Articles

pH Dependence of Kinetic Parameters for Oxalacetate Decarboxylation and Pyruvate Reduction Reactions Catalyzed by Malic Enzyme†

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Received December 5, 1985; Revised Manuscript Received February 28, 1986

ABSTRACT: Both chicken liver NADP–malic enzyme and *Ascaris suum* NAD–malic enzyme catalyze the metal-dependent decarboxylation of oxalacetate. Both enzymes catalyze the reaction either in the presence or in the absence of dinucleotide. The presence of dinucleotide increases the affinity of oxalacetate for the chicken liver NADP–malic enzyme, but this information could not be obtained in the case of *A. suum* NAD–malic enzyme because of the low affinity of free enzyme for NAD. The kinetic mechanism for oxalacetate decarboxylation by the chicken liver NADP–malic enzyme is 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. An enzyme residue is required protonated for oxalacetate decarboxylation (by both enzymes) and pyruvate reduction (by the NAD–malic enzyme), but the β-carboxyl of oxalacetate must be unprotonated for reaction (by both enzymes). The pK of the enzyme residue of the chicken liver NADP–malic enzyme decreases from a value of 6.4 in the absence of NADP to about 5.5 with Mg^{2+} and 4.8 with Mn^{2+} in the presence of NADP. The pK value of the enzyme residue required protonated for either oxalacetate decarboxylation or pyruvate reduction for the *A. suum* NAD–malic enzyme is about 5.5–6.0. Although oxalacetate binds equally well to protonated and unprotonated forms of the NADP–enzyme, the NAD–enzyme requires that oxalacetate or pyruvate selectively bind to the protonated form of the enzyme. Both enzymes prefer Mn^{2+} over Mg^{2+} for oxalacetate decarboxylation. The NAD–malic enzyme also prefers Mn^{2+} for pyruvate reduction, and the rate of this reaction is $^1/_{600}$th the rate of the oxidative decarboxylation of malate (using Mg^{2+} as the divalent metal ion). The kinetic mechanism for pyruvate reduction is rapid equilibrium ordered with NADH adding first. A comparison of the absolute values of V_{max} for the oxidative decarboxylation of L-malate and the decarboxylation of oxalacetate suggests that decarboxylation of the oxalacetate intermediate is the main rate-determining step in the malic enzyme reaction. Since the NAD–malic enzyme from *A. suum* catalyzes the metal-dependent decarboxylation of oxalacetate and reduction of pyruvate, the present classification for this enzyme (EC 1.1.1.39) should be changed to EC 1.1.1.38.

Three distinct malic enzymes (EC 1.1.1.38–40) catalyze the metal-dependent oxidative decarboxylation of L-malate as depicted in eq 1. Two of the enzymes also catalyze the

$$\text{NAD(P)} + \text{L-malate} \rightleftharpoons \text{CO}_2 + \text{pyruvate} + \text{NAD(P)H} \quad (1)$$

decarboxylation of oxalacetate and the reduction of α-keto acids such as pyruvate and ketomalonate as shown in eq 2 and 3.

$$\text{oxalacetate} \xrightarrow{M^{2+},\ \text{NAD(P)}} \text{CO}_2 + \text{pyruvate} \quad (2)$$

$$\text{H}^+ + \text{NAD(P)H} + \alpha\text{-keto acid} \rightleftharpoons \alpha\text{-hydroxy acid} + \text{NAD(P)} \quad (3)$$

Malic enzymes have been purified from a number of sources including animals, plants, insects, parasites, and microorganisms, and their 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 the synthesis of fatty acids and other functions and the production of pyruvate for gluconeogenesis. A divalent cation is needed as an activator in all of the malic enzyme reactions.

All of the above reactions are catalyzed by pigeon liver malic enzyme (Hsu, 1970; Tang & Hsu, 1973) which has been purified in crystalline form by Hsu and Lardy (1967). Reaction 1 is catalyzed at neutral and high pH with the most favorable pH being 7.5 (Veiga-Salles & Ochoa, 1950). In addition, the pigeon liver malic enzyme catalyzes reaction 2 at a rate similar to reaction 1. The reaction is optimal at low pH and requires the presence of a divalent metal ion and NADP (Schimerlik & Cleland, 1977b). Therefore, Hsu (1970) suggested that oxalacetate is an intermediate in the oxidative decarboxylation of malate.

For reaction 3, oxalacetate is the best substrate for the NADP–malic enzyme from pigeon liver. The maximum velocity is about 10% of the rate of the reductive carboxylation of pyruvate (Schimerlik & Cleland, 1977b). 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 & Hsu, 1973).

†This research was supported by National Institutes of Health Grants AI-12331 (B.G.H.) and GM-36799 (P.F.C.), by Robert A. Welch Foundation Grants B-1031 (P.F.C.) and B-997 (B.G.H.), by an Organized Faculty Research Grant from the Texas College of Osteopathic Medicine (P.F.C.), and by Grant BRSG S07 RR07195-05 awarded by the Biomedical Research Grant Program, Division of Research Resources, National Institutes of Health (P.F.C.). P.F.C. is the recipient of an NIH Research Career Development Award (AM-01155).

0006-2960/86/0425-3752$01.50/0

Each of the malic enzymes studied thus far is distinguished by its dinucleotide specificity and oxalacetate decarboxylase activity. There are three different classifications of malic enzyme. The first classification is EC 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 EC 1.1.1.39 [L-malate:NAD oxidoreductase (decarboxylating)] which includes malic enzyme from the group D streptococcus (London & Meyer, 1969), cauliflower (MacRae, 1971), potato tuber (Grover et al., 1981), and *Ascaris suum* (Saz & Hubbard, 1957; Fodge et al., 1972). Finally, the third type is EC 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 the dung beetle *Catharsius* (Imbuga & Pearson, 1982). The class EC 1.1.1.38 exists only at low levels in vertebrates (Sauer, 1973a,b; Lin & Davis, 1974), but more activity is found in certain invertebrate tissues.

The *A. suum* NAD–malic enzyme (EC 1.1.1.39) has a steady-state random kinetic mechanism for reaction 1 (Park et al., 1984). In addition, the location of rate-limiting steps along the reaction path has been determined (Kiick et al., 1986).

The present study shows that the *A. suum* enzyme catalyzes the decarboxylation of oxalacetate and, thus, its classification should be changed to EC 1.1.1.38. In addition, it has been shown that NAD is not required for the decarboxylation reaction. For the chicken liver NADP–malic enzyme, NADP is also not required for oxalacetate decarboxylation at all pH values tested, but the presence of the dinucleotide does increase the affinity of enzyme for oxalacetate. Data are presented for the pH dependence of the kinetic parameters of reaction 2 (chicken liver and *A. suum* malic enzyme) and reaction 3 (*A. suum* malic enzyme). A chemical mechanism is proposed for these reactions.

Materials and Methods

Chemicals. The dinucleotides NAD and NADP were from Boehringer-Mannheim. L-Malate, NADH, oxalacetate (OAA),[1] DTT, liver alcohol dehydrogenase, and yeast aldehyde dehydrogenase were from Sigma. Pyruvate was from United States Biochemical Corp. Perdeuterioethanol was from Merck. An OAA solution was made fresh daily in ice-cooled water, titrated to pH 5.0 with KOH, and allowed to establish tautomeric equilibrium for 1 h (Dolin, 1968; Pogson & Wolfe, 1972). It was then stored in an ice bath. Only the keto form of oxalacetate is acted upon by the malic enzyme (Schimerlik & Cleland, 1977b).

The concentration of OAA was determined enzymatically by using 15 units of malate dehydrogenase and 0.3 mM NADH. A pyruvate solution was prepared by using the same procedure as for the OAA 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 concentrations of NAD, NADP, and NADH were determined spectrophotometrically by using extinction coefficients of 17 800 M^{-1} cm^{-1} at 259 nm for NAD, 18 000 M^{-1} cm^{-1} for NADP at 259 nm, and 6220 M^{-1} cm^{-1} for the reduced dinucleotide at 340 nm. All other chemicals and reagents were obtained from commercial sources and were of the highest purity available.

Enzyme Assays. Mitochondrial NAD–malic enzyme from *Ascaris suum* was purified 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 units/mg 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 *A. 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, and freeze–thawing had no apparent effect on activity. In later studies (for example, those for reduction of pyruvate to give lactate), large concentrations of enzyme were used. As a result, 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, 40 mM β-mercaptoethanol, 3 mM EDTA, and 10% glycerol and stored according to conditions identical with those given above for *A. suum* malic enzyme. The chicken liver enzyme was assayed in the direction of oxidative decarboxylation by using a mixed buffer system (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). The enzyme concentration (catalytic sites) is expressed in units of nanomolar.

All assays were carried out at 25 °C with a Gilford 2600 spectrophotometer, a Hewlett-Packard 7225B Graphics Plotter, and a 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 water bath prior to assay.

The decarboxylation of OAA was monitored by using the disappearance of the enolic OAA absorbance at 282.5 nm in the presence of Mn^{2+} and at 263.5 nm in the presence of Mg^{2+}. In the case of the chicken liver enzyme, the 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. For the *A. suum* enzyme, the velocity was also linearly dependent on enzyme concentration over the range 550–1360 nM with Mg^{2+} as the divalent metal ion. Oxalacetate was simultaneously added to four cuvettes 30 s before enzyme, and a background rate was recorded for each point. The reported velocities are corrected for the background rate resulting from nonenzymatic OAA decarboxylation catalyzed by the divalent metal ion. Examples of time courses at several different concentrations of the *A. suum* enzyme are shown in Figure 1. Dissolved CO_2 does not affect the enzymatic velocities even at 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+}, and 1 mM DTT in the presence and absence of NADP with 180 nM (using Mn^{2+}) or 360 nM (using Mg^{2+}) enzyme with OAA concentration

[1] Abbreviations: OAA, oxalacetate; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; LDH, lactate dehydrogenase; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid.

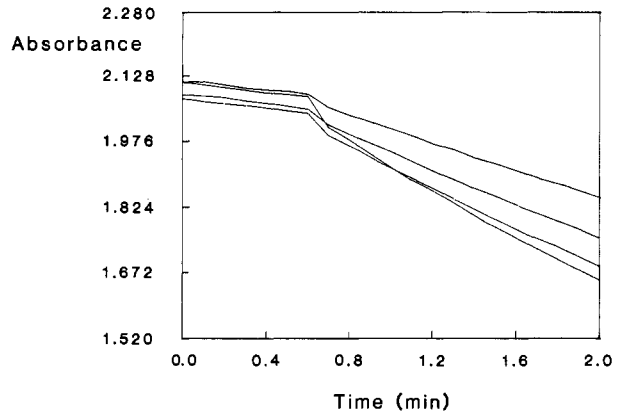

FIGURE 1: Time courses for oxalacetate decarboxylation by the *A. suum* NAD–malic enzyme with Mg^{2+} as the metal ion. Data were obtained at pH 4.5, 100 mM mixed buffer with 20 mM $MgSO_4$ and 1.7 mM OAA. Enzyme was added at the break in the curves. The enzyme concentrations from top to bottom are 550, 820, 1090, and 1360 nM, respectively.

varied from 0.1 to 3 mM. The apparent K_m for OAA is about 0.2 mM with Mn^{2+} and 1 mM with Mg^{2+} in the presence of NADP. In the absence of dinucleotide, a K_m value of 3 mM is obtained when either metal ion is used. The highest OAA concentration used was 3 mM since the absorbance is too high at higher OAA concentrations. As a result, there is some error in the V_{max} values obtained in the absence of NADP. A typical assay for the *A. suum* malic enzyme contained mixed buffer (see pH Studies) from pH 4.0 to 6.5, 20 mM Mg^{2+} or Mn^{2+}, and 1 mM DTT in the absence and presence of NAD with 260 nM (using Mn^{2+}) or 1060 nM (using Mg^{2+}) enzyme with OAA concentration varied from 0.25 to 2.5 mM.

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

Metal Chelate Correction. The concentrations of the reactants malate, dinucleotide, and pyruvate added to the reaction mixture were corrected for the concentration of the metal–ligand chelate complex according to Park et al. (1984).

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

Pyruvate reduction studies were carried out by using 100 mM Mes titrated to the desired pH with KOH. All points in pH studies were duplicated at least 2 times. The points shown were the average of the duplicate values of saturation curves obtained by using four or more substrate concentrations. The standard error is within the size of the point. The pH in all experiments was measured with a Markson pH meter standardized at the given temperature to ±0.01 pH unit. 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 was not affected by increases in ionic strength to at least 3.5 M. At pH values around 5.0, enzyme sometimes precipitated, but the precipitate was always redissolved upon addition of saturating Mn^{2+} or Mg^{2+}.

Deuterium Isotope Effects. Isotope effects were obtained by direct comparison of initial velocities. The concentration of NADH(D) was maintained at 0.35 mM while Mg^{2+} (100 mM) or Mn^{2+} (80 mM) was maintained at a fixed saturating concentration. In all cases, the concentration of pyruvate was varied from 1 to 6 mM (corrected for metal chelate complex formation).

Both NADH and A-side NADD were prepared in parallel reactions using a modification of the method of Viola et al. (1979). The reaction mixture contained the following in a 5-mL volume: 50 mM Taps (titrated with KOH), pH 9, 20 mM NAD, 100 mM ethanol (or ethanol-d_6), 1 mM DTT, 25 units of equine liver alcohol dehydrogenase, and 50 units of yeast aldehyde dehydrogenase. The reaction was allowed to proceed for 50 min with continuous monitoring of pH and adjustment to pH 9.0 with dilute KOH when necessary. After 50 min, reaction mixtures were filtered to remove enzymes by using an Amicon microconcentrator (Centricon 10). The resulting solutions were used without further purification. The estimated concentration of ethanol after dilution was 1–2 mM, and this concentration of ethanol has no effect on the malic enzyme reaction.

Data Processing. Reciprocal initial velocities were plotted as a function of reciprocal substrate concentrations, and all plots were linear. Data were fitted by using the appropriate rate equation whenever possible 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 by using the appropriate rate equation. Data conforming to a straight line were fitted by using the equation for a straight line ($y = mx + b$). Saturation curves for oxalacetate and pyruvate that were used to obtain the pH profiles were fitted by using eq 4. Data conforming to rapid

$$v = \frac{VA}{K_a + A} \tag{4}$$

equilibrium ordered addition of A prior to B were fitted by using eq 5. Data for deuterium isotope effects by direct

$$v = \frac{VAB}{K_{ia}K_b + K_bA + AB} \tag{5}$$

comparison of initial velocities were fitted by using eq 6 and 7, respectively. In eq 4–7, K_a and K_b are the apparent Mi-

$$v = \frac{VA}{K_a(1 + F_iE_{V/K}) + A(1 + F_iE_V)} \tag{6}$$

$$v = \frac{VA}{(K_a + A)(1 + F_iE_V)} \tag{7}$$

chaelis constants for A and B, A and B are reactant concentrations, K_{ia} is the dissociation constant for A, and V is the maximum velocity. In eq 6 and 7, F_i is the fraction of deuterium in the labeled compound while E_V and $E_{V/K}$ are the isotope effects minus 1 for the respective parameters. Equation 6 allows independent isotope effects on V and V/K while eq 7 assumes the isotope effects on V and V/K are identical. Data

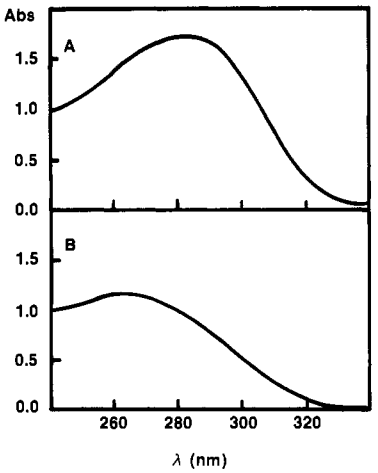

FIGURE 2: Absorption spectra for the enol of oxalacetate in the presence of metal ion. (A) Spectrum obtained in 100 mM mixed buffer, pH 4.5, 1 mM OAA, and 20 mM $MnSO_4$. The λ_{max} is 282.5 nm. (B) Spectrum obtained in 100 mM mixed buffer, pH 4.5, 1 mM OAA, and 20 mM $MgSO_4$. The λ_{max} is 263.5 nm.

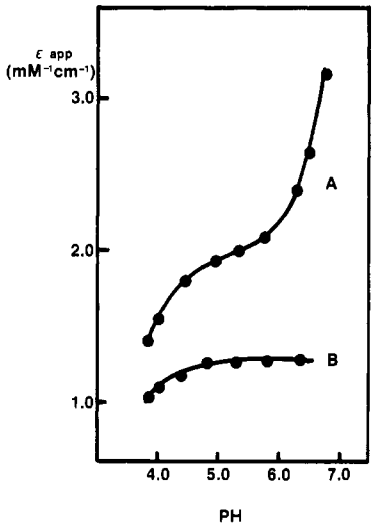

FIGURE 3: pH dependence of the effective equilibrium molar extinction coefficient of the enol of oxalacetate in the presence of metal ion. All data were obtained in 100 mM buffer with 20 mM metal ion. (A) $MnSO_4$; (B) $MgSO_4$.

for pH profiles which decreased with a slope of 1 at low pH and a slope of −1 at high pH were fitted by using eq 8, while data for pH profiles which decreased with a slope of −1 at high pH were fitted by using eq 9. In eq 8 and 9, K_1 and K_2 are

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

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

the acid dissociation constants of enzyme or substrate functional groups that must be unprotonated and protonated, respectively, for activity, while the hydrogen ion concentration is H, and C is the pH-independent value of y.

RESULTS

pH Dependence of the Extinction Coefficient of Enolic Oxalacetate in the Presence of Metal Ion. To determine λ_{max} for the enolic oxalacetate in the presence of metal ion, the absorption spectrum was obtained from 240 to 340 nm. Results are shown in Figure 2. The λ_{max} in the presence of Mn^{2+} is 282.5 nm, while that in the presence of Mg^{2+} is 263.5 nm. The effective extinction coefficient for enolic oxalacetate was

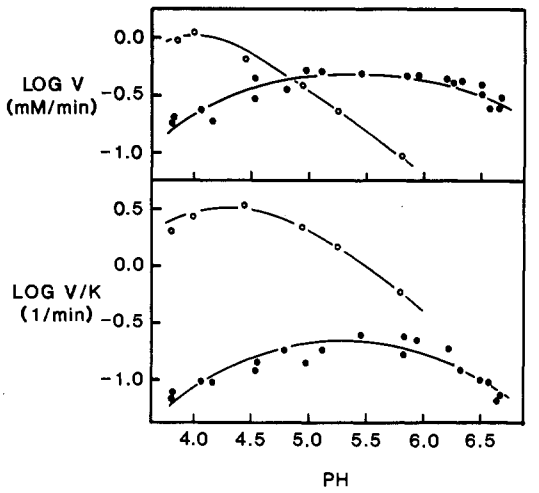

FIGURE 4: pH dependence of the kinetic parameters for oxalacetate decarboxylation by chicken liver NADP-malic enzyme with Mn^{2+} in the presence and absence of NADP. The theoretical curves are from the fit obtained by using eq 8. Closed circles represent reaction mixtures containing 100 mM mixed buffer, 0.1–3 mM OAA, 20 mM $MnSO_4$, and 180 nM enzyme while open circles represent mixtures containing 100 mM mixed buffers, 0.1–3 mM OAA, 20 mM $MnSO_4$, (below pH 4.93) 0.025–0.2 mM NADP or (above pH 5.0) 0.05 mM NADP, and 180 nM enzyme.

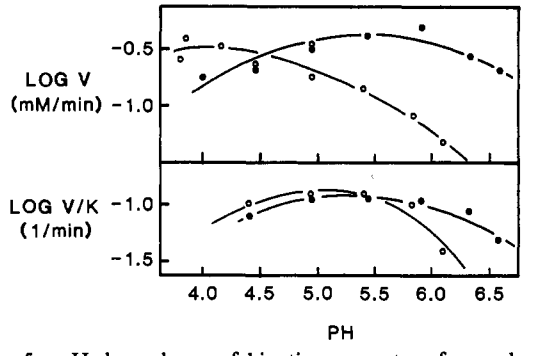

FIGURE 5: pH dependence of kinetic parameters for oxalacetate decarboxylation by chicken liver NADP-malic enzyme with Mg^{2+} in the presence and absence of NADP. The theoretical curves are from a fit obtained by using eq 8. Closed circles represent reaction mixtures containing 100 mM mixed buffer, 0.1–3 mM OAA, 20 mM $MgSO_4$, and 360 nM enzyme. Open circles represent reaction mixtures containing 100 mM mixed buffer, 0.1–3 mM OAA, 20 mM $MgSO_4$, (below pH 4.5) 0.01–0.2 mM NADP or (above pH 4.5) 0.05 mM NADP, and 360 nM enzyme.

obtained as a function of pH with each metal ion at a concentration of 20 mM and an OAA concentration of 0.1 mM. In the case of Mg^{2+}, the effective extinction coefficient decreases below pH 5, while for Mn^{2+}, in addition to the decrease below pH 5, the extinction coefficient increases above pH 6.0 (Figure 3). The presence of NADP, NAD, and DTT did not affect ϵ_{app} vs. pH. The nonenzymatic decarboxylation of oxalacetate catalyzed by Mn^{2+} gave a final absorbance of 0.05 after several hours at 282.5 nm. In addition, no absorbance at 282.5 nm was obtained under any conditions in the absence of oxalacetate.

pH Dependence of Oxalacetate Decarboxylation for Chicken Liver NADP-Malic Enzyme. As shown in Figures 4 and 5, NADP is not required, but divalent metal ion is required for the enzyme-catalyzed decarboxylation of oxalacetate at all pH values tested. However, the presence of the dinucleotide does increase the affinity of enzyme for oxalacetate by about 8.4-fold with Mn^{2+}, and about 2.2-fold when Mg^{2+} is used as the divalent metal ion (Table II). No change in the maximum rate or the K_m for oxalacetate is observed

Table I: Summary of pK Values[a]

parameters	pK_a ± SE	pK_b ± SE
NADP–Malic Enzyme		
V_{OAA} (Mn^{2+})	4.18 ± 0.09	6.69 ± 0.12
V_{OAA} (Mn^{2+}, NADP)		4.7 ± 0.12
V_{OAA} (Mg^{2+})	4.29 ± 0.21	6.59 ± 0.27
V_{OAA} (Mg^{2+}, NADP)	4.04 ± 0.25	5.42 ± 0.14
V/K_{OAA} (Mn^{2+})	4.18 ± 0.11	6.43 ± 0.11
V/K_{OAA} (Mn^{2+}, NADP)		5.08 ± 0.14
V/K_{OAA} (Mg^{2+})	4.32 ± 0.19	6.39 ± 0.13
V/K_{OAA} (Mg^{2+}, NADP)	4.48 ± 0.53	5.57 ± 0.37
NAD–Malic Enzyme		
V/K_{OAA} (Mn^{2+})	4.22 ± 0.09	5.34 ± 0.09
V/K_{OAA} (Mn^{2+}, NAD)	4.13 ± 0.37	5.16 ± 0.23
V/K_{OAA} (Mg^{2+})	4.15[b]	5.5[b]
V/K_{pyr} (Mn^{2+})		6.09 ± 0.22
V/K_{pyr} (Mg^{2+})		6.09 ± 0.24

[a] The subscript OAA is for OAA decarboxylation, and the subscript pyr is for pyruvate reduction. [b] Estimated value.

Table II: Absolute Values of Kinetic Parameters

parameter	
NADP–Malic Enzyme	
V_{OAA}/E_t (Mn^{2+}) (s^{-1})	47
V_{OAA}/E_t (Mn^{2+}, NADP) (s^{-1})	88
V_{OAA}/E_t (Mg^{2+}) (s^{-1})	37
V_{OAA}/E_t (Mg^{2+}, NADP) (s^{-1})	21
$V/K_{OAA}E_t$ (Mn^{2+}) (M^{-1} s^{-1})	1.9×10^4
$V/K_{OAA}E_t$ (Mn^{2+}, NADP) (M^{-1} s^{-1})	3.0×10^5
$V/K_{OAA}E_t$ (Mg^{2+}) (M^{-1} s^{-1})	1.1×10^4
$V/K_{OAA}E_t$ (Mg^{2+}, NADP) (M^{-1} s^{-1})	1.3×10^4
NAD–Malic Enzyme	
V_{OAA}/E_t (Mn^{2+}) (s^{-1})	28
V_{OAA}/E_t (Mn^{2+}, NAD) (s^{-1})	30
$V/K_{OAA}E_t$ (Mn^{2+}) (M^{-1} s^{-1})	2.8×10^4
$V/K_{OAA}E_t$ (Mn^{2+}, NAD) (M^{-1} s^{-1})	4.0×10^4
$V/K_{OAA}E_t$ (Mg^{2+}) (M^{-1} s^{-1})	1.2×10^3
$V/K_{OAA}E_t$ (Mg^{2+}, NAD) (M^{-1} s^{-1})	6.3×10^2
V_{pyr}/E_t (Mn^{2+}) (s^{-1})	0.09
V_{pyr}/E_t (Mg^{2+}) (s^{-1})	0.06
$V/K_{pyr}E_t$ (Mn^{2+}) (M^{-1} s^{-1})	81
$V/K_{pyr}E_t$ (Mg^{2+}) (M^{-1} s^{-1})	16

Table III: Summary of Dinucleotide K_i Values

NADP–Malic Enzyme Oxalacetate Decarboxylation		
	$K_{i,NADP}$ (μM)	
pH	Mn^{2+}	Mg^{2+}
4.0	110 ± 11	4.0 ± 0.1
4.45	36 ± 7	1.2 ± 0.1
4.93	15 ± 2	
NAD–Malic Enzyme Pyruvate Reduction		
	$K_{i,NADH}$ (μM)	
pH	Mn^{2+}	Mg^{2+}
5.7		80 ± 20
6.64	290 ± 50	

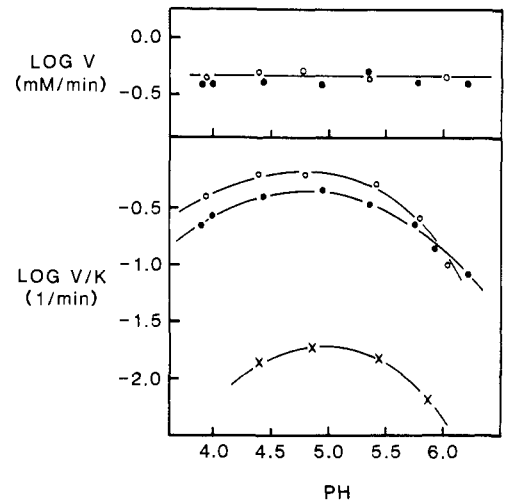

FIGURE 6: pH dependence of the kinetic parameters for oxalacetate decarboxylation by *A. suum* NAD–malic enzyme with Mn^{2+} (in the presence or absence of 0.1 mM NAD) or Mg^{2+}. The theoretical curve for the V/K values is from a fit obtained by using eq 8. For V, the line represents the average value of the parameter. Open circles represent reaction mixtures containing 100 mM mixed buffer, 0.25–2.5 mM OAA, 20 mM $MnSO_4$, 0.1 mM NAD, and 260 nM enzyme. Closed circles represent reaction mixtures containing 100 mM mixed buffer, 0.25–2.5 mM OAA, 20 mM $MnSO_4$, and 260 nM enzyme. The symbol (×) represents reaction mixtures containing 100 mM mixed buffer, 0.25–2.5 mM OAA, 20 mM $MgSO_4$, and 1060 nM enzyme.

when Mn^{2+} is replaced by Mg^{2+} in the absence of NADP at any pH value. A decrease is observed in both V and the V/K for oxalacetate below the pK for the β-carboxyl of oxalacetate and above the pK for an enzyme residue (Figures 4 and 5). When Mn^{2+} in the presence of NADP was used, there was a slight decrease below pH 4. Data were difficult to obtain below this pH as a result of an increasing K_m for oxalacetate most likely due to protonation of the β-carboxyl of OAA. In the absence of dinucleotide (Figures 4 and 5), the pK for the enzyme residue is 6.4–6.6 with either metal ion and is observed in both V and V/K_{OAA} pH profiles.

With NADP bound, the pK of the enzyme residue decreases to 4.8 with Mn^{2+} (Figure 4) and to 5.5 with Mg^{2+} (Figure 5). Initial velocity studies obtained by varying OAA concentration at different fixed levels of NADP give a pattern which intersects on the ordinate (figure not shown). Thus, 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 the K_i values for NADP is listed in Tables I, II, and III, respectively.

pH Dependence of Oxalacetate Decarboxylation for A. suum NAD–Malic Enzyme. As for the chicken liver enzyme, the dinucleotide NAD is not required for the metal-dependent enzyme-catalyzed decarboxylation of oxalacetate at all pH values tested. The presence of 60 mM malate completely inhibits oxalacetate decarboxylation by the *A. suum* malic enzyme while DTT had no effect on oxalacetate decarboxylation. Pyruvate kinase (EC 2.7.1.40), which has oxalacetate decarboxylase activity (Creighton & Rose, 1976), was not found in the *A. suum* enzyme preparation. The maximum velocity with Mn^{2+} is pH independent while the V/K for oxalacetate decreases at high and low pH using either Mn^{2+} or Mg^{2+} (Figure 6). As for the NADP–malic enzyme, the V/K_{OAA} profile most likely decreases below the pK for the β-carboxyl of oxalacetate and above a pK of 5.4 which represents an enzyme residue. The kinetic mechanism for oxalacetate decarboxylation for the NAD–malic enzyme could not be determined since the K_i for NAD is too high over the pH range tested. In the absence of dinucleotide, Mn^{2+} increases the oxalacetate affinity for enzyme by about 24-fold compared with Mg^{2+} (Table II). The V_{max} profile could not be obtained with Mg^{2+} as the divalent metal ion since the K_m for oxalacetate is too high. A summary of pK values and absolute values of kinetic parameters is listed in Tables I and II, respectively.

pH Dependence of the Metal-Dependent Reduction of Pyruvate by A. suum NAD–Malic Enzyme. With either Mg^{2+} or Mn^{2+}, the V_{max} for pyruvate reduction is pH independent although slightly higher with Mn^{2+} (Figure 7). The V/K for pyruvate using either Mg^{2+} or Mn^{2+} decreases above a pK of

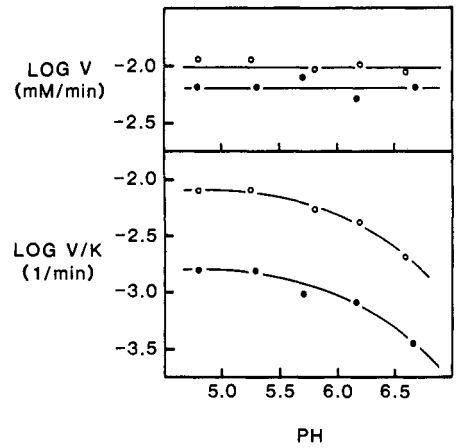

FIGURE 7: pH dependence of the kinetic parameters for pyruvate reduction by the *A. suum* NAD–malic enzyme in the presence of Mn^{2+} or Mg^{2+}. The theoretical curve for V/K is from a fit obtained by using eq 9. For V, the line represents the average value of the parameter. Open circles represent reaction mixtures containing 100 mM Mes buffer, 1–4 mM pyruvate, 0.35 mM NADH, 80 mM $MnSO_4$, and 1.76 μM enzyme. Closed circles represent reaction mixtures containing 100 mM Mes buffer, 1–4 mM pyruvate, 0.35 mM NADH, 100 mM $MgSO_4$, and 1.76 μM enzyme.

about 6 (Figure 7). With Mn^{2+}, the V/K is about 5-fold higher than that obtained with Mg^{2+} (Table II). In addition, replacing Mg^{2+} with Mn^{2+} increases the affinity of enzyme for pyruvate by about 3.6-fold (Table II). The V_{max} of this reaction is $^1/_{600}$th the rate of oxidative decarboxylation of malate using Mg^{2+} (Park et al., 1984). Initial velocity studies obtained by varying pyruvate concentration at different fixed levels of NADH gave a pattern which intersected on the ordinate (figure not shown). Thus, the kinetic mechanism appears to be rapid equilibrium ordered at all pH values tested, with NADH adding to enzyme first. A summary of pK values, absolute values of kinetic parameters, and K_i values for NADH is listed in Tables I, II, and III, respectively.

Deuterium Isotope Effects for Pyruvate Reduction by A. suum NAD–Malic Enzyme. Deuterium isotope effects were obtained at two pH values (above and below the pK observed in the $V/K_{pyruvate}$ profiles) with Mg^{2+} and Mn^{2+}. In all cases, isotope effects were present on V and V/K. Using the criteria of the lowest value of σ (sum of squares of residuals divided by degrees of freedom where degrees of freedom are equal to number of points minus the number of parameters) and the lowest standard errors on all parameters, we fit the data best by the equation which assumed equal isotope effects on V and V/K.

With Mg^{2+} at pH 5.5, $^DV = {}^D(V/K_{pyr}) = 1.69 \pm 0.10$, while at pH 6.25, $^DV = {}^D(V/K_{pyr}) = 1.75 \pm 0.12$. With Mn^{2+} at pH 5.5, $^DV = {}^D(V/K_{pyr}) = 1.56 \pm 0.04$, while at pH 6.25, $^DV = {}^D(V/K_{pyr}) = 1.54 \pm 0.05$. In all cases, the isotope effects are pH independent and relatively small.

Discussion

pH Dependence of the Effective Extinction Coefficient of the Metal–Oxalacetate Complex. The effective extinction coefficient of oxalacetate is a result of the enolic form of the compound (Kosicki, 1962). The metal ion increases the absorbance of oxalacetate as a result of shifting the keto–enol equilibrium in favor of the enol.

The decrease in the extinction coefficient at low pH in the case of both Mg^{2+} and Mn^{2+} is a result of the protonation of the β-carboxyl of oxalacetate, reducing the percentage of enolic species (Tate et al., 1964). The increase in the extinction coefficient in the case of Mn^{2+} is most likely a result of a shift in the equilibrium toward enol and enolate species.

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 & Cleland, 1977b). However, the present study shows that NADP is not required at any of the pH values tested. The velocity vs. enzyme concentration curve is linear in the presence and absence of NADP (figure not shown). The presence of the dinucleotide does increase the affinity of enzyme for oxalacetate by about 8-fold with Mn^{2+} and about 2-fold with Mg^{2+} (Table II). The enzyme prefers Mn^{2+} over Mg^{2+} for oxalacetate decarboxylation. Since the dinucleotide is not required for oxalacetate binding and subsequent decarboxylation, the kinetic mechanism for the NADP–malic enzyme has some degree of randomness compared to the original report by Hsu and Lardy (1967) but is still most likely predominantly ordered.

The kinetic mechanism for oxalacetate decarboxylation in the presence of NADP is rapid equilibrium ordered at pH values below 5.0, with NADP adding to enzyme first as indicated by an initial velocity pattern that intersects on the ordinate (figure not shown). The K_i for NADP increases by a factor of 10 per pH unit below pH 5.0 (Table III). 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 which is 4.2 under the conditions used in these studies. In addition, the pH profiles decrease 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 & 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 residue is 6.4 in the absence of dinucleotide and decreases to about 5.5 with Mg^{2+} and 4.8 with Mn^{2+} in the presence of dinucleotide. This phenomenon is probably due to a conformational change upon dinucleotide binding which results in a perturbation of the pK for this enzyme residue.

The pH dependencies of V and 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.

pH Dependence of Kinetic Parameters for Oxalacetate Decarboxylation by A. suum NAD–Malic Enzyme. Since the initial characterization of the *A. suum* NAD–malic enzyme by Saz and Hubbard (1957), it has been placed in classification EC 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 in the presence of NAD. The addition of 1 mM DTT had no effect on the decarboxylation reaction while 60 mM malate completely inhibits the reaction, indicating that the decarboxylation reaction makes use of the same active site as that used for malate oxidative decarboxylation. Although 0.1 mM NAD slightly increased V and V/K for oxalacetate

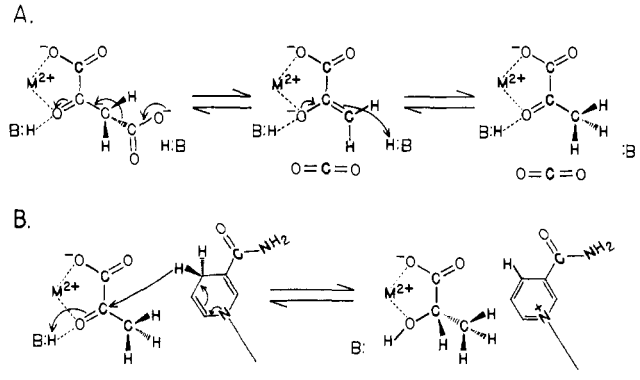

FIGURE 8: (A) Proposed reaction mechanism for oxalacetate decarboxylation by either the NAD- or the NADP-dependent malic enzymes. (B) Proposed reaction mechanism for pyruvate reduction for the *A. suum* NAD–malic enzyme.

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

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 the correctly protonated form of enzyme, as was also found for the oxidative decarboxylation reaction (Kiick et al., 1986).

In summary, the enzyme classification for the *Ascaris suum* NAD–malic enzyme should be changed to EC 1.1.1.38 (oxalacetate-decarboxylating). Data presented here are consistent with oxalacetate as an intermediate in the malic enzyme reaction, as originally proposed by Hsu and Lardy (1967), and work carried out for the NADP enzyme from pigeon liver (Schimerlik & Cleland, 1977b) and chicken liver (Hermes et al., 1982). The enzyme residue is postulated to perform the same role as that for the chicken liver enzyme (Figure 8A). The pK of 5.5 is essentially for the free enzyme form since NAD was not present at a saturating concentration. This pK agrees well with that obtained from the V/K_{NAD} pH profile for the malate oxidative decarboxylation reaction but is somewhat higher than the value of 4.9 obtained from the V/K_{malate} profile (Kiick et al., 1986). Since the predominant enzyme form present under V/K_{NAD} conditions is E–Mg–malate and for V/K_{malate} conditions is E–Mg–NAD, the presence of NAD must cause a decrease in the pK of the catalytic group that abstracts the proton from the 2-hydroxyl of L-malate concomitant with hydride transfer. Such a decrease was seen with the NADP–enzyme. Thus, as shown in Figure 8A, the enzyme residue must be protonated to hydrogen bond to the keto oxygen. The divalent metal ion then provides the necessary polarization of the carbonyl to facilitate decarboxylation. A second enzyme residue acts as a general acid, donating a proton to enolpyruvate to form pyruvate. This second enzyme residue was not observed in these studies since it has a pK of about 9.0 (Kiick et al., 1986) and thus is in its correct protonation state over the entire pH range covered.

pH Dependence of Kinetic Parameters for the Metal-Dependent Reduction of Pyruvate by Ascaris suum NAD–Malic Enzyme. The turnover number for pyruvate reduction is very low (Table II). As a result, high enzyme concentrations (4 units/mL) were used in each cuvette. Contaminating lactate dehydrogenase activity was detected under these conditions as non-metal-dependent activity. In addition, since NADPH is degraded at low pH, a minus metal ion plus EDTA blank was run for each reaction.

The pH dependence of kinetic parameters was obtained in order to identify the pK for the acid–base catalyst responsible for the malic enzyme catalyzed reduction of pyruvate. The V for pyruvate reduction is pH independent as is the V for decarboxylation of oxalacetate and the oxidative decarboxylation of malate with this enzyme. Therefore, pyruvate binds to only the correctly protonated form of enzyme as do malate and oxalacetate. The V/K for pyruvate decreases above the pK for an enzyme residue. This residue must be protonated for reaction (Figure 8B) and is the same residue which must be unprotonated to abstract a proton from the 2-hydroxyl of malate in the oxidative decarboxylation reaction. In the case of pyruvate reduction, the enzyme residue acts as a general acid, donating a proton to the keto oxygen concomitant with reduction. Divalent metal ion again is postulated to provide for polarization of the carbonyl. The pK of this residue is ca. 6.0 which is within experimental error the same as that obtained from the V/K_{OAA} profile for oxidative decarboxylation, particularly in view of the very low rate of pyruvate reduction and the difficulties of measurement of the reaction. The reaction proceeds slightly better with Mn^{2+} as can be seen in Table II.

The kinetic mechanism is rapid equilibrium ordered with NADH adding first as suggested by the initial velocity pattern that intersects on the ordinate (figure not shown). The rate of this reaction is $^1/_{600}$th the rate of the oxidative decarboxylation of malate (using Mg^{2+}). As a result, most likely every step prior to catalysis becomes rapid equilibrium.

Deuterium Isotope Effects. Although the maximum rate for pyruvate reduction is about $^1/_{600}$th the rate of oxidative decarboxylation, hydride transfer still does not appear to be rate limiting since isotope effects are small, 1.5–1.8. There is always the possibility that these values are intrinsic effects indicative of a late transition state, but the test of this hypothesis will have to await determination of the tritium isotope effects. An alternative explanation may be that the conformational change required to set up the enzyme active site for catalysis is not as facile with pyruvate as with oxalacetate.

Comparison of NAD- and NADP-Malic Enzymes. Both the NAD- and NADP-dependent enzymes catalyze the decarboxylation of oxalacetate. The pH dependence of the V/K for oxalacetate is qualitatively similar for both enzymes. Quantitatively, however, the pK for the enzyme residue is higher in the absence of dinucleotide with the chicken liver enzyme than that obtained for the *Ascaris* enzyme. In addition, there does not appear to be as much sensitivity to the metal ion with the NAD–enzyme. Although the data could not be obtained with the NAD–enzyme, we would predict that in the presence of saturating NAD, the pK for the enzyme residue would decrease to about 4.9.

The absolute values of the kinetic parameters are remarkably similar (Table II) with both enzymes preferring Mn^{2+} over Mg^{2+}. The only major difference is the 10-fold difference in V/K_{OAA} with Mg^{2+}. When the values in Table II are compared with those obtained for oxidative decarboxylation, the results are even more interesting. The V/E_t for malate oxidative decarboxylation for the NADP–enzyme with Mn^{2+} as the divalent metal ion is 39 s^{-1} while $V/K_{malate}E_t$ is 5×10^5 M^{-1} s^{-1}, and with Mg^{2+}, V/E_t is 28 s^{-1} while $V/K_{malate}E_t$ is 10^5 M^{-1} s^{-1} (Schimerlik & Cleland, 1977b). These values can be compared with the values in the presence of Mn^{2+} and NADP of 88 s^{-1} and 3×10^5 M^{-1} s^{-1} and in the presence of Mg^{2+} and NADP or 21 s^{-1} and 1.3×10^4 M^{-1} s^{-1}. Thus, decarboxylation

of oxalacetate is most likely a major rate-limiting step along the reaction pathway. In agreement with this, observed ^{13}C-isotope effects of about 3.5% are obtained for this reaction (Hermes et al., 1982).

Similarly, the values obtained for the NAD–malic enzyme reaction for malate oxidative decarboxylation with Mg^{2+} are a V/E_t of 38 s^{-1} and a $V/K_{malate}E_t$ of 4×10^4 M^{-1} s^{-1} while with Mn^{2+} the value for V/E_t is 65 s^{-1} and $V/K_{malate}E_t$ is 3×10^5 M^{-1} s^{-1}. Unfortunately, data in the presence of dinucleotide are not available, but the value of 28 s^{-1} for V/E_t for oxalacetate decarboxylation with Mn^{2+} as the divalent metal ion suggests a phenomenon similar to that observed for the NADP–enzyme. One of the largest differences appears to reside in the binding constant for the dinucleotide as shown in Table III.

ACKNOWLEDGMENTS

We thank Rita Huber and Wanda Ford for typing the manuscript.

Registry No. NADP, 53-59-8; NADH, 58-68-4; EC 1.1.1.40, 9028-47-1; EC 1.1.1.38, 9080-52-8; D_2, 7782-39-0; pyruvate, 127-17-3; oxalacetate, 328-42-7.

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