total concentration of any reactant was calculated according to the following equation (Park et al., 1984):

$$[L]_t = [L]_f + \frac{[L]_f [M^{2+}]_f}{K_{ML}} \quad (2)$$

where $[L]_t$ is the total final concentration of ligand added to the reaction mixture, $[L]_f$ is the desired uncomplexed concentration of the ligand, $[M^{2+}]_f$ is the desired uncomplexed concentration of divalent metal, and K_{ML} is the equilibrium constant for dissociation of the metal-ligand chelate complex [$K_{ML} = (M)(L)/(ML)$]. The total concentration of divalent metal to be added under any given set of concentration conditions for a solution containing i ligands was calculated according to the following equation:

$$[M^{2+}]_t = [M^{2+}]_f + \sum \frac{[Li]_f [M^{2+}]_f}{K_{MLi}} \quad (3)$$

where $[M^{2+}]_t$ is the total final concentration of divalent metal to be added to the reaction mixture, $[Li]_f$ is the desired uncomplexed concentration of the i th ligand, K_{MLi} is the equilibrium constant for dissociation of a chelate complex between divalent metal and the i th ligand, and $[M^{2+}]_f$ has the same definition as above. Dissociation constants used in the above calculations are listed in Table I.

TABLE I
DISSOCIATION CONSTANT^a

Ligand/Metal	Mg ²⁺	Mn ²⁺
NAD	19.5	12.9
NADH	"	"
NADP	"	"
Malate	25.1	6.3
Pyruvate	158.5	55.0
Tartronate	6.8	1.7
Oxalate	1.7	0.6
	58 ^b	40 ^b
L-Lactate	117.5	64.6
D-Lactate	"	"
Thio-glycolate	--	0.0417
	--	0.0275 ^b
Alanine	10.9	3.4
		55 ^b
Glycolate	120.2	26.3

^aK_d for M:L unit = mM

^bK_d for M:L₂ unit = mM²

(Martell & Smith, 1977)

In some malate analog experiments, no stability constant was available and the analog concentration was not corrected for metal chelate formation. In the case of oxalacetate decarboxylation, the concentrations of metal and oxalacetate was not corrected for chelate complex formation. All other reaction components gave negligible corrections under concentration conditions used.

pH Studies

Oxalacetate decarboxylation studies were carried out using a mixed buffer system which contained 25 mM each of acetic acid, glycine, Mes, and Hepes adjusted to desired pH with KOH. This buffer, which was chosen for the pH studies, chelates metal very weakly (Schimerlik and Cleland, 1977a), and does not show appreciable inhibition of the reaction. Doubling the buffer concentration also gave no inhibition.

Pyruvate reduction studies were carried out using 100 mM MES titrated to the desired pH by KOH. The pH of all experiments was measured with a Markson pH meter standardized at the given temperature to ± 0.01 pH units with buffers from MCB calibrated from 0 to 95° C. In all cases, negligible pH changes were observed before and after reaction. Since total substrate and inhibitor concentrations were sometimes high, substrate and inhibitor stock solutions were titrated to the approximate pH value of the assay. Enzyme is insensitive to increases in ionic strength to at least 2.1 M.

Effective Equilibrium Extinction Co-
efficient of the Enolic Oxalacetate
in the Presence of Metal

The keto-enol equilibrium constant and protonation state for oxalacetate are pH dependent. Therefore, the change in absorbance of the enolic oxalacetate at 282.5 nm (in the presence of Mn^{2+}) and at 263.5 nm (in the presence of Mg^{2+}) was recorded and the effective extinction coefficient was calculated by dividing the absorbance change by the oxalacetate concentration which was enzymatically calibrated with malate dehydrogenase and NADH.

Data Processing

Reciprocal initial velocities were plotted as a function of reciprocal substrate concentrations and all plots were linear. Data were fitted using the appropriate rate equation whenever possible by the least-squares method (Wilkinson, 1961) and by using the FORTRAN programs of Cleland (1979). The points in the figures are the experimentally determined values, while the curves are calculated from fits of the data using the appropriate rate equation. Data conforming to a straight line were fitted using the equation for a straight line. Single reciprocal plots used to obtain the pH profiles were fitted using eq. 4.

$$v = \frac{VA}{K + A} \quad (4)$$

Data conforming to rapid equilibrium ordered addition of A prior to B were fitted using eq. 5.

$$v = \frac{VAB}{K_{ia} K_b + K_b A + AB} \quad (5)$$

Data for linear competitive and linear noncompetitive inhibition were fitted using eqs. 6 and 7, respectively.

$$v = \frac{VA}{K(1 + \frac{I}{K_{is}}) + A} \quad (6)$$

$$v = \frac{VA}{K(1 + \frac{I}{K_{is}}) + A(1 + \frac{I}{K_{ii}})} \quad (7)$$

In equations 4-7, K is the apparent Michaelis constant, A and B are reactant concentrations, and I is the inhibitor concentration, while K_b is the Michaelis constant for B and K_{ia} , K_{is} , and K_{ii} are dissociation constant for A , and inhibition constants for slope, and intercept, respectively. V is the maximum velocity. Data for pH profiles which decreased with a slope of 1 at low pH and a slope of -1 at high pH were fitted using eq. 8. However, data for pH profiles which decreased with a slope of -1 at high pH were fitted using eq. 9.

$$\log y = \log \frac{C}{1 + H/K_1 + K_2/H} \quad (8)$$

$$\log y = \log \frac{C}{1 + K_2/H} \quad (9)$$

In eqs. 8 and 9, K_1 and K_2 are the acid dissociation constants of enzyme or substrate functional groups that must be deprotonated and protonated, respectively, for activity, the hydrogen-ion concentration is H , and C is the pH independent value of y .

CHAPTER III

RESULTS

Absorption Spectra and pH Dependence of the Extinction Coefficient of the Enolic Oxal- acetate in the Presence of Metal

In order to determine $\lambda_{\max}$ for the enolic oxalacetate in the presence of metal, wavelength was scanned from 240 nm to 340 nm. Results are shown in Fig. 5. The $\lambda_{\max}$ in the case of Mn^{2+} is 282.5 nm, while that in the case of Mg^{2+} is 263.5 nm. The effective extinction coefficient for enolic oxalacetate was obtained as a function of pH with each metal ion at a concentration of 20 mM. In the case of Mg^{2+} , the effective extinction coefficient decreases below pH 5. For Mn^{2+} , in addition to the decrease below pH 5, the extinction coefficient increases above pH 6.0 (Fig. 6). The presence of NADP, NAD and DTT did not affect E_{app} vs. pH. In separate experiments (Fig. 7), the enolic oxalacetate absorbance increases hyperbolically with metal concentration due to a shift in the keto-enol tautomeric equilibrium (Fig. 8). Experiments carried out at pH 6.7 with 1 mM oxalacetate and 20 mM Mn^{2+} showed that nonenzymatic oxalacetate decarboxylation catalyzed by divalent metal gave a final absorbance of 0.05 after several hours. Thus, the enolic oxalacetate in the presence of metal significantly contributes to absorbance.

Figure 5

Absorption spectra at pH 4.5 for enolic oxalacetate in the presence of metal.

Data were obtained in 100 mM mixed buffer with 1 mM OAA.

A. Absorption spectra in the presence of 20 mM MnSO_4 .

$$\lambda_{\text{max}} = 282.5 \text{ nm}$$

B. Absorption spectra in the presence of 20 mM MgSO_4 .

$$\lambda_{\text{max}} = 263.5 \text{ nm}$$

WAVELENGTH SCAN

Figure 6

pH Dependence of equilibrium effective molar extinction coefficient of the enolic oxalacetate in the presence of metal. All data were obtained in 100 mM mixed buffer with 20 mM metal.

A. MnSO_4

B. MgSO_4

Figure 7

Absorbance of enolic oxalacetate vs. Mn^{2+} concentration as a function of pH. All spectra were obtained in 100 mM mixed buffer, 1 mM OAA, various concentrations of Mn^{2+} as indicated.

A. pH 6.73

B. pH 5.76

C. pH 5.07

D. pH 4.02

Figure 8

Keto-enol tautomeric equilibrium and gem-diol form of oxalacetate in the solution depending on pH in the presence of metal.

a. $K_T = \frac{[\text{keto}]}{[\text{enol}]}$ Keto-enol tautomeric equilibrium constant

$$K_{T_1} = 19.10$$

$$K_{T_2} = 8.17$$

$$K_{T_3} = 6.09$$

$$K_{T_{3M}} = 3.1 \quad (\text{in the presence of } \text{Mg}^{2+})$$

b. pK = negative log of acid dissociation constant

$$\text{pK}_1 = 2.24 \qquad \text{pK}_2 = 3.90$$

$$\text{pK}'_1 = 1.89 \qquad \text{pK}'_2 = 3.73$$

$$\text{pK}'_3 = 12.18 \qquad \text{pK}'_{3M} = 8.11 \quad (\text{in the presence of } \text{Mg}^{2+})$$

c. $K_s = \frac{[\text{LM}]}{[\text{L}][\text{M}]}$ = stability constant of metal-ligand complex

$$\text{Log } K_s = 2.20\text{M}^{-1} \quad \text{Log } K'_s = 1.91\text{M}^{-1} \quad \text{Log } K''_s = 4.82\text{M}^{-1}$$

Above values are from Tate et al. (1964a,b).

pH Dependence of Oxalacetate Decarboxylation
for Chicken Liver NADP-Malic Enzyme

The nucleotide (NADP) is not required but divalent metal is required for the enzyme catalyzed decarboxylation of oxalacetate at all pH values tested. However, the presence of the nucleotide increases the affinity of enzyme for oxalacetate by about 8.4-fold with Mn^{2+} as the metal, and about 2.2-fold when Mg^{2+} is used as the divalent metal. No change in maximum rate or K_m for oxalacetate is observed when Mn^{2+} is replaced by Mg^{2+} in the absence of NADP at any pH value. Results are qualitatively consistent with those reported by Schimerlik and Cleland (1977b). All profiles shown in Fig. 9-12 exhibit a decrease below the pK for the β -carboxyl of oxalacetate which is 3.8 (Martell and Smith, 1977) and above the pK for an enzyme base. Even with Mn^{2+} as the metal in the presence of NADP, there was a slight decrease below pH 4. Data were not obtained below this pH because of an increasing K_m for oxalacetate as a result of protonation of β -carboxyl. In the absence of nucleotide (Figures 9 and 11), the pK for the enzyme base is 6.4-6.6 with either metal and is observed in either V or V/K_{OAA} pH profiles.

With NADP bound, the pK decreases to 4.8 with Mn^{2+} (Fig. 10) and 5.5 with Mg^{2+} as the metal (Fig. 12). The kinetic mechanism appears to be rapid equilibrium ordered at pH values below pH 5.0 with NADP adding to enzyme first. The K_i for NADP increases by a factor of 10 per pH unit below pH 5.0. A summary of pK values, absolute values of kinetic parameters and K_i values for NADP are listed in Tables II, III, and IV.

Figure 9

pH Dependence of kinetic parameters for oxalacetate decarboxylation by chicken liver NADP-malic enzyme in the presence of Mn^{2+} . The data at each pH were fitted using eq. 4 and the resulting values of V/K for oxalacetate and V as a function of pH were fitted using eq. 8. The theoretical curves drawn are from the fit to the appropriate equation.

Reaction mixtures contained 100 mM mixed buffer, 0.1 mM - 3 mM oxalacetate, 20 mM MnSO_4 , 178 nm enzyme.

NADP-MALIC ENZYME
OXALACETATE DECARBOXYLATION
(Mn^{2+})

Figure 10

pH Dependence of kinetic parameters for oxalacetate decarboxylation by chicken liver NADP-malic enzyme in the presence of Mn^{2+} and NADP.

The data at each pH were fitted using eq. 4 and the resulting values of V/K for oxalacetate and V as a function of pH were fitted using eq. 9. The theoretical curves drawn are from the fit using the appropriate equation.

Reaction mixtures contained 100 mM mixed buffer, 0.1-3 mM oxalacetate, 20 mM MnSO_4 , below pH 4.93 0.025-0.2 mM NADP, above pH 5.0 0.05 mM NADP, 178 nM enzyme.

NADP-MALIC ENZYME
OXALACETATE DECARBOXYLATION
(Mn^{2+} , NADP)

Figure 11

pH Dependence of kinetic parameters for oxalacetate decarboxylation by chicken liver NADP-malic enzyme in the presence of Mg^{2+} .

The data at each pH were fitted using eq. 4 and the resulting values of V/K for oxalacetate and V as a function of pH were fitted using eq. 8. The theoretical curves drawn are from the fit to the appropriate equation.

Reaction mixtures contained 100 mM mixed buffer, 0.1-3 mM oxalacetate, 20 mM MgSO_4 , 355 nM enzyme.

NADP-MALIC ENZYME
OXALACETATE DECARBOXYLATION
(Mg^{2+})

Figure 12

pH Dependence of kinetic parameters for oxalacetate decarboxylation by chicken liver NADP-malic enzyme in the presence of Mg^{2+} and NADP.

The data at each pH were fitted using eq. 4 and the resulting values of V/K for oxalacetate and V as a function of pH were fitted using eq. 8. The theoretical curves drawn are from the fit to the appropriate equation.

Reaction mixtures contained 100 mM mixed buffer, 0.1-3 mM oxalacetate, 20 mM MgSO_4 , below pH 4.45 0.01-0.2 mM NADP, above pH 4.5 0.05 mM NADP, 355 nM enzyme.

NADP-MALIC ENZYME
OXALACETATE DECARBOXYLATION
(Mg^{2+} , NADP)

TABLE II
SUMMARY OF pK VALUES

NADP-Malic Enzyme		
Parameter	$pK_a \pm \text{S.E.}$	$pK_b \pm \text{S.E.}$
$V_{\text{OAA}} (\text{Mn}^{2+})$	4.18 ± 0.09	6.69 ± 0.12
$V_{\text{OAA}} (\text{Mn}^{2+}, \text{NADP})$	—	4.7 ± 0.02
$V_{\text{OAA}} (\text{Mg}^{2+})$	4.29 ± 0.21	6.59 ± 0.27
$V_{\text{OAA}} (\text{Mg}^{2+}, \text{NADP})$	4.04 ± 0.25	5.42 ± 0.14
$V/K_{\text{OAA}} (\text{Mn}^{2+})$	4.18 ± 0.11	6.43 ± 0.11
$V/K_{\text{OAA}} (\text{Mn}^{2+}, \text{NADP})$	—	5.08 ± 0.04
$V/K_{\text{OAA}} (\text{Mg}^{2+})$	4.32 ± 0.19	6.39 ± 0.13
$V/K_{\text{OAA}} (\text{Mg}^{2+}, \text{NADP})$	4.48 ± 0.53	5.57 ± 0.37
NAD-Malic Enzyme		
$V/K_{\text{OAA}} (\text{Mn}^{2+})$	4.22 ± 0.09	5.34 ± 0.09
$V/K_{\text{OAA}} (\text{Mn}^{2+}, \text{NAD})^a$	4.13 ± 0.37	5.16 ± 0.23
$V/K_{\text{OAA}} (\text{Mg}^{2+})$	4.15^b	5.5^b
$V/K_{\text{PYR}} (\text{Mn}^{2+})$	—	6.09 ± 0.02
$V/K_{\text{PYR}} (\text{Mg}^{2+})$	—	6.09 ± 0.04

^aFigure not shown

^bEstimated value

(Note subscript OAA is for OAA decarboxylation and
subscript PYR is for pyruvate reduction.)

TABLE III

ABSOLUTE VALUES OF KINETIC PARAMETERS

<u>Parameter</u>	<u>NADP-Malic Enzyme</u>
V_{OAA}/E_t (Mn^{2+})	47 S^{-1}
V_{OAA}/E_t (Mn^{2+} , NADP)	87.6 S^{-1}
V_{OAA}/E_t (Mg^{2+})	37.4 S^{-1}
V_{OAA}/E_t (Mg^{2+} , NADP)	21 S^{-1}
$V/K_{\text{OAA}}E_t$ (Mn^{2+})	$1.87 \times 10^4 \text{ M}^{-1} \text{ S}^{-1}$
$V/K_{\text{OAA}}E_t$ (Mn^{2+} , NADP)	$2.97 \times 10^5 \text{ M}^{-1} \text{ S}^{-1}$
$V/K_{\text{OAA}}E_t$ (Mg^{2+})	$1.1 \times 10^4 \text{ M}^{-1} \text{ S}^{-1}$
$V/K_{\text{OAA}}E_t$ (Mg^{2+} , NADP)	$1.33 \times 10^4 \text{ M}^{-1} \text{ S}^{-1}$
	<u>NAD-Malic Enzyme</u>
V_{OAA}/E_t (Mn^{2+})	28.23 S^{-1}
V_{OAA}/E_t (Mn^{2+} , NAD) ^a	30.25 S^{-1}
$V/K_{\text{OAA}}E_t$ (Mn^{2+})	$2.82 \times 10^4 \text{ M}^{-1} \text{ S}^{-1}$
$V/K_{\text{OAA}}E_t$ (Mn^{2+} , NAD) ^a	$3.99 \times 10^4 \text{ M}^{-1} \text{ S}^{-1}$
$V/K_{\text{OAA}}E_t$ (Mg^{2+})	$1.18 \times 10^3 \text{ M}^{-1} \text{ S}^{-1}$
$V/K_{\text{OAA}}E_t$ (Mg^{2+} , NAD) ^a	$6.32 \times 10^2 \text{ M}^{-1} \text{ S}^{-1}$
V_{PYR}/E_t (Mn^{2+})	0.091 S^{-1}
V_{PYR}/E_t (Mg^{2+})	0.064 S^{-1}
$V/K_{\text{PYR}}E_t$ (Mn^{2+})	$80.68 \text{ M}^{-1} \text{ S}^{-1}$
$V/K_{\text{PYR}}E_t$ (Mg^{2+})	$15.73 \text{ M}^{-1} \text{ S}^{-1}$

^aFigure not shown

TABLE IV
SUMMARY OF NUCLEOTIDE K_i VALUES

<u>NADP-Malic Enzyme Oxalacetate Decarboxylation</u>		
<u>pH</u>	<u>K_i NADP (μM)</u>	
	<u>Mn²⁺</u>	<u>Mg²⁺</u>
4.0	110.0 $\pm$ 10.9	3.7 $\pm$ 0.1
4.45	36.2 $\pm$ 6.9	1.2 $\pm$ 0.1
4.93	15.1 $\pm$ 1.9	
<u>NAD-Malic Enzyme Pyruvate Reduction</u>		
<u>pH</u>	<u>K_i NADH (μM)</u>	
	<u>Mn²⁺</u>	<u>Mg²⁺</u>
5.7	—	78 $\pm$ 19
6.64	288 $\pm$ 53	—

pH Dependence of Oxalacetate Decarboxylation
for the *Ascaris suum* NAD-Malic Enzyme

As for the chicken liver enzyme, the nucleotide, NAD, is not required for the metal-dependent enzyme catalyzed decarboxylation of oxalacetate at all pH values. The presence of 60 mM malate completely inhibits oxalacetate decarboxylation by *Ascaris suum* malic enzyme while DTT did not have any effect on oxalacetate decarboxylation. Pyruvate kinase (E.C. 2.7.1.40), which has oxalacetate decarboxylation activity (Creighton and Rose, 1976), was not found in the *Ascaris suum* enzyme preparation. The maximum velocity with Mn^{2+} is pH independent (Fig. 13) while V/K for oxalacetate decreases at high and low pH using either Mn^{2+} or Mg^{2+} (Figures 13 and 14). Like the NADP-malic enzyme, the V/K_{OAA} profile most likely decreases below the pK for the β -carboxyl of oxalacetate and above the pK for an enzyme base. The pK of this base is ca. 5.4. The kinetic mechanism could not be determined since K_i for NAD was too high in the pH range tested. In the absence of nucleotide, Mn^{2+} increases the oxalacetate affinity for enzyme by about 24-fold compared with Mg^{2+} . The V_{max} profile was not obtained using Mg^{2+} as the divalent metal. The double reciprocal plots intersected at or near the origin suggesting the oxalacetate K_m was too high. A summary of pK values and absolute values of kinetic parameters are listed in Tables II and III.

Figure 13

pH Dependence of kinetic parameters for oxalacetate decarboxylation by Ascaris suum NAD-malic enzyme in the presence of Mn^{2+} .

The data at each pH were fitted using eq. 4 and the resulting values of V/K for oxalacetate as a function of pH were fitted using eq. 8. The theoretical curve for V/K is drawn from the fit using the appropriate equation. For V , the line represents the average value of the parameter.

Reaction mixtures contained 100 mM mixed buffer, 0.25-2.5 mM oxalacetate, 20 mM MnSO_4 , 264 nM enzyme.

NAD-MALIC ENZYME
OXALACETATE DECARBOXYLATION
(Mn^{2+})

Figure 14

pH Dependence of kinetic parameters for oxalacetate decarboxylation by Ascaris suum NAD-malic enzyme in the presence of Mg^{2+} .

The data at each pH were fitted using eq. 4 and the resulting values of V/K for oxalacetate as a function of pH were fitted using eq. 8. The theoretical curve drawn is from the fit using the appropriate equation.

Reaction mixtures contained 100 mM mixed buffer, 0.25-2.5 mM oxalacetate, 20 mM MgSO_4 , 1056 nM enzyme.

NAD-MALIC ENZYME
OXALACETATE DECARBOXYLATION
(Mg^{2+})

pH Dependence of the Metal-Dependent Reduction of
Pyruvate by the *Ascaris suum* NAD-Malic Enzyme

Using either Mg^{2+} or Mn^{2+} , the $V_{\max}$ is pH independent although slightly higher with Mn^{2+} (Figures 15 and 16). The V/K for pyruvate using either Mg^{2+} or Mn^{2+} decreases above a pK of about 6 (Figures 15 and 16). With Mn^{2+} , the V/K was about 5-fold higher than that obtained with Mg^{2+} . In addition, replacing Mg^{2+} with Mn^{2+} increases the affinity of enzyme for pyruvate by about 3.6-fold. The $V_{\max}$ of this reaction is 1/600 the rate of oxidative decarboxylation of malate (using Mg^{2+}). The kinetic mechanism appears to be rapid equilibrium ordered at pH values tested with NADH adding to enzyme first. Because of the large amount of enzyme used in this reaction, enzyme sometimes precipitated at pH values around 5, but this precipitate disappeared after adding saturating metal. A summary of pK values, absolute values of kinetic parameters, and K_i values for NADH are listed in Tables II, III and IV.

Inhibition Studies by Analogs of Malate

In order to find a tight binding inhibitory analog of malate, inhibition studies were carried out with malate as the variable substrate. In all instances, the K_i values are reported, except for the cases where metal-ligand stability constants were not known. In these cases, an apparent K_i value using a fixed concentration of metal was used correcting only for Mg-NAD and Mg-Malate. Examples of inhibition patterns using tartronate and oxalate are shown in Figures 17 and 18. Summary of K_i values is given in Table V.

Figure 15

pH Dependence of kinetic parameters for pyruvate reduction by Ascaris suum NAD-malic enzyme in the presence of Mn^{2+} . The data at each pH were fitted using eq. 4 and the resulting values of V/K for oxalacetate as a function of pH were fitted using eq. 9. The theoretical curve drawn for V/K is from the fit using the appropriate equation. For V , the line represents the average value of the parameter.

Reaction mixtures contained 100 mM Mes buffer, 1-4 mM pyruvate, 0.35 mM NADH, 80 mM MnSO_4 , 1758 nM enzyme.

NAD-MALIC ENZYME
PYRUVATE REDUCTION
(Mn^{2+})

Figure 16

pH Dependence of kinetic parameters for pyruvate reduction by Ascaris suum NAD-malic enzyme in the presence of Mg^{2+} . Data were analyzed as discussed in legend to Fig. 15.

Reaction mixtures contained 100 mM Mes buffer, 1-4 mM pyruvate, 0.35 mM NADH, 100 mM MgSO_4 , 1758 nm enzyme.

NAD-MALIC ENZYME
PYRUVATE REDUCTION
(Mg^{2+})

Figure 17

Competitive inhibition by tartronate vs. malate at pH 6.5. The data were fitted using eq. 6 to produce the theoretical curves as shown.

Reaction mixtures contained 100 mM Mes, pH 6.5, 2 mM NAD, 18 mM Mn^{2+} , 0.4-4 mM malate, 0.07-0.28 mM tartronate, 8.8 nM Ascaris suum enzyme. All substrate and inhibitor concentrations were corrected for the amount of metal-chelate formation as described under Materials and Methods (metal-chelate correction).

Figure 18

Noncompetitive inhibition by oxalate vs. malate at pH 6.5. The data were fitted using eq. 7 to produce the theoretical curves as shown. Reaction mixtures contained 100 mM Mes, pH 6.5, 2 mM NAD, 320 mM Mg^{2+} , 1.17-11.7 mM malate, 25-100 μ M oxalate, 8.8 nM Ascaris suum enzyme. All substrate and inhibitor concentrations were corrected for the amount of metal-chelate formation as described under Materials and Methods (metal chelate correction).

SUMMARY OF K_i VALUES FOR INHIBITING ANALOGS OF MALATE^a

<u>Analog</u> s	K_i (mM)	
	<u>Mg</u> ²⁺	<u>Mn</u> ²⁺
Tartronate	1.4	0.05
Oxalate ^b	0.08	0.007
L-Lactate	3.2	0.7
D-Lactate	32.9	14.9
Thioglycolate	7.7 ^c	0.02
Thiomalate ^c	2.0	0.39
L-Alanine	5.1	2.7
β -Chloroalanine ^c	5.5	1.1
DL-Isoserine ^c	NI ^d	75
Glycolate	55.3	6.2
Thiolactate ^c	11.7	2.7
β -Hydroxypyruvate ^c	43.0	5.5
β -Chlorolactate ^c	11.1	1.6
DL-Nitrolactate ^c	0.5	0.17
P-Hydroxyphenylpyruvate ^c	2.9	0.3
Acetopyruvate ^c	0.9	0.3
β -Fluoropyruvate ^c	20.3	1.3

^aAll data collected at pH 6.5, 100 mM Mes and 25° C.

^bInhibition by oxalate is noncompetitive vs. malate.

The K_{ii} is 0.29 mM with Mg²⁺ as the metal and 0.027 mM with Mn²⁺ as the metal.

^cInhibitor not corrected for Inhibitor-metal chelate.

^dNo inhibition.

CHAPTER IV

DISCUSSION

Interpretation of pH Dependence of the Extinction Coefficient

Solid-free oxalacetic acid is entirely composed of trans-enol form (Gruber et al., 1956; Banks, 1961). The infrared spectrum for the trans-enol does not exhibit the OH stretch for normal hydroxyl ($2.8\ \mu$), because of the strongly hydrogen-bonded hydroxyl. Several samples of solid-free oxalacetic acids have an identical infrared spectrum, an identical powder diffraction pattern. Although it has been previously reported (Wohl, 1907) that the low melting form is the cis-enol and the high melting form is the trans-enol, variation in the melting point is probably a result of contaminating keto form (Banks, 1961). The keto form cannot be crystallized. Once solid-free oxalacetic acid is dissolved in water, keto-enol tautomeric equilibrium is established. The amount of each isomeric component (trans, cis-enol; keto; gem-diol) depends on the pH and the nature and amount of metal in solution among other things. At pH 7.4, 74.3% is keto, 17.8% is enol and 7.8% is the hydrated gem-diol (Pogson and Wolfe, 1972). The keto form is the substrate for malic enzyme (Schimerlik and Cleland, 1977b). The keto form has two acid dissociable groups while the enol form has three. The enolic form of oxalacetic acid has dramatically

increased absorption at 280 nm due to the conjugate π - π^* bond system of the enolic form (Kosicki, 1962). Metal ions increase absorbance by increasing the proportion of enol (Fig. 7) relative to the overall oxalacetate concentration by shifting the keto-enol tautomeric equilibrium in favor of enol form. That is, the metal preferentially binds to the enol form.

As shown in Fig. 5, the λ_{max} for enolic oxalacetate in the presence of Mn^{2+} is 282.5 nm and that for the enolic oxalacetate in the presence of Mg^{2+} is 263.5 nm. The equilibrium molar extinction coefficient of oxalacetate has been determined in the pH range 3.8-6.7 as shown in Fig. 6. Protonation of the β -carboxyl of the enolic oxalacetate reduces the percentage of enolic species which is the chromophore (Tate et al., 1964a) by shifting the keto-enol tautomeric equilibrium toward the keto form and consequently favoring the gem-diol form at low pH. This appears to be the reason for decreasing the E_{app} as the pH decreases below 5 as shown in Fig. 6. There is a sharp increase in E_{app} for enolic oxalacetate in the presence of Mn^{2+} . This is most likely due to ionization of the enolic hydroxyl group and Mn^{2+} stabilizes the trianion form of enolic oxalacetate. The highest E_{app} value is $3.2 \text{ mM}^{-1} \text{ cm}^{-1}$ when almost all oxalacetate exists as the trianion form of the enol. The value obtained in Fig. 6 is in excellent agreement with the molar extinction coefficient of oxalacetic acid in ether ($3.32 \text{ mM}^{-1} \text{ cm}^{-1}$), in

which solvent the acid is known to be the protonated enol (Banks, 1961; Meyer, 1912; Hantzsch, 1915).

As in the case of Mn^{2+} , it is expected that the E_{app} for enolic oxalacetate in the presence of Mg^{2+} will also exhibit a sharp increase at pH values about 8 (Tate et al., 1964b). In other words, between pH 5 and 7 in the case of Mn^{2+} , the dianion and trianion forms of enolic oxalacetate compete for Mn^{2+} but in the case of Mg^{2+} the dianion form of the enol is the predominant form (the overall predominant species is still the keto form). The pK for the enolic hydrogen is about 12 (Tate et al., 1964a) in the absence of metal.

Doubling the Mg^{2+} concentration from 20 mM to 40 mM inhibits the decarboxylation of oxalacetate by chicken liver malic enzyme at 4.0-5.0. This is most likely a result of depletion of the enzymatically active uncomplexed keto form of oxalacetate as a result of build-up of the enolic oxalacetate. The increase in E_{app} is consistent with this result. To be a valid assay keto-enol tautomerism must not affect the kinetics of oxalacetate disappearance. In this study, because metal increases spontaneous keto-enol tautomerism, the disappearance of oxalacetate is not affected by tautomerism (Dolin, 1968; Loewus et al., 1955).

pH Dependence of Kinetic Parameters for Oxalacetate Decarboxylation by Chicken Liver NADP-Malic Enzyme

The chicken liver NADP-malic enzyme used in the present studies and the pigeon liver malic enzyme are kinetically

identical as shown by Cook and Cleland (1981). It has previously been reported that the pigeon liver NADP-malic enzyme requires NADP for oxalacetate decarboxylation at least below pH 5.5 (Schimerlik and Cleland, 1977b). However, the present study shows that NADP is not required at all pH values tested. The velocity vs. enzyme concentration curve is linear in the presence and absence of NADP. The presence of the nucleotide does increase the affinity of enzyme for oxalacetate by about 8-fold with Mn^{2+} and about 2-fold with Mg^{2+} . The enzyme prefers Mn^{2+} over Mg^{2+} for oxalacetate decarboxylation.

The kinetic mechanism appears to be rapid equilibrium ordered at pH values below 5.0 with NADP adding to enzyme first. The K_i for NADP increases by a factor of 10 per pH unit below pH 5.0 (Table IV). Thus, it appears that the 2' phosphate of NADP must be unprotonated for optimum binding as suggested by Schimerlik and Cleland (1977b). All pH profiles for oxalacetate decarboxylation decrease below the pK for the β -carboxyl of oxalacetate (pK=3.8) and above the pK for an enzyme residue. These data suggest that only the dianion form of oxalacetate is a substrate and an enzyme residue is required to be protonated for oxalacetate decarboxylation. The same group has been shown to be required unprotonated for malate oxidative decarboxylation (Schimerlik and Cleland, 1977b). Thus, the group must accept a proton from the 2-hydroxyl of malate concomitant with hydride transfer. It would then be protonated to hydrogen-bond to the oxalacetate intermediate. The pK of the enzyme base decreases from a

value of 6.4 in the absence of nucleotide to about 5.5 with Mg^{2+} and 4.8 with Mn^{2+} in the presence of nucleotide. This phenomena is probably due to conformational perturbation of the pK for this enzyme base. Purging the reaction mixtures with dry nitrogen did not affect the rate of oxalacetate decarboxylation.

The pH dependence of $\log V$ and $\log V/K$ for oxalacetate exhibit identical pK values. Thus, oxalacetate binds equally well to protonated and unprotonated forms of the enzyme, but only the protonated form of the enzyme is catalytically competent. This mechanism requires that the observed pK is the true pK.

pH Dependence of Kinetic Parameters for Oxalacetate Decarboxylation by the *Ascaris suum* NAD-Malic Enzyme

Since the initial identification of the *Ascaris suum* NAD-malic enzyme by Saz and Hubbard (1957) it has been placed in classification E.C. 1.1.1.39 which indicates a failure to decarboxylate oxalacetate. However, the present study shows that this enzyme does catalyze the decarboxylation of oxalacetate either in the absence or presence of NAD. The addition of 1 mM DTT had no effect on the decarboxylation reaction while 60 mM malate completely inhibits the decarboxylation of oxalacetate. As a side reaction, pyruvate kinase (E.C. 2.7.1.40) (Creighton and Rose, 1976) catalyzes the decarboxylation of oxalacetate, but pyruvate kinase activity was not detected in the NAD-malic enzyme preparation. Although 100 μ M NAD did slightly increase V and V/K for oxalacetate

(Table III), the K_i for NAD from E:NAD is too high to saturate with NAD since the increase in absorbance as a result of the nucleotide made it impossible to monitor the reaction. In the absence of nucleotide, Mn^{2+} increases the oxalacetate affinity for enzyme by about 24-fold compared with Mg^{2+} .

As for the chicken liver malic enzyme, the V/K profiles for oxalacetate decrease below the β -carboxyl of oxalacetate and above the pK for an enzyme residue. The V profile with Mn^{2+} or Mg^{2+} is pH independent, suggesting that oxalacetate binds to only correctly protonated form of enzyme, as was also found for the oxidative decarboxylation reaction (Kiick, 1985).

In summary, the enzyme classification for the Ascaris suum malic enzyme should be changed to E.C. 1.1.1.38 (oxalacetate decarboxylating). Data presented here are consistent with oxalacetate as an intermediate in the reaction, as has already been shown for the NADP enzyme from chicken liver (Hermes et al., 1982). A proposed reaction mechanism for oxalacetate decarboxylation for the NAD and NADP enzyme is shown in Fig. 19A.

pH Dependence of Kinetic Parameters for the Metal-
Dependent Reduction of Pyruvate by the
Ascaris suum NAD-Malic Enzyme

The turnover number for this reaction is very low (Table III). As a result, high enzyme concentrations (4 U/ml) in each cuvette) were used. Contaminating lactate

Figure 19

- A. Proposed reaction mechanism for oxalacetate decarboxylation for the NAD and NADP malic enzyme.
- B. Proposed reaction mechanism for pyruvate reduction for Ascaris suum NAD malic enzyme.

A.

B.

dehydrogenase activity was detected under these conditions as non-metal-dependent activity. In addition, NADH is degraded at low pH. Thus, a blank was run for each cuvette.

At pH 5.0, in some instances after adding high concentrations of enzyme to the reaction mixture in the absence of metal, some of the protein precipitated. However, this precipitate disappeared after adding a saturating level of metal (100 mM Mg^{2+} or 80 mM Mn^{2+}). This phenomenon may result from an isoelectric precipitation. Although Fodge *et al.* 1972) reported a value of 6.63 for the pI, this value will depend on other solutes.

To identify the acid-base catalyst responsible for the malic enzyme catalyzed pyruvate reduction, the pH dependence of kinetic parameters was obtained. The V for pyruvate reduction is pH independent as is the V for decarboxylation of oxalacetate and the oxidative decarboxylation of malate by this enzyme. Therefore, pyruvate binds to only correctly protonated form of enzyme. The V/K for pyruvate decreases above the pK for an enzyme base. This base must be protonated for reaction and is the same base which must be unprotonated to abstract a proton from the 2-hydroxyl of malate in the oxidative decarboxylation reaction. The pK of this base is ca. 6.0 which is within experimental error the same as that obtained from the V/K_{OAA} profile for oxidative decarboxylation. The reaction proceeds slightly better with Mn^{2+} as can be seen in Table III.

The kinetic mechanism appears to be rapid equilibrium ordered with NADH adding first (Table IV). The rate of this reaction is 1/600 the rate of the oxidative decarboxylation of malate (using Mg^{2+}). As a result, most likely every step prior to catalysis becomes rapid equilibrium. In Fig. 19B, a chemical mechanism is proposed for pyruvate reduction.

Interpretation of Inhibition Data

The purpose of this study is to determine which functional group(s) is(are) responsible for malate binding and what stereochemistry is involved in substrate/inhibitor binding. Oxalate is the tightest binding inhibitor found to date. Oxalate is an analog of enolpyruvate which is thought to be an intermediate of the oxidative decarboxylation of malate. Because of the structural similarity between E:NADH:Mg and E:NAD:Mg, oxalate binds to both. Thus, one obtains noncompetitive inhibition.

In Table V, all $\text{app}K_i$ values were corrected to K_i using the following equation (Park et al., 1984):

$$\text{app}K_i = K_i \left(1 + \frac{\text{mal}}{K_{\text{mal}}} + \frac{K_{\text{IM}}}{M} \right)$$

K_{mal} : Michaelis constant for malate

K_{IM} : Dissociation constant of metal from
ternary complex (E:NAD:M)

M: Metal concentration

Upon examination of all inhibitor structures and inhibition constants (Fig. 20), the following general comments can be made.

1. The malate binding site is specific for the L-isomer.
2. A negative charge at C-3 and C-4 contributes to binding.
3. There is no steric hindrance around C-3 as shown by para-hydroxyphenylpyruvate.
4. A hydroxyl rather than a thiol at C-2 contributes to binding because of the hydrogen bonding ability.

Figure 20

Structure and inhibition constant of inhibitory analogs of malate using Mn^{2+} or Mg^{2+} .

All data collected at pH 6.5, 100 mM Mes and 25° C.

a. Inhibition by oxalate is noncompetitive vs. malate.

The K_{ii} is 0.29 mM with Mg^{2+} as the metal and 0.027 mM with Mn^{2+} as the metal.

b. Inhibitor not corrected for inhibitor-metal chelate.

$\begin{array}{c} \text{COO}^- \\ \\ \text{COO}^- \end{array}$		$\begin{array}{c} \text{COO}^- \\ \\ \text{HO}-\text{C}-\text{H} \\ \\ \text{COO}^- \end{array}$		$\begin{array}{c} \text{COO}^- \\ \\ \text{HO}-\text{C}-\text{H} \\ \\ \text{CH}_3 \end{array}$		$\begin{array}{c} \text{COO}^- \\ \\ \text{HS}-\text{C}-\text{H} \\ \\ \text{H} \end{array}$	
Oxalate ^a		Tartronate		L-Lactate		Thioglycolate	
Mg	0.08	1.4		3.2		7.7 ^b	
Mn	0.007	0.05		0.7		0.02	
$\begin{array}{c} \text{COO}^- \\ \\ \text{H}_3\text{N}^+-\text{C}-\text{H} \\ \\ \text{CH}_3 \\ \\ (\text{CH}_2\text{Cl}) \end{array}$		$\begin{array}{c} \text{COO}^- \\ \\ \text{HS}-\text{C}-\text{H} \\ \\ \text{CH}_3 \end{array}$		$\begin{array}{c} \text{COO}^- \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{CH}_3 \end{array}$		$\begin{array}{c} \text{COO}^- \\ \\ \text{HO}-\text{C}-\text{H} \\ \\ \text{H} \end{array}$	
(B-Chloro) L-Alanine ^b		Thiolactate ^b		D-Lactate		Glycolate	
Mg	5.1 (5.5)	11.7		32.9		55.3	
Mn	2.7 (1.1)	2.7		14.9		6.2	
$\begin{array}{c} \text{COO}^- \\ \\ \text{HS}-\text{C}-\text{H} \\ \\ \text{H}-\text{C}-\text{H} \\ \\ \text{COO}^- \end{array}$		$\begin{array}{c} \text{COO}^- \\ \\ \text{HO}-\text{C}-\text{H} \\ \\ \text{H}-\text{C}-\text{H} \\ \\ \text{Cl} \end{array}$		$\begin{array}{c} \text{COO}^- \\ \\ \text{O}=\text{C} \\ \\ \text{H}-\text{C}-\text{H} \\ \\ \text{OH} \end{array}$		$\begin{array}{c} \text{COO}^- \\ \\ \text{HO}-\text{C}-\text{H} \\ \\ \text{H}-\text{C}-\text{H} \\ \\ \text{NH}_3^+ \end{array}$	
Thiomalate ^b		B-Chlorolactate ^b		B-Hydroxypyruvate ^b		DL-Isoserine ^b	
Mg	2.0	11.1		43.0		No inhibition	
Mn	0.39	1.6		5.5		75.0	
$\begin{array}{c} \text{COO}^- \\ \\ \text{HO}-\text{C}-\text{H} \\ \\ \text{H}-\text{C}-\text{H} \\ \\ \text{NO}_2 \end{array}$		$\begin{array}{c} \text{COO}^- \\ \\ \text{O}=\text{C} \\ \\ \text{H}-\text{C}-\text{H} \\ \\ \text{O}=\text{C} \\ \\ \text{CH}_3 \end{array}$		$\begin{array}{c} \text{COO}^- \\ \\ \text{O}=\text{C} \\ \\ \text{H}-\text{C}-\text{H} \\ \\ \text{C}_6\text{H}_4 \\ \\ \text{OH} \end{array}$		$\begin{array}{c} \text{COO}^- \\ \\ \text{O}=\text{C} \\ \\ \text{H}-\text{C}-\text{H} \\ \\ \text{F} \end{array}$	
DL-Nitrolactate ^b		Acetopyruvate ^b		P-Hydroxyphenyl pyruvate ^b		B-Fluoropyruvate ^b	
Mg	0.5	0.9		2.9		20.3	
Mn	0.17	0.3		0.3		1.3	

BIBLIOGRAPHY

- Allen, B. L. and Harris, B. G. (1981) Mol. Biochem. Parasitol. 2, 367.
- Atkins, J. F., Lewis, J. B., Anderson, C. W. and Gesteland, R. F. (1975) J. Biol. Chem. 250, 5688.
- Banks, B.E.C. (1961) J. Chem. Soc., 5043.
- Cleland, W. W. (1979) Methods in Enzymol. 63, 103.
- Cook, P. F. and Cleland, W. W. (1981) Biochemistry 20, 1790.
- Creighton, D. J. and Rose, I. A. (1976) J. Biol. Chem. 251, 61.
- Dolin, M. I. (1968) J. Biol. Chem. 243, 3916.
- Fernandez, M. J., Medrano, L., Ruiz-Amil, M. and Losada, M. (1967) Eur. J. Biochem. 3, 11.
- Fodge, D. W., Gracy, R. W. and Harris, B. G. (1972) Biochim. Biophys. Acta 268, 271.
- Frenkel, R. (1975) Current Topics in Cellular Regulation, Vol. 9, 157.
- Grover, S. D., Canellas, P. F. and Wedding, R. T. (1981) Arch. Biochem. Biophys. 209, 396.
- Gruber, W., Pfeleiderer, G. and Wieland, T. (1956) Biochem. Zeitschr. 328, 245.
- Hantzsch, A. (1915) Ber. Chem. Ges. 48, 1407.
- Hermes, J. D., Roeske, C. A., O'Leary, M. H. and Cleland, W. W. (1982) Biochemistry 21, 5106.
- Hsu, R. Y. (1970) J. Biol. Chem. 245, 6675.
- Hsu, R. Y. and Lardy, H. A. (1967) J. Biol. Chem. 242, 520.
- Imbuga, M. O. and Pearson, D. J. (1982) Insect Biochem. 12, 55.
- Klick, D. M. (1985) Ph.D. Dissertation, North Texas State University, Denton, TX.

- Korkes, S., del Campillo, A. and Ochoa, S. (1950) J. Biol. Chem. 187, 891.
- Kosicki, G. W. (1962) Can. J. Chem. 40, 1280.
- Lin, R. C. and Davis, E. J. (1974) J. Biol. Chem. 249, 3867.
- Loewus, F. A., Tchen, T. T. and Vennesland, B. (1955) J. Biol. Chem. 212, 787.
- London, J. and Meyer, E. Y. (1969) J. Bacteriol. 98, 705.
- MacRae, A. R. (1971) Biochem. J. 122, 495.
- Martell, A. E. and Smith, R. M. (1977) Critical Stability Constants, Vol. 1, 3, 5, Plenum Press, New York.
- Meyer, K. H. (1912) Ber. Chem. Ges. 45, 2860.
- Ochoa, S. (1952) The Enzymes. Vol. 2, 929, Academic Press, New York.
- O'Farrell, P. H. (1975) J. Biol. Chem. 250, 4007.
- Park, S. H., Kiick, D. M., Harris, B. G. and Cook, P. F. (1984) Biochemistry 23, 5446.
- Pogson, C. I. and Wolfe, R. G. (1972) Biochem. Biophys. Res. Comm. 46, 1048.
- Sauer, L. A. (1973a) Biochem. Biophys. Res. Commun. 50, 524.
- Sauer, L. A. (1973b) FEBS Lett. 33, 251.
- Saz, H. J. and Hubbard, J. A. (1957) J. Biol. Chem. 225, 921.
- Schimerlik, M. I. and Cleland, W. W. (1977a) Biochemistry 16, 565.
- Schimerlik, M. I. and Cleland, W. W. (1977b) Biochemistry 16, 576.
- Scrutton, M. (1971) Methods in Microbiology, Vol. 6A, 479, Academic Press, New York.
- Tang, C. L. and Hsu, R. Y. (1973) Biochem. J. 135, 287.
- Tate, S. S., Grzybowski, A. K. and Dotta, S. P. (1964a) J. Chem. Soc. 1372.
- Tate, S. S., Grzybowski, A. K. and Dotta, S. P. (1964b) J. Chem. Soc. 1381.

Wilkinson, G. N. (1961) Biochem. J. 80, 324.

Veiga Salles, J. B. and Ochoa, S. (1950) J. Biol. Chem. 187, 849.

Wohl, A. (1907) Ber. Chem. Ges. 40, 2282.