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pH Variation of the Kinetic Parameters and the Catalytic Mechanism of Malic Enzyme†

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ABSTRACT: The pH variation of the kinetic parameters for the oxidative decarboxylation of L-malate and decarboxylation of oxalacetate catalyzed by malic enzyme has been used to gain information on the catalytic mechanism of this enzyme. With Mn^{2+} as the activator, an active-site residue with a pK of 5.4 must be protonated for oxalacetate decarboxylation and ionized for the oxidative decarboxylation of L-malate. With Mg^{2+} as the metal, this pK is 6, and, at high pH, V/K for L-malate decreases when groups with pKs of 7.8 and 9 are deprotonated. The group at 7.8 is a neutral acid (thought to be water coordinated to Mg^{2+}), while the group at 9 is a cationic acid such as lysine. The V profile for reaction of malate shows these pKs displaced outward by 1.4 pH units, since the rate-limiting step is normally TPNH release, and the chemical reaction, which is pH sensitive, is 25 times faster. TPN binding is decreased by ionization of a group with pK 9.3 or protonation of a group with pK 5.3. The pH variation of the K_m for Mg shows that protonation of a group with pK 8.7 (possibly SH) decreases metal binding in the presence of malate by a factor of 1400, and in the absence of malate by a factor of 20. A catalytic mechanism is proposed in which hydride transfer is accompanied by transfer of a proton to the group with pK 5.4–6, and enolpyruvate is protonated by water coordinated to the Mg^{2+} (pK 7.8) after decarboxylation and release of CO_2.

Pigeon liver malic enzyme catalyzes reaction 2 at low pH and reaction 1 (the normal physiological one) at neutral and high pH. In addition to these rapid reactions, reaction 3 is catalyzed at a very slow rate (10% of the reverse rate of reaction 1 with oxalacetate, and only 1% with pyruvate).

$$\text{TPN}^{1} + \text{L-malate} \overset{Mg^{2+}\text{ or }Mn^{2+}}{\longleftrightarrow} CO_2 + \text{pyruvate} + \text{TPNH} \quad (1)$$

$$\text{Oxalacetate} \overset{\text{TPN, }Mg^{2+}\text{ or }Mn^{2+}}{\longrightarrow} CO_2 + \text{pyruvate} \quad (2)$$

$$\text{TPNH} + \alpha\text{-keto acid} \overset{Mg^{2+}\text{ or }Mn^{2+}}{\longrightarrow} \alpha\text{-hydroxy acid} + \text{TPN} \quad (3)$$

Reaction 1 has an ordered kinetic mechanism (Hsu et al., 1967), with TPN adding first before malate, and product release in the order CO_2, pyruvate, and TPNH. The studies reported in the two previous papers of this issue (Schimerlik and Cleland, 1977; Schimerlik et al., 1977) establish the specificity for binding of substrates and inhibitors, and indicate which steps in reaction 1 are rate limiting under different conditions. With this information in hand, it becomes possible to interpret the pH variation of the kinetic parameters and draw conclusions concerning the chemistry of the catalytic reaction. In this paper, we present pH studies on reactions 1 and 2, and propose a chemical mechanism for catalysis of these reactions.

Materials and Methods

Pigeon liver malic enzyme was isolated by the procedure of Hsu and Lardy (1967).

The pH profiles of reactions 1 and 2 were run in a buffer 25 mM in glycine, cacodylate, acetate, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) adjusted to pH by addition of KOH. The high-pH profiles of V and V/K for L-malate were also run in a cationic buffer system 25 mM in Tris and glycine or a neutral-acid buffer system 25 mM in diethylmalonate and phenolsulfonate in the presence and absence of 20% (v/v) dimethylformamide to determine whether the basic pKs resulted from neutral or cationic groups. Experiments to determine the enthalpy of ionization of basic groups were also run in the cationic buffer system at 15, 25, and 35 °C. Bovine serum albumin was included at 67 μg/ml in all experiments.

Reaction 1 was followed at 340 nm using a Beckman DU monochrometer with deuterium lamp, a Gilford optical density converter, and a 10-mV recorder with adjustable zero and multispeed drive. Full-scale sensitivity of 0.05–0.10 OD and a chart speed of 0.2–6.0 in./min were used. The temperature of the cell compartment was maintained with thermospacers and cuvets were equilibrated in a temperature bath before addition of enzyme via microliter syringe or adder-mixer.

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[1] Abbreviations used are: TPN, triphosphopyridine nucleotide; TPNH, reduced TPN; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; NMR, nuclear magnetic resonance.

Low-pH profiles using Mn^{2+} as the metal, as well as initial-velocity studies of L-malate vs. TPN with Mg^{2+} at pH 5–8, were run in 10-cm cells containing 30 ml, while all other experiments were run in 1.0-cm cells containing 3.0 ml.

Reaction 2 was followed by disappearance of the metal enolate absorbance at 281 nm. This method was linear with enzyme concentration for the range 0.05–0.5 unit/ml. Oxalacetate was added immediately before enzyme to 1-cm cuvets, 3.0 ml total volume, equilibrated to 25 °C in a temperature bath and a blank was run for each point. The reported velocities are the enzymatic velocities minus that of the blank.

Control experiments indicated that the enzyme was stable under these assay conditions at pH 7 and 4; however, the enzyme was inactivated at pHs greater than 9.5. Initial velocities from time courses at pHs greater than 9.5 were taken from the tangent to the curve extrapolated to time zero. Dimethylformamide was not inhibitory in the forward direction of reaction 1 until 30% (v/v); thus, 20% (v/v) was used with the neutral acid or cationic acid buffers in solvent perturbation studies.

pHs were measured with a Radiometer 26 pH meter standardized at the given temperature to ±0.01 pH unit with buffers from Beckman calibrated from 0 to 95 °C.

Data Processing. The nomenclature used in this paper is that of Cleland (1963a). When reciprocal velocities were plotted vs. reciprocal substrate concentrations, linear plots were obtained in all cases. Data conforming to sequential initial velocity, equilibrium ordered initial velocity, linear noncompetitive inhibition, linear competitive inhibition, single reciprocal plots, bell-shaped pH curves, half-bell-shaped pH curves with activity at acidic or basic pH, a pH curve with two plateaus, and a pH curve with two pK's and loss of activity upon ionization of either group were fitted to eq 4–13.

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \tag{4}$$

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

$$v = \frac{VA}{K\left(1 + \frac{I}{K_{is}}\right) + A\left(1 + \frac{I}{K_{ii}}\right)} \tag{6}$$

$$v = \frac{VA}{K\left(1 + \frac{I}{K_{is}}\right) + A} \tag{7}$$

$$v = \frac{VA}{K + A} \tag{8}$$

$$\log Y = \log\left(\frac{C}{1 + \frac{H}{K_1} + \frac{K_2}{H}}\right) \tag{9}$$

$$\log Y = \log\left(\frac{C}{1 + \frac{K_2}{H}}\right) \tag{10}$$

$$\log Y = \log\left(\frac{C}{1 + \frac{H}{K_1}}\right) \tag{11}$$

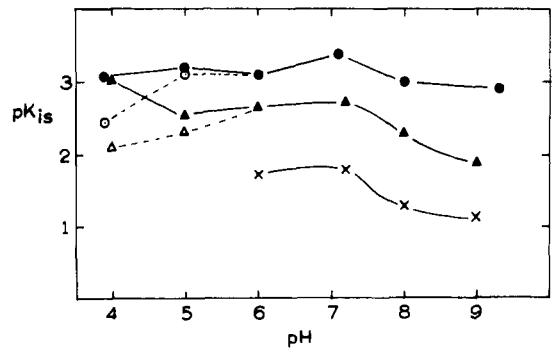

FIGURE 1: pH profiles for binding of inhibitors. Circles, oxalacetate; triangles, mesotartrate with malate as variable substrate (TPN, 0.3–1 mM; $MgSO_4$, 20 mM; data fitted to eq 7). Crosses, mesotartrate vs. pyruvate (TPNH, 0.2 mM; CO_2, 7.2, 3.4, 4.9, 1 mM at pH 6, 7.2, 8, 9; $MgSO_4$, 20 mM; data fitted to eq 6). Closed symbols, corrected for binding of only the dianion; open symbols, uncorrected.

$$pK_m = \log\left(\frac{c + d\left(\frac{K_1}{H}\right)}{1 + \frac{K_1}{H}}\right) \tag{12}$$

$$\log Y = \log\left(\frac{C}{(1 + K_3/H)(1 + K_4/H)}\right) \tag{13}$$

In eq 4 and 5, K_a and K_{ia} are the Michaelis and dissociation constants of A, and K_b the Michaelis constant of B, while in eq 6–8 K is the apparent Michaelis constant and K_{is} and K_{ii} are the apparent slope and intercept inhibition constants. V equals the maximum velocity in equations 4–8. In equations 9–11, K_1 and K_2 are acid dissociation constants of groups that must be deprotonated and protonated, respectively, for activity, H is the hydrogen-ion concentration, and C is the value of Y attained at the optimum state of protonation. In eq 12, log c and log d are the low- and high-pH values of pK_m, H is the hydrogen-ion concentration, and K_1 is the dissociation constant of the ionizable group. In eq 13, K_3 and K_4 are dissociation constants of groups that must both be protonated for activity, and C is the value of Y when both are protonated.

Experimental data were fitted to eq 4–13 by the least-squares method assuming equal variances for the values of v, pK_m, or log Y in eq 4–13 (Wilkinson, 1961), and using a digital computer and the Fortran programs of Cleland (1963b, 1967). The points in all plots are the experimental values, while the curves are calculated from the fits to the corresponding rate equations.

Results

pH Profiles for Inhibitors. The pH variation of pK_{is} for oxalacetate as a competitive inhibitor vs. malate, and for mesotartrate with either malate or pyruvate as the variable substrate is shown in Figure 1. The observed K_{is} values at low pH have been corrected by assuming that only the dianions were bound. This gives a flat profile for oxalacetate, but appears to overcorrect for mesotartrate; possibly, one or both of the monoanionic forms of the latter is also inhibitory. The curves in Figure 1 are drawn by eye, since there are not sufficient points for a fit to eq 12, but the ionization of a group with a pK about 8 clearly causes the binding of mesotartrate to become looser by a factor of 4–6.

pH Profiles for Oxalacetate Decarboxylation with Mn^{2+}. The decarboxylation of oxalacetate at low pH is catalyzed by both Mg^{2+} and Mn^{2+}, but the K_m for Mg^{2+} is so high (over 100 mM at pH 4.5) that kinetic studies were not practical.

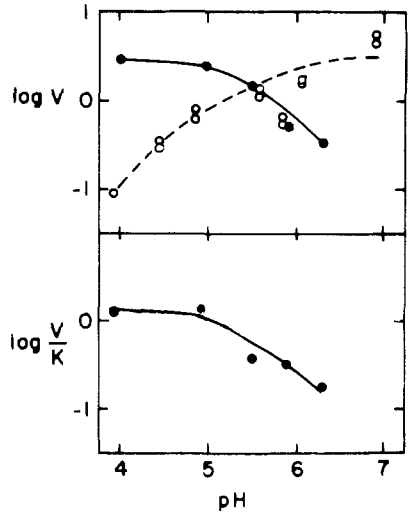

FIGURE 2: (Top) pH variation of V for reaction 1 (open circles, dashed line) fitted to eq 11, pK = 5.4, and reaction 2 (closed circles, solid lines) fitted to eq 10, pK = 5.4. Velocities for reaction 2 were multiplied by 3.4 to place the pH profiles on the same scale. (Bottom) pH variation of V/K for the dianion form of oxalacetate in reaction 2 fitted to eq 10, pK = 5.4. Mn^{2+} was the metal in these experiments.

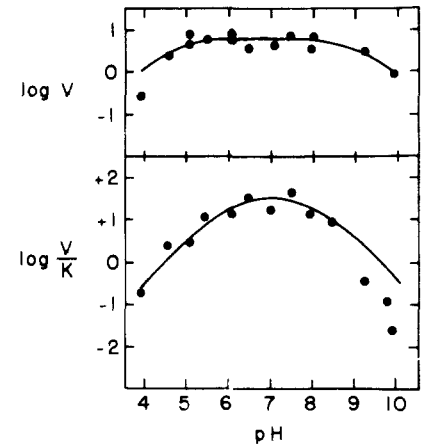

FIGURE 3: (Top) pH variation of V for reaction 1, fitted to eq 9, pK_1 = 4.6, pK_2 = 9.2. (Bottom) pH variation of V/K for malate, fitted to eq 9, pK_1 = 6, pK_2 = 8. Mg^{2+} was the metal in these experiments.

Thus, Mn^{2+} was used for studying reaction 2, and for comparing the kinetics of reactions 1 and 2. The pH profile of reaction 2 was determined from a combination of initial-velocity patterns for oxalacetate and TPN (pH 3.9–5.5) and reciprocal plots vs. oxalacetate at pH 5.9 (50 μM TPN) and 6.3 (30 μM TPN). The pattern at pH 3.9 is equilibrium ordered with TPN adding first, and the data were fitted to eq 5, while at pH 4.9 and 5.5 the patterns were sequential and were fitted to eq 4. At pH 5.9 and 6.3, the data were fitted to eq 8. All data were obtained at 20 mM $MnSO_4$, which is not saturating (K_m = 16 mM at pH 4.5 with 330 μM each of TPN and oxalacetate), and thus the V values are lower than if they were corrected to saturating metal ion. The V profile shown in Figure 2 was fitted to eq 10, giving a pK of 5.45 ± 0.10.

Although TPN seems necessary for the reaction at pH values of 5.5 and below, and its dissociation constants (K_i values) agree with those determined at similar pH values from malate–TPN initial-velocity patterns for reaction 1 with 20 mM $MgSO_4$ (see below and Figure 5), at pH 5.9 with 1 mM oxalacetate the rate in the absence of added TPN was 90% of that in the presence of saturating TPN. Thus, either the K_i has become low enough that enzyme-bound TPN is sufficient for activity, or the reaction really does not require TPN at pH values above 6. The latter possibility seems more likely.

The K_m values for oxalacetate, corrected for activity of only the dianion, were nearly constant in the range of 0.3–0.8 mM, and agreed well with the K_{is} values as a competitive inhibitor vs. malate of reaction 1 (Figure 1). The V/K profile shown in Figure 2 was fitted to eq 10, giving a pK of 5.4 ± 0.3.

pH Profile for Malate Oxidation with Mn^{2+}. The low-pH profile for reaction 1 was obtained with Mn^{2+} as the metal from a series of reciprocal plots with either L-malate as variable substrate at 20 mM $MnSO_4$ or with $MnSO_4$ as variable substrate with L-malate at 1 mM (except 5 mM at pH 3.93). TPN was saturating (500 μM below pH 5, and 333 μM above pH 5). Since the K_m for malate is below 100 μM over this pH range, except at the low end where it is 200 μM at pH 4.43 and 2 mM at pH 3.93, the V values from the $MnSO_4$ reciprocal plots were not corrected for lack of saturation with L-malate. The K_m for $MnSO_4$ undergoes drastic changes with pH, however (see Figure 6), rising from 40 mM at pH 7 to 180 mM at pH 5.54, and then dropping to 2.7 mM at pH 3.93. The V values from the malate reciprocal plots were thus corrected to saturating Mn^{2+} by multiplying by $(1 + K_{Mn}/Mn^{2+})$, where Mn^{2+} is 20 mM, and K_{Mn} the apparent Michaelis constant for $MnSO_4$ at each pH. Although this correction factor was as large as 10 at pH 5.54, the two sets of V values were nearly identical, as can be seen from Figure 2, where both are plotted as open circles. The full set of values was fitted to eq 11, giving a pK of 5.40 ± 0.05. Thus, the pK of the group, which must be ionized for reaction 1 to take place, is equal to the pK of the group which must be protonated to catalyze reaction 2.

pH Profile for Malate Oxidation with Mg^{2+}. Reaction 1 with Mg^{2+} was examined over the pH range 3.9–10. The malate and TPN initial-velocity pattern at pH 3.9 was equilibrium ordered with TPN adding first (the same results were obtained when Mn^{2+} was the metal) and the data were fitted to eq 5. Data for the V profile were obtained either from L-malate and $MgSO_4$ initial-velocity patterns at saturating TPN concentrations (1 mM below pH 4.5, 500 μM from pH 4.5 to 5.5, 333 μM from pH 5.5 to 8, 667 μM from pH 8.5 to 9.5, and 3.3 mM from pH 9.5 to 10) which were fitted to eq 4, or from reciprocal plots vs. L-malate at saturating TPN fitted to eq 8 and corrected to metal saturation as described above. The pH profile for V in the forward direction of reaction 1 (Figure 3) was obtained from a fit to eq 9 giving pK_1 = 4.55 ± 0.27 and pK_2 = 9.22 ± 0.37.

The log V/K_b vs. pH profile determined from the above data (Figure 3) falls off at high and low pH and was fitted to eq 9 giving pK_1 = 6.0 ± 0.4 and pK_2 = 8.0 ± 0.4. As can be seen from Figure 3, however, the slope at high pH appears to approach a limiting slope of two, rather than one, suggesting that the ionization of at least two groups affects V/K_b. The data in this case are not numerous enough to attempt to determine the two pK's, but, for six detailed profiles run from pH 6.3 to 10, significant fits were obtained on the assumption of two pK's (Table I and Figure 4).

pH Profiles for TPN. Values of pK_i for TPN were taken from TPN and L-malate initial-velocity patterns (20 mM Mg^{2+}) fitted to eq 4 or at pH 3.9 to eq 5. The pH profile in Figure 5 decreases at high and low pH with pK_1 = 5.3 ± 0.1 and pK_2 = 9.3 ± 0.1 from a fit to eq 9. With Mn^{2+} as the metal, very similar values were seen at pH 3.9–5.5 for reaction 2, and at pH 3.9–7 for reaction 1. It thus appears that the dissociation constant of TPN does not vary with the reaction

TABLE I: Apparent pK Shifts Induced by 20% (v/v) Dimethylformamide in Neutral and Cationic Acid Buffers.

	Neutral Acid Buffer[a]			Cationic Acid Buffer[b]		
pH Profile	−DMF	+DMF	Δ	−DMF	+DMF	Δ
V[c]	9.98 ± 0.32	8.93 ± 0.27	−1.05 ± 0.59	9.31 ± 0.10	9.62 ± 0.17	+0.31 ± 0.27
V/K_{malate}[d]	8.29 ± 0.04	7.75 ± 0.02[e]	−0.54 ± 0.06	7.84 ± 0.04	8.51 ± 0.02[e]	+0.67 ± 0.06
	9.47 ± 0.06	7.75 ± 0.02[e]	−1.72 ± 0.08	8.76 ± 0.04	8.51 ± 0.02[e]	−0.25 ± 0.06

[a] 25 mM diethylmalonate and phenolsulfonate. [b] 25 mM Tris and glycine. [c] pK values calculated from fits to eq 10. [d] pK's calculated from fits to eq 13. [e] Significant fits obtained to eq 13 only when the two pK's were assumed to be identical. Identity of the pK's was confirmed by examination of the least-squares surface in the vicinity of the fitted values.

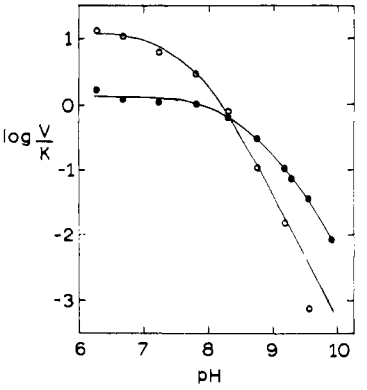

FIGURE 4: Effect of dimethylformamide on the V/K profile for malate in a neutral acid buffer. Closed circles are without and open circles with 20% dimethylformamide. Data fitted to eq 13, except that the point at pH 9.55 with dimethylformamide was not included in the analysis, since its omission decreased the residual least square by a factor of 3.4.

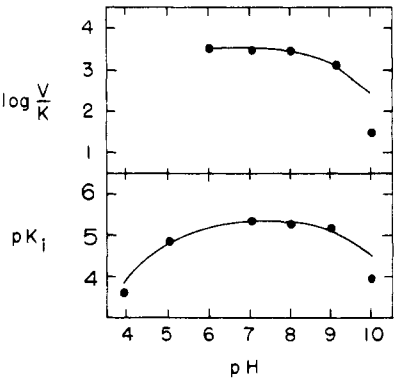

FIGURE 5: (Top) pH variation of V/K for TPN. Data for pH 6–10 were fitted to eq 10, pK = 8.9. Mg^{2+} was the metal. (Bottom) pH variation of K_i for TPN fitted to eq 9, pK_1 = 5.3, pK_2 = 9.3. Mg^{2+} was the metal.

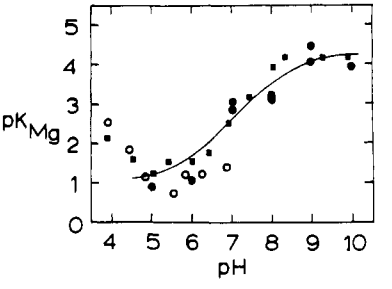

FIGURE 6: pH variation of Michaelis constant for metal. Open circles, Mn^{2+}. Closed circles, Mg^{2+}, from initial-velocity patterns with malate and $MgSO_4$. Closed squares, Mg^{2+}, from reciprocal plots for $MgSO_4$. The line is a fit to eq 12 of the Mg^{2+} data, except at pH 3.9. pK = 8.7.

being catalyzed (as required by the ordered addition of TPN before the other substrate), or with the nature of the metal ion.

In an ordered mechanism, $(V/K)/E_t$ for TPN should be the bimolecular rate constant for addition of TPN to free enzyme (or, actually, to the enzyme–metal complex in this case). The pH profile of V/K for TPN in reaction 1 with Mg as the metal clearly decreases at high pH (Figure 5), and the data between pH 6 and 10 were fitted to eq 10, giving pK = 8.9 ± 0.1. At pH 3.9, the equilibrium-ordered initial-velocity pattern implies that the K_m for TPN has become zero, and thus that V/K should be infinite. What has really happened, however, is that V has decreased 20-fold from its value at pH 7, while the K_i for TPN has increased 20-fold, so that, if V/K does not change, the K_m (which is nearly the same as the K_i at pH 7) would become 400-fold lower than the K_i at pH 3.9. Such a value would appear to be zero, and thus account for the apparent equilibrium-ordered pattern. This analysis does not prove that V/K for TPN stays constant at low pH but, if it decreases very much, the K_m for TPN should have stayed large enough to be observable.

pH Profiles for Metal Ions. In contrast with the great differences in K_m seen between Mg^{2+} and Mn^{2+} in catalyzing reaction 2, for reaction 1 the affinities of the two metal ions were nearly the same, although high levels of Mn^{2+} could not be used above pH 7.5 because of the formation of a precipitate. It should be pointed out, however, that Mn^{2+} shows strong negative cooperativity, while Mg^{2+} does not (Hsu et al., 1976; Schimerlik et al., 1977), and the values reported here are for the high K_m region of Mn^{2+}. K_m values for Mg^{2+} were determined either from reciprocal plots with $MgSO_4$ as the variable substrate (saturating TPN; malate, 1.5 × K_m), or from initial-velocity patterns for $MgSO_4$ and malate at saturating TPN (Figure 6). The combined data (except at pH 3.9) were fitted to eq 12, which gave a pK of 8.7 ± 0.1, and a limiting K_m at high pH of 53 ± 10 μM, and at low pH of 74 ± 17 mM. Below pH 5, the K_m again begins to decrease. This behavior is also shown by Mn^{2+} (open circles in Figure 6), and the K_m's are similar in value to those for Mg^{2+}. No attempt was made to fit this part of the pH profile.

The initial-velocity patterns with $MgSO_4$ and malate also yielded K_i values for Mg^{2+} (that is, dissociation constants from E–TPN, with malate not present). These were 50–100 μM at high pH, and seemed to increase as a result of the same pK at 8.7, but leveled off at a value of 1–2 mM at pH 7 and below. The errors in determining these values were greater than for the K_m's, and they have not been analyzed further.

Identification of Groups Ionizing at High pH by Solvent Perturbation. To determine whether the groups responsible for the basic pK's in the V and V/K_{malate} pH profiles for reaction 1 are neutral or cationic acids, these profiles were run

in neutral and cationic acid buffers in the presence or absence of 20% dimethylformamide (Table I). Since the pH values are measured before the perturbing solvent was added, cationic acid pK's should appear to be shifted to low pH in a neutral acid buffer and neutral acid pK's should be shifted to higher pH in a cationic acid buffer when the dielectric constant of the medium is lowered upon addition of an organic solvent (Herries et al., 1962). If only the V profile were looked at one would be tempted to assign the pK to a cationic acid, since dimethylformamide lowered the apparent pK in the neutral acid buffer more than it raised it in the cationic acid buffer. However, the pK's in the neutral acid buffer in the absence of dimethylformamide are inexplicably higher than those in the cationic acid buffer, thus casting doubt on the validity of this interpretation. Further, the V/K profiles in this case clearly showed two pK's, and dimethylformamide caused these values, which originally were 0.9–1.2 units apart, to become identical in both buffers (Figure 4). This behavior is predicted only when the lower pK results from a neutral acid and the higher one from a cationic acid. The pK's in the neutral acid buffer are again higher than those in the cationic acid buffer, although dimethylformamide seems to overcome this effect, whatever it is, and restore the pK's to values consistent with those seen in the cationic acid buffer.

Temperature Studies. The enthalpies of ionization of the groups with low and high pK's were calculated from an Arrhenius plot of pK vs. $1/T$ for pK's determined at 15, 25, and 35 °C in the V vs. pH profile for reaction 1. The values for the low- and high-pH pK's were 22 000 ± 4800 and 9300 ± 3000 cal/mol. The V/K profiles from pH 6.3 to 10 were also determined at these three temperatures in the cationic acid system. All three profiles showed two pK's with the first in the range 7.6–7.8, and the second in the range 8.8–9.2, but the accuracy of determining two pK's was not considered sufficient to permit evaluation of ΔH_{ion} values.

Discussion

Interpretation of pK_i and pK_m Profiles. One expects to see in the pH profile of pK_i for an inhibitor or for TPN (where K_i is its dissociation constant from E–TPN) a break in the curve and a drop in pK_i whenever a group on either the enzyme or the molecule binding to it must be in a specific ionization state for binding to occur. If the improper state of ionization leads to no binding, the profile changes to a line with slope of +1 when protonation prevents binding, or −1 when deprotonation prevents binding, and the breaks occur at the true pK values of the ionizing groups. Thus, TPN binding is prevented when a group with pK 5.3 becomes protonated, or when a group with pK 9.3 is ionized. Since these pK values do not correspond to those of TPN, they must represent groups on the enzyme. The group with pK 9.3 is probably a lysine, which when protonated binds the pyrophosphate or ribose 2′-phosphate groups of TPN. Since this pK is also seen in the V/K profile for TPN, the effect is on the bimolecular rate constant for addition of TPN to the enzyme (which equals $(V/K_{TPN})/E_t$ in the ordered mechanism), and thus TPN combines only when this group is protonated.

The group with pK 5.3, on the other hand, seems to affect the unimolecular rate constant for TPN release. The equilibrium-ordered initial-velocity pattern at pH 3.9 for both reactions 1 and 2 shows that this rate constant has become much greater than V/E_t, and the analysis presented under Results above suggests that $(V/K)/E_t$ (the bimolecular rate constant) does not decrease at low pH. Possibly, a general conformation change in the protein at this pH is responsible (see the discussion below concerning metal binding at low pH).

Similar pH behavior is expected for pK_i or pK_m profiles for metal activators whose addition to the enzyme will be at equilibrium in the steady state. The pH variation in the K_m for Mg^{2+} (Figure 6) suggests that it is chelated by a group with a pK of 8.7. This pK seems too high for histidine; however, it is consistent with a sulfhydryl group. Protection of the active site sulfhydryl by Mn^{2+} from modification by Ellman's reagent (Tang and Hsu, 1974) or *p*-mercuriphenylsulfonate and Hg^{2+} (Rutter and Lardy, 1958) support this view, as does the recent finding that the catalytic Zn in alcohol dehydrogenase is coordinated to two cysteines (Lange et al., 1975). In the absence of more definitive experimental evidence, however, this must remain only a suggestion.

When this group is protonated, the binding of Mg^{2+} is not totally prevented, but is decreased by a factor of about 20 in the absence of malate (when there is presumably enough room for the group to move out of the way), and a factor of 1400 in the presence of malate. The factor of 20 is reasonable for the contribution to binding strength of a single ligand, but the factor of 1400 suggests that steric hindrance becomes a real difficulty when the active site is filled and this group is protonated. A further complication arises below pH 5, where in the presence of malate the binding of both Mg^{2+} and Mn^{2+} increases, and is still increasing at pH 3.9. Presumably, a general conformational change in the enzyme at pH 5 relaxes the steric hindrance caused by protonation of the presumed sulfhydryl group, since the K_m for Mg^{2+} is only sixfold higher than its K_i at pH 3.9. This conformational change at pH 5 may be the same one that loosens TPN binding (see above).

Interpretation of V/K and V Profiles of Malate and Oxalacetate. As discussed above, the pK_i profile for an inhibitor shows the effects of ionizations on binding only, but has the virtue of giving correct pK values. In contrast, V/K and V profiles show the effect of ionizations on catalysis as well as on binding, and thus a comparison of the two types of profile is very useful in identifying the groups responsible for catalysis, as opposed to those only involved in binding. The V/K profile shows pKs of groups in free enzyme (in this case, E–TPN–metal) or free substrate, and usually gives the correct pK values. As discussed by Cleland (1977), however, when the substrate will combine with incorrectly protonated enzyme and is also sticky (that is, dissociates more slowly or at the same rate as the catalytic reaction occurs), the pK is displaced outwards on the profile by $\log_{10}(1 + k_3/k_2)$, where k_3 is the net-rate constant for the catalytic reaction, and k_2 is the rate constant for dissociation of the substrate. On the basis of the analysis in the previous paper of rate limiting steps in the reverse reaction, however, it seems unlikely that malate is sticky, and thus correct pK's are probably seen (Schimerlik et al., 1977).

The breaks that appear in the V profile reflect the pK values of the catalytic groups with the substrate present, and will differ from those seen in the V/K profile whenever the substrate prefers to bind to one protonation state of the enzyme. (In the extreme case where binding occurs only with the correctly protonated form of enzyme, the pK is seen in the V/K, but not in the V profile.) In addition, when a non-pH-dependent slow step follows the catalytic reaction and is normally rate limiting, the pK's are displaced outwards in the V profile to the point where the catalytic reaction becomes rate limiting. This phenomenon does not affect the V/K profile, which reflects steps only through the release of the first product.

When we compare the V/K_{malate} profiles in Figures 2, 3, and 4 with the pK_i profiles in Figure 1, it is obvious that the V/K

profiles reflect the ionizations of groups responsible for catalysis. There is no sign of a pK of 5.4–6 in the pK_i profiles for oxalacetate or mesotartrate, and thus the protonation state of this group is not important for binding of these molecules. For oxalacetate, this may reflect binding of the keto form to protonated enzyme, and the enol or *gem*-diol forms to deprotonated enzyme, but for mesotartrate one is forced to conclude that the protonation state of this group is not important. This is somewhat surprising, since this group appears to be the one that accepts the proton from the OH of malate during dehydrogenation, and thus one would expect it to have some effect on binding. It is possible that mesotartrate does not bind exactly as does malate, since it is not oxidized, and that other inhibitors might show some effect.

In any case, the reciprocal effect of this pK on reactions 1 and 2 (Figure 2) strongly implies that the group which is protonated for oxalacetate decarboxylation must be ionized for the oxidative decarboxylation of L-malate. The extremely high enthalpy of ionization observed for this pK (22 000 ± 5000 cal/mol) suggests that a conformational change in the enzyme accompanies the protonation of this group, and thus the nature of this group can not be determined from temperature studies. An attempt was made to identify this group by employing solvent perturbation in a neutral acid buffer (20 mM each in acetate, formate, and cacodylate) from pH 4.15 to 5.81. Because of the high K_m for Mg^{2+}, initial-velocity patterns with $MgSO_4$ and malate at saturating TPN were run at each pH value with and without the addition of 10% dimethylformamide. Considerable scatter was present in the values of V and V/K for malate, but no indication was seen in the data for a shift of the pK to lower pH. These data suggest that the pK results from a neutral acid, but a definite assignment can not be made.

At high pH, the V/K profile for malate shows two pK's, and the effects of dimethylformamide suggest that the one at pH 7.8 is a neutral acid and that at pH 9 a cationic acid. The latter is probably a lysine, and may represent a conformation change upon deprotonation which destroys catalytic activity, since it does not appear to affect mesotartrate or oxalacetate binding (Figure 1). The ionization state of the neutral acid with pK 7.8, on the other hand, does appear to affect mesotartrate binding by a factor of 4–6, although it has little effect on oxalacetate binding.

The pK's in the V profile in Figure 3 (see also Table I) are displaced outward by about 1.4 pH units from the values seen in the corresponding V/K profile, which presumably represents the true pK values for the E–TPN complex. This displacement suggests that the catalytic reaction at neutral pH is 25 times faster than the rate-limiting step, which is TPNH release (Schimerlik et al., 1977), and that it becomes rate limiting only when slowed down to the same rate as TPNH release. This picture agrees with that deduced from the deuterium-isotope effects on V (Schimerlik et al. 1977).

Identification of Catalytic Groups. The pH profiles discussed above show that a group with pK 5.4 (with Mn^{2+}) or 6 (with Mg^{2+}) must be protonated for decarboxylation of oxalacetate and deprotonated for oxidative decarboxylation of malate, and thus probably is hydrogen bonded in active catalytic complexes to the oxygen on carbon 2 of the substrate. Since the distance from the metal to carbons 1 and 2 of pyruvate in a dead-end, E–TPN–pyruvate–Mn^{2+} complex was 5.4–5.6 Å, Hsu et al. (1976) have considered this group to be a water or hydroxyl in the coordination sphere of the catalytic metal. However, this is an extremely low pK for such a bound water, particularly when it is freely exchangeable with solvent,

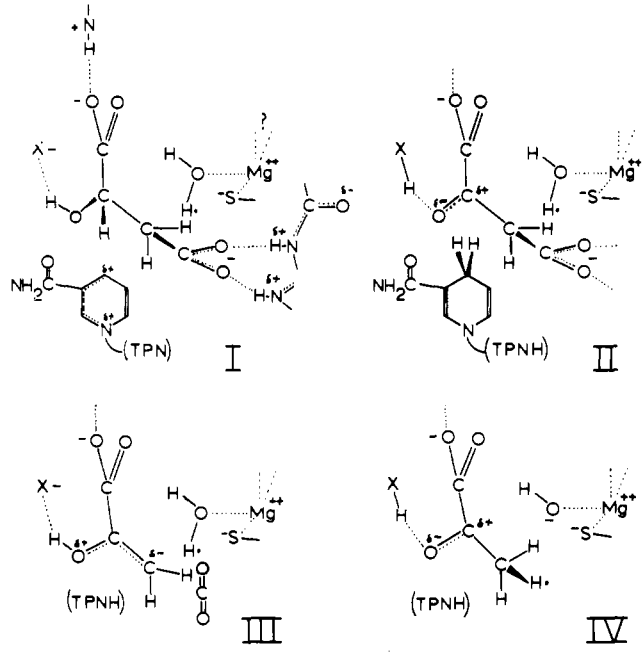

FIGURE 7: A possible catalytic mechanism for malic enzyme. The hydrogen transferred from water to enolpyruvate is marked with a dot. See text for further details and discussion.

as shown by the NMR results (Hsu et al., 1976). We prefer to consider the metal-bound water to be the neutral acid with pK around 8, which must be protonated for activity and presumably donates a proton to enolpyruvate following decarboxylation and CO_2 release. The distance to carbon 3 was not measured by Hsu et al. (1976), and would clearly provide a test of this hypothesis. If metal-bound water were the group with pK 6, then some other neutral acid, such as a sulfhydryl group, would have to protonate enolpyruvate and be the group with pK 8. We consider this unlikely, but it can not be ruled out on the basis of present evidence.

Proposed Chemical Mechanism of Catalysis. The evidence presented in this paper and the previous two in this issue (Schimerlik and Cleland, 1977; Schimerlik et al., 1977) may be interpreted in terms of a chemical mechanism for catalysis (Figure 7). In I of Figure 7, the 1-carboxyl of malate is shown bound by an ion pair bond to a positively charged group, which is probably arginine, since the binding of oxalacetate does not decrease at high pH, as it should if a lysine were involved (Figure 1). The 4-carboxyl group is shown forming induced dipolar bonds to amide groups of either Gln or Asn or the peptide chain, while the 2-hydroxyl is hydrogen bonded to the X group (possibly carboxyl) with pK 5.4–6 that must be deprotonated for malate to be oxidized and protonated for oxalacetate to be decarboxylated. TPN is shown with its A side down, facing the hydrogen that is transfered as a hydride. It thus lies above the plane of carbons 1–3 of malate. Since dehydrogenase reactions seem to involve carbonium ion induced hydride shifts, TPN is shown with part of the positive charge in the 4 position of the nicotinamide ring (this charge distribution being favored by burying N-1 in a completely hydrophobic environment in the E–TPN complex). The other catalytic group shown is a water coordinated to Mg^{2+}. The metal is shown lying below the plane of carbons 1–3 of malate, as required to produce retention of configuration during decarboxylation (Rose, 1970). The drawing is roughly to scale for bond distances between the carbons of malate and the Mg^{2+} and its coordinated water. The placing of the 4-carboxyl of

malate below the plane of carbons 1-3 means that during hydride transfer, which converts carbon 2 of malate from tetrahedral to planar, carbon 2 will drop down into the plane of carbons 1 and 3 and the oxygen attached to carbon 2, thus narrowing the bond angle between carbons 2, 3, and 4 and facilitating subsequent decarboxylation. Placing carbon 4 above the plane (the other possibility, since it must occupy an out of plane position in order to be decarboxylated) would mean that hydride transfer would not facilitate decarboxylation, which seems unlikely.

The first step in the reaction is hydride transfer to give the E-TPNH-oxalacetate complex II. Since the X group has become protonated, carbon 2 of the oxalacetate will have considerable positive charge, and either hydride transfer from TPNH (the reverse of the first step) or decarboxylation to CO_2 and enolpyruvate (enzyme form III) may occur. The isotope-effect studies of Schimerlik et al. (1975) and Schimerlik et al. (1977) show that at pH 7-8 reverse hydride transfer is 6-8 times faster than decarboxylation. The E-TPNH-enolpyruvate-CO_2 complex now loses CO_2, and the enolpyruvate is protonated by the metal-bound water to give ketopyruvate, protonated X, and MgOH (form IV). Since the decarboxylation occurs with retention of configuration (Rose, 1970), the metal-bound water must lie close to the 4-carboxyl as shown.

Several points deserve comment. First, the decarboxylation of oxalacetate at low pH is readily understood, since enzyme in which X is protonated would adsorb ketooxalacetate to give a complex resembling II, except that it would contain TPN in place of TPNH. The different K_m's and specificities for metal for reactions 1 and 2 show, however, that the conformational change that accompanies protonation of X alters the affinity of the enzyme for metal. Metal is, thus, presumably required for the decarboxylation of oxalacetate in order to establish the correct conformation of the enzyme (it is also required for oxidation of malate-β-amide, where no decarboxylation occurs, and for the reduction of α-keto acids), and to protonate enolpyruvate before it dissociates, rather than to induce decarboxylation directly as it does in the nonenzymatic reaction.

Second, the slow reduction of keto acids in the presence of TPNH (reaction 3) involves hydride transfer from TPNH to the carbonyl carbon in a complex similar to IV. The reason that this is normally so slow, and that the enzyme does not act efficiently as a lactate dehydrogenase, is probably that the geometry is not quite correct for hydride transfer in IV, when the metal ion is liganded by OH rather than by water. Thus, only after pyruvate is enolized to give $MgOH_2$ and after enolpyruvate attacks CO_2 to give oxalacetate can hydride transfer from TPNH occur with any speed.

The mechanism outlined in Figure 7 requires that E-TPNH, in order for pyruvate to adsorb to give IV, must exist at least to some extent with X protonated and the metal-bound water as MgOH. The proportion of this form to that shown in I of Figure 7 will be constant in the neutral pH range, and only below pH 6 will both groups be protonated, or above 8 could both be deprotonated.[2] The proportion in the XH, MgOH form present at neutral pH will depend on the separation of the pK's, and if we take these to be 6 and 8 the proportion is 1 part in 100. This seems small, but is supported by the low observed bimolecular rate constant for combination of pyruvate with E-TPNH. The values of $(V/K)/E_t$ for TPN, TPNH, pyruvate, and CO_2 from Schimerlik et al. (1977), assuming the active subunit to have a molecular weight of 65 000 (Nevaldine et al., 1974) are 7.0×10^6, 2.6×10^6, 7.6×10^2, and 1.9×10^4 M^{-1} s^{-1}. In an ordered mechanism, these values for TPN, TPNH, and pyruvate are the bimolecular rate constants for addition to the enzyme, and the value for CO_2, which does not vary appreciably with pH, corresponds to such a bimolecular rate constant at pH 8.5-9, where V and K for CO_2 both appear to have become infinite (Schimerlik et al., 1977). Since pyruvate is a small rigid molecule, one might expect its rate of combination with E-TPNH to be diffusion limited (that is, to approach 10^9 M^{-1} s^{-1}), while, in fact, it is 9000 times less than the value for TPN. This certainly shows that most of the enzyme is not in the correct configuration to react with pyruvate.[3] Lack of correct protonation is probably one factor, while existence of E-TPNH mainly in the more open form from which TPNH can be released, as opposed to the closed catalytically active form from which it can not, probably is responsible for the rest of the difference.

The $(V/K)/E_t$ value for CO_2, while 25 times that of pyruvate, is also far from the diffusion-limited value. Probably, CO_2 can react with E-TPNH-pyruvate only when the pyruvate has been enolized and the proton transferred to form $MgOH_2$. The proportion of the E-TPNH-pyruvate complex in which pyruvate is enolized thus must be quite small, which is reasonable in view of how much higher the pK of the methyl protons of pyruvate should be than that for the $MgOH_2$ group, even when allowance is made for the carbonyl oxygen of pyruvate being hydrogen bonded to XH.

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[2] If K_1 is the acid dissociation constant of X and K_2 that for $MgOH_2$, the distribution among the various forms will be: XH, $MgOH_2$: $(H/K_1')/\Delta$; X, $MgOH_2$: $(K_1/K_1')/\Delta$; XH, MgOH: $(K_2/K_1')/\Delta$; X, MgOH: $(K_2'/H)/\Delta$, where $\Delta = 1 + H/K_1' + K_2'/H$ and $K_1' = K_1 + K_2$ and $K_2' = 1/(1/K_1 + 1/K_2)$. Whenever $K_1 \gg K_2$, as in the present case, $K_1' = K_1$ and $K_2' = K_2$, so that the ratio of the XH, MgOH and X, $MgOH_2$ forms is given by K_2/K_1.

[3] The alternate explanation that enolpyruvate is the substrate and thus only a small part of pyruvate can react with the enzyme is unlikely, since the enzyme catalyzes the enolization of pyruvate (Bratcher, 1974), and direct release of enolpyruvate should not require a protonated catalytic group so that the pK of 7.8 should not be seen in the V/K profile.

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Evidence that the Catalytic and Regulatory Functions of Carbamyl-Phosphate Synthetase from *Escherichia coli* Are Not Dependent on Oligomer Formation†

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ABSTRACT: Carbamyl-phosphate synthetase from *Escherichia coli* is an allosteric enzyme which undergoes reversible association reactions in phosphate buffer. The positive allosteric effectors, ornithine, inosine 5′-monophosphate (IMP), and ammonia, facilitate oligomer formation, whereas uridine 5′-monophosphate (UMP), a negative effector, prevents or decreases oligomer formation. When the enzyme is immobilized by reaction with activated Sepharose, under conditions where the enzyme exists only as a monomer, nearly full catalytic activity is retained and the effects of ornithine, IMP, and UMP on the catalytic activity as a function of MgATP concentration are not significantly altered. Gel-filtration chromatography on Sephadex G-200 of catalytic quantities of the enzyme in the presence of all substrates showed that the elution volume was the same as that measured for the enzyme under conditions where it is known to exist in the monomer form. The specific activity of the enzyme does not increase when the concentration of the enzyme is increased 100-fold from a concentration at which the enzyme exists as monomer to a level at which the enzyme exists predominantly as oligomer. These results indicate that the monomer form of the enzyme is the principle active species and that oligomer formation is not directly related to enzyme activity or enzyme regulation.

Carbamyl-phosphate synthetase from *Escherichia coli* is an allosteric enzyme composed of two nonidentical subunits of different molecular weight (Anderson and Meister, 1966; Matthews and Anderson, 1972; Trotta et al., 1971, 1974). The enzyme is subject to feedback inhibition by UMP[1] and is activated by ornithine and also by IMP if phosphate is absent (Anderson and Meister, 1966; Pierard, 1966; Anderson and Marvin, 1970). The allosteric effectors and all substrates, except L-glutamine, apparently bind to site(s) on the heavy subunit; the light subunit functions as a glutamine-binding subunit, releasing NH_3, which binds and reacts at a site on the heavy subunit (Trotta et al., 1971).

The enzyme undergoes reversible association reactions in the presence of phosphate buffer (Anderson and Marvin, 1970; Trotta et al., 1974). The enzyme exists as a monomer when the concentration of the enzyme is as low as the concentrations normally employed in kinetic assays. Oligomer formation is dependent on enzyme concentration and is facilitated by the presence of the positive allosteric effectors, ornithine, IMP, and ammonia, and also by the substrate MgATP, whereas the presence of UMP, a negative allosteric effector, prevents or decreases the formation of oligomer.

When the existence of this monomer–oligomer equilibrium was first reported, it was suggested that allosteric regulation could possibly be explained in terms of the state of association of the enzyme, i.e., that oligomer formation was necessary for full activity (Anderson and Marvin, 1968). More recent studies have suggested the alternative scheme shown in Figure 2 in which the monomer is the principle active species and allosteric regulation is the result of stabilization of different conformational states of the monomer by different allosteric effectors; according to this scheme, oligomer formation is not directly related to enzyme activity or enzyme regulation, but is simply facilitated by formation of the active conformational states of the enzyme (Anderson and Marvin, 1970). As pointed out by Trotta et al. (1974), however, sufficient evidence has not been reported which would clearly establish the state of association of the active form of the enzyme and thus distinguish between these two possible schemes. The purpose of the present study was to obtain evidence that the catalytic activity of this enzyme and the allosteric effects of ornithine, UMP, and IMP are not dependent on oligomer formation. A preliminary report of this work has been published (Anderson, 1976).

Material and Methods

Carbamyl-phosphate synthetase was isolated from *E. coli* B by the procedure described by Anderson et al. (1970), as modified by Matthews and Anderson (1972). Sepharose 4B, Sephadex G-200, and Blue Dextran 2000 were products from Pharmacia. Cyanogen bromide was obtained from Aldrich Chemical Co. All other biochemical reagents were obtained from Sigma Chemical Co.

Carbamyl-phosphate synthetase was immobilized by reaction with activated Sepharose 4B. The Sepharose was activated by reacting 1 g of cyanogen bromide with Sepharose 4B

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[1] Abbreviations used are: UMP and IMP, uridine and inosine 5′-monophosphates; ADP and ATP, adenosine di-, and triphosphates; EDTA, (ethylenedinitrilo)tetraacetic acid.