Determination of the Rate-Limiting Steps for Malic Enzyme by the Use of Isotope Effects and Other Kinetic Studies†

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ABSTRACT: Isotope effects have been measured with Mg^{2+} as the activator and L-malate labeled with deuterium or tritium at carbon 2 as the substrate over the pH range 4–10. Comparison of the nearly pH-independent deuterium-isotope effect on V/K_{malate} of 1.5 with the tritium effect of 2.0 by the method of Northrop (Northrop, D. B. (1975), *Biochemistry 14,* 2644) gives limits on the true effect of deuterium substitution on the bond-breaking step of 5–8 in the forward reaction and 4–6.5 in the reverse direction. Comparison of the deuterium effect on V/K with the ^{13}C-isotope effect of 1.031 reported by Schimerlik et al. (Schimerlik, M. I., Rife, J. E., and Cleland, W. W. (1975), *Biochemistry 14,* 5347) allows the deduction that at pH 8 reverse hydride transfer is six to eight times faster than decarboxylation, which is thus largely rate limiting for the catalytic reaction. The absence of a deuterium-isotope effect on V at pH 7–8 and comparison of the K_i of pyruvate as an uncompetitive inhibitor of the forward reaction and a substrate for the reverse reaction indicate that at neutral pH the release of TPNH from enzyme-reduced triphosphopyridine nucleotide (E-TPNH) is the rate-limiting step in the forward direction. The observation of a deuterium effect on V that approaches 3 at pH 4 and 10 shows, however, that, at very low and very high pH, hydride transfer may become partly rate limiting. In the reverse reaction, the probable rate-limiting step at pH 7 is the isomerization of E–TPNH, while at pH 8.5 and above V becomes too large to measure and appears infinite. Substitution of Co^{2+}, Ni^{2+}, or low levels of Mn^{2+} for Mg^{2+} gives similar deuterium-isotope effects, although the values of V and K_{malate} vary considerably with metal. The kinetics of Mn^{2+} show pronounced negative cooperativity, with K_m values of 7 μM and 5 mM for concentration ranges from 4 to 100 μM and over 1 mM.

In the preceding paper of this issue, we reported studies with alternate substrates and inhibitors of pigeon liver malic enzyme, which define the mode of binding of L-malate and support the concept of an ordered kinetic mechanism in which L-malate prevents TPN[1] release from the enzyme, while pyruvate prevents TPNH release. Before the pH studies, to be reported in the following paper of this issue, can be interpreted in terms of a chemical mechanism, however, it is necessary to know the relative rates of the various steps in this kinetic mechanism, and, in particular, what steps are rate limiting under different conditions. In this paper, we present isotope-effect studies, which, together with the ^{13}C-isotope effect already reported by Schimerlik et al. (1975) and several other types of kinetic experiments, allow this information on relative rates to be determined.

Materials and Methods

Pigeon liver malic enzyme was isolated by the procedure of Hsu and Lardy (1967), and substrates were from Sigma, except for DL-malate-d_3 which was from Aldrich. Malate-*2-d* was synthesized by deamination of aspartate-*2-d* (Schimerlik et al., 1975), and malate-*2-t* was synthesized by the same procedure, except that the aspartate exchange was run in 1.5 ml of water containing 1 Ci of tritium, instead of D_2O. The concentrations of L-malate in DL-malate-d_3 and malate-*2-d* or malate-*2-t* were determined by enzymatic analysis with malic enzyme in the presence of excess TPN.

Unless otherwise specified, all experiments were run in a buffer 25 mM each in acetic acid, cacodylic acid, glycine, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) adjusted to the desired pH by addition of KOH. Bovine serum albumin was included at a final level of 67 μg/ml. The tritium-isotope discrimination studies were run in 50 mM triethanolamine hydrochloride, pH 7.4, or 25 mM glycine, pH 9.4, and to completion in 50 mM Tris-HCl, pH 8.5.

In order to determine the tritium-isotope effect on V/K, the reaction was either run to completion (to calculate the specific activity of TPNH when all the L-malate-*2-t* had been consumed) or stopped at 25–30% of completion by rapid addition of 0.5 ml of CCl_4, followed by vigorous vortexing. After removal of denatured protein, tritiated TPNH was separated from unreacted L-malate-*2-t* and D-malate-*2-t* on a 1.5 × 3 cm DEAE-cellulose column equilibrated at 4 °C with 50 mM Tris-HCl, pH 8.7, by elution with a linear gradient of 0–0.3 M NaCl in the same buffer. The malate appeared just prior to the tritiated TPNH; thus, the initial tritiated TPNH fractions showing a high specific activity were discarded, and the remaining fractions, showing a constant specific activity, were used to give the TPNH specific activity in cpm/μmol.

The $MgSO_4$ concentration was 20 mM for the tritium-isotope discrimination experiments and all experiments were run below pH 7, while 5 mM $MgSO_4$ was used in all experiments above pH 7.

All reactions were followed using a Beckman DU monochrometer with a 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 0.6–6.0 in./min were used. The cell compartment was maintained at 25 °C by means of thermospacers.

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[1] Abbreviations used are: TPN, triphosphopyridine nucleotide; TPNH, reduced TPN; DEAE, diethylaminoethyl; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

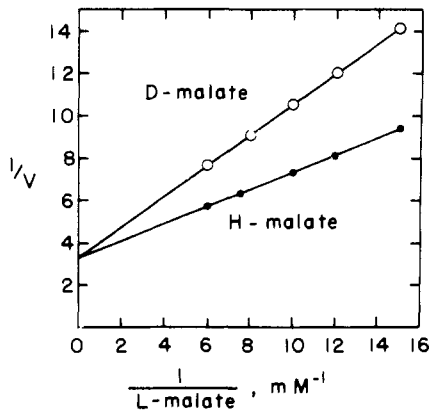


FIGURE 1: Deuterium-isotope effect at pH 7.0. TPN, 333 μM. Closed circles are L-malate and open circles are DL-malate-d_3.

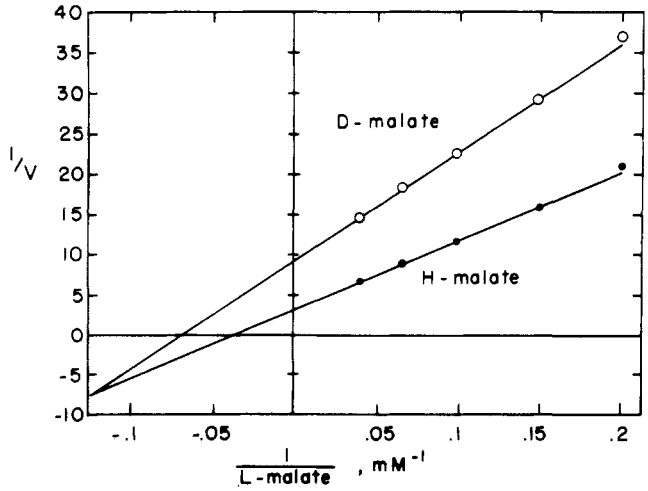


FIGURE 2: Deuterium-isotope effect at pH 9.7. TPN, 3.33 mM. Closed circles are L-malate and open circles are DL-malate-d_3.

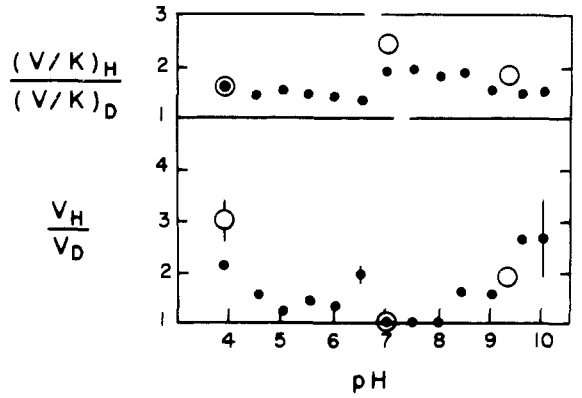


FIGURE 3: pH profile of deuterium-isotope effects on V and V/K_{malate}. Closed circles are DL-malate-d_3 (average of two experiments) and open circles are L-malate-*2-d*. Where standard errors are significant, they are indicated by error bars.

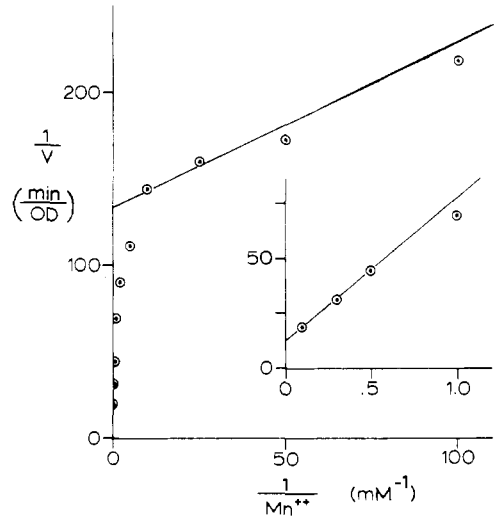


FIGURE 4: Negative cooperativity in the kinetics of Mn^{2+} at pH 7.0. L-malate, 3 mM; TPN, 100 μM. The apparent K_m values for the two portions of the curve were 7 ± 1 μM and 5 ± 1 mM.

The inhibition of pyruvate vs. L-malate was run in 10-cm cells containing 30 ml, while all other experiments were run in 1.0-cm cuvets, 3.0 ml total volume. The reaction was started by the addition of enzyme, via a microliter syringe, to the 30-ml reaction mixture equilibrated to 25 °C in a temperature bath, or by adder-mixer to the 1.0-cm cuvets, which were also at 25 °C.

Data Processing. The nomenclature used in this paper is that of Cleland (1963a). Reciprocal velocities were plotted vs. reciprocal substrate concentrations and linear plots were obtained in all cases. Data conforming to sequential initial velocity and linear uncompetitive inhibition were fitted to eq 1 and 2. The initial-velocity data at pH 8.5 and 9.0 in the reverse direction were fitted to eq 3.

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (1)$$

$$v = \frac{VA}{K + A(1 + I/K_{ii})} \quad (2)$$

$$v = \frac{(V/K_b)AB}{K_{ia} + A + ((V/K_b)B)/(V/K_a)} \quad (3)$$

In eq 1, K_a and K_{ia} are the Michaelis and dissociation constants for A, K_b is the Michaelis constant for B, and V the maximum velocity. In eq 2, K is the apparent Michaelis constant for A and K_{ii} is the apparent intercept inhibition constant for I. In eq 3, only V/K_a, V/K_b, and K_{ia} can be determined, since V, K_a, and K_b have all become too large to measure. The data for kinetic deuterium-isotope effect experiments were fitted to eq 4

$$v = \frac{V_H A}{K_H\left(1 + f\left[\frac{(V/K)_H}{(V/K)_D} - 1\right]\right) + A\left(1 + f\left[\frac{V_H}{V_D} - 1\right]\right)} \quad (4)$$

where f is the fraction of deuterium in the substrate (0 for normal L-malate and 1.0 for malate-*2-d*) and A is the concentration of whichever form of L-malate was used. When there was no isotope effect on V, a similar equation was used in which the second denominator term was simply A.

Experimental data were fitted to eq 1–4 by the least-squares method assuming equal variances for the velocities (Wilkinson, 1961) and using a digital computer and the Fortran programs of Cleland (1963b, 1967). The points in the double-reciprocal plots are the experimentally determined values, while the lines drawn through the points are calculated from the fits of these data to the corresponding rate equations.

Results

Deuterium-Isotope Effects. There was no detectable deuterium-isotope effect on V between pH 7 and 8 with either

TABLE I: Deuterium-Isotope Effects at pH 7 with Metals Other Than Mg^{2+}.[a]

Metal	Concn[b] (mM)	$\frac{(V/K)_H}{(V/K)_D}$	$\frac{V_H}{V_D}$	K_{malate}[c] (μM)	$\frac{V_{Me^{2+}}}{V_{Mg^{2+}}}$[d]
Co^{2+}	4 ($25 \times K_m$)	1.9 ± 0.3	1.12 ± 0.07	16 ± 3	0.18
Ni^{2+}	10 ($5.3 \times K_m$)	1.34 ± 0.09	1.0	370 ± 40	0.42
Mn^{2+}	0.05 ($7 \times K_{m1}$, $0.01 \times K_{m2}$)	1.91 ± 0.09	1.0	40 ± 2	0.13[e]
Mn^{2+}	10 ($2 \times K_{m2}$)	1.5 ± 0.3	1.4 ± 0.1	90 ± 20	1.36

[a] From reciprocal plots with L-malate and DL-malate-d_3 with 100 μM TPN. Data fitted to eq 4, or to the similar equation with the second denominator term consisting of A when there was only a V/K effect. [b] Ratio of metal concentration to K_m for this metal (at 5 mM L-malate and 100 μM TPN). For Mn^{2+}, K_{m1} is that seen in the range 4–100 μM, while K_{m2} is that seen above 1 mM. Negative cooperativity was not observed for Ni^{2+} in the range 2–20 mM, or for Co^{2+} in the range 0.2–4 mM. [c] Michaelis constant for L-malate with this metal. The value for 20 mM Mg^{2+} is 290 ± 20 μM. [d] Relative V values with 5 mM L-malate, 100 μM TPN, and metal as varied reactant. [e] The comparison is with the apparent V from data in the range 4–100 μM Mn^{2+}.

DL-malate-d_3 or malate-*2-d* (Figure 1); however, a small V isotope effect was found at pH values lower than 7, gradually increasing to nearly 3 at pH 3.9, and at pH values greater than 8, increasing to 3 above pH 9.5 (Figure 2). A deuterium-isotope effect on V/K_{malate} was found at all pH values between 3.9 and 10 and appeared relatively pH independent (although the values were slightly higher between pH 7–8) with an average value of about 1.5. This value agrees with the value of 1.45 ± 0.03 determined by the equilibrium-perturbation method by Schimerlik et al. (1975). The pH variation of the deuterium-isotope effects on V and V/K is summarized in Figure 3.

While Mg^{2+} is certainly the normal activator in vivo, a number of other metals can replace it; Mn^{2+} in particular has been used extensively for studies on this enzyme. While Mg^{2+} gives normal noncooperative kinetics,[2] reciprocal plots for Mn^{2+} show extreme negative cooperativity, as seen in Figure 4. Hsu et al. (1976) have recently reported similar results, and have also showed by direct binding studies that the tight sites correspond to two of the four subunits, and the loose sites to the other two. Since it is possible that the rate-limiting steps might be different when Mg^{2+} is replaced with a different metal, or in the tight as opposed to the loose binding regions for Mn^{2+}, deuterium isotope effects on V and V/K, using malate-*2-d* and several metals, including both high and low Mn^{2+}, are shown in Table I.

Tritium-Isotope Effects. With malate-*2-t*, the specific activity of the TPNH for a reaction run to 25–30% completion was 5.84 ± 0.12 × 10^4 cpm/μmol at pH 9.4, and 5.86 ± 0.12 × 10^4 cpm/μmol at pH 7.4, compared to an average specific activity of 1.08 ± 0.02 × 10^5 cpm/μmol for two reactions run to completion at pH 8.5. The tritium-isotope effect on V/K was calculated from eq 5

$$\frac{(V/K)_H}{(V/K)_T} = \frac{\log (1-f)}{\log \left(1 - \frac{fR_f}{R_0}\right)} \quad (5)$$

where f equals the fractional conversion of substrate to product; R_0, the specific activity of TPNH when the reaction was run to completion; and R_f, the specific activity of the TPNH at f fractional conversion to product. The resulting value of $(V/K)_H/(V/K)_T$ of 2.02 ± 0.06 at both pH 7.4 and 9.4 again indicates invariance of the V/K isotope-effect with pH.

[2] There may be slight negative cooperativity with Mg^{2+}. The kinetics above 500 μM are certainly normal, but in the range 4–500 μM the Michaelis constant for Mg^{2+} appears three- to fourfold lower, and the extrapolated V about half of the true value. More precise determination is difficult because of the low rates in this range of Mg^{2+}.

TABLE II: Comparison of K_i Values for Pyruvate as a Substrate and Inhibitor.[a]

	Vary L-Malate, UC Inhibition by Pyruvate	Pyruvate, CO_2 Initial-Velocity Pattern[b]	
pH	K_{ii} (mM)	$K_{i\ pyruvate}$ (mM)	Ratio
7.1	19 ± 1[c]	20 ± 4	1.0 ± 0.2
8.5	62 ± 3[d]	31 ± 3	2.0 ± 0.3

[a] 5 mM $MgSO_4$. [b] TPNH, 200 μM. [c] Corrected to infinite TPN by dividing experimental value by $(1 + K_a/A)$, where K_a is Michaelis constant for TPN (4 μM) and A is TPN concentration (10 μM). [d] TPN, 667 μM.

Dissociation Constant of Pyruvate Measured in Forward and Reverse Reactions. Initial-velocity patterns for pyruvate and CO_2 at saturating TPNH (200 μM) were intersecting and the data at pH 7.1 were fitted to eq 1. However, the data at pH 8.5 and 9 fitted eq 3 (that is, V and both Michaelis constants were infinite). Despite the changes in Michaelis constants, V/K for pyruvate was the same at pH 7 and 8.5, and only slightly less at pH 9, and the same was true for V/K for CO_2. The dissociation constant for pyruvate also changed only slightly (Table II).

The patterns for product inhibition by pyruvate vs. L-malate at pH 7.1 and 8.5 were uncompetitive and the data were fitted to eq 2. The resulting K_{ii} values are shown in Table II.

Discussion

Intrinsic Deuterium Isotope Effect. Northrop (1975) discovered that, when K_{eq} was not affected by substitution of deuterium in the transfered atom, the intrinsic deuterium-isotope effect on a bond-breaking step involving C–H cleavage could be determined by comparison of the deuterium- and tritium-isotope effects on V/K according to eq 6:

$$\frac{(V/K)_H/(V/K)_D - 1}{(V/K)_H/(V/K)_T - 1} = \frac{a-1}{a^{1.44}-1} \quad (6)$$

where $a = k_H/k_D$ for the bond-breaking step, and $a^{1.44} = k_H/k_T$. For malic enzyme, however, deuterium substitution in the transferred hydrogen atom causes an appreciable change in K_{eq} (Schimerlik et al., 1975):

$$\beta = \frac{K_{eq\ D}}{K_{eq\ H}} = 0.82 \pm 0.04 \quad (7)$$

The intrinsic deuterium-isotope effects on the bond-breaking step are now different in forward and reverse directions (a and βa), and application of eq 6 does not give a unique solution for a or βa. However, if an apparent value of a is calculated from eq 6 with the observed V/K isotope effects, and an apparent value of βa is obtained from eq 6 by first multiplying the observed $(V/K)_H/(V/K)_D$ value by β, and the observed $(V/K)_H/(V/K)_T$ value by $\beta^{1.44}$, we can calculate the following limits on the true values of a and βa:

$$\frac{(\text{app } \beta a)}{\beta} \geq a \geq (\text{app } a) \quad (8)$$

$$(\text{app } \beta a) \geq \beta a \geq \beta(\text{app } a) \quad (9)$$

These equations apply when $\beta < 1$; when $\beta > 1$, the inequalities are reversed.

Assuming a pH-invariant V/K isotope effect, average values of 1.47 ± 0.08 and 2.02 ± 0.06 for the ^2H and ^3H isotope effects were used with eq 6 to give (app a) $= 4.9 \pm 2.4$ and (app βa) $= 6.5 \pm 1.3$. Eq 8 and 9 then give the following limits for the intrinsic effects:

$$7.9 \pm 1.6 \geq (k_H/k_D)_{forward} \geq 4.9 \pm 2.4 \quad (10)$$

$$6.5 \pm 1.3 \geq (k_H/k_D)_{reverse} \geq 4.0 \pm 2.0 \quad (11)$$

The range of values yielded by this analysis is broad and the errors are large; this results from the small size of the observed V/K effects and the relatively large value of β. For reactions where the observed V/K effects are larger, the standard errors would be smaller, and the range of values resulting from the fact that $\beta \neq 1$ would be narrower. The values are comparable to those seen in favorable cases with other dehydrogenases, however.

Interpretation of Deuterium- and ^{13}C-Isotope Effects on V/K. With the knowledge that the intrinsic deuterium-isotope effect in the forward direction probably lies in the range of 5–8, we are in a position to interpret the observed deuterium effects on V and V/K shown in Figure 3 and Table I, and compare them with the ^{13}C effect on V/K_{malate} of 1.031 at pH 7.8–8.0 reported by Schimerlik et al. (1975).

Equation 12 represents a model for the kinetic mechanism of pigeon liver malic enzyme

$$E_1 \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} E_2 \underset{k_4}{\overset{k_3B}{\rightleftharpoons}} E_3 \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E_4 \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E_5 \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}}$$

$$E_6 \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} E_7 \underset{k_{14}P}{\overset{k_{13}}{\rightleftharpoons}} E_8 \underset{k_{16}Q}{\overset{k_{15}}{\rightleftharpoons}} E_9 \underset{k_{18}R}{\overset{k_{17}}{\rightleftharpoons}} E_1 \quad (12)$$

where A, B, P, Q, R, and E_1 are TPN, L-malate, CO_2, pyruvate, TPNH, and free enzyme, respectively. k_7 and k_8 are the rate constants for hydride transfer and k_9 and k_{10} those for decarboxylation and carboxylation in the forward and reverse reactions. k_5, k_6, k_{11}, and k_{12} represent rate constants for conformational changes from EAB (E_3) to an enzyme form poised for catalysis (E_4), and from the enzyme form immediately following catalysis (E_6) to one from which product release takes place (E_7). Using the method of net-rate constants (Cleland, 1975), and assuming an unequal deuterium-isotope effect on k_7 and k_8:

$$\frac{(V/K_b)_H}{(V/K_b)_D} = \frac{(k_{7H}/k_{7D}) + c_f + c_r/\beta}{(1 + c_f + c_r)} \quad (13)$$

$$c_f = \frac{k_{7H}}{k_6}\left(1 + \frac{k_5}{k_4}\right) \quad (14)$$

$$c_r = \frac{k_{8H}}{k_9}\left(1 + \frac{k_{10}}{k_{11}}\left(1 + \frac{k_{12}}{k_{13}}\right)\right) \quad (15)$$

$$\beta = \frac{K_{eq\ D}}{K_{eq\ H}} = \frac{k_{7D}k_{8H}}{k_{8D}k_{7H}} \quad (16)$$

where c_f and c_r are commitments to catalysis in forward and reverse directions. If c_f and c_r are small, then the observed $(V/K)_H/(V/K)_D$ equals the true isotope effect on the bond-breaking step; however, as Northrop (1975) showed, a high commitment to catalysis in the forward direction, corresponding to high ratios of k_5/k_4 or k_{7H}/k_6, or in the reverse direction (which means a slow step follows the bond breaking one so that it comes to equilibrium), corresponding to high ratios of k_{12}/k_{13}, k_{10}/k_{11}, or k_{8H}/k_9, will lower the observed V/K isotope effect.

When the ^{13}C isotope effect on V/K is measured, k_9 and k_{10} are the bond-breaking and -making steps, and β is essentially equal to 1.[3] Equations 13–15 now become:

$$\frac{(V/K_b)_{12}}{(V/K_b)_{13}} = \frac{k_{9(12)}/k_{9(13)} + c_f' + c_r'}{1 + c_f' + c_r'} \quad (17)$$

$$c_f' = \frac{k_9}{k_8}\left(1 + \frac{k_7}{k_6}\left(1 + \frac{k_5}{k_4}\right)\right) \quad (18)$$

$$c_r' = \frac{k_{10}}{k_{11}}\left(1 + \frac{k_{12}}{k_{13}}\right) \quad (19)$$

Note that eq 15 contains k_8/k_9, while eq 18 contains k_9/k_8.

The deuterium effect on V/K of 1.5 is rather small compared to the true effect of 5–8 on k_7, while the ^{13}C effect seen by Schimerlik et al. (1975) is nearly half of the maximum values known for decarboxylation. If we take an average value of 6.5 for the true value of k_{7H}/k_{7D}, and 1.07 for the true ^{13}C-isotope effect on k_9, we can use eq 13–19 to provide possible limits on k_9/k_8, $(k_7/k_6)(1 + k_5/k_4)$, and $(k_{10}/k_{11})(1 + k_{12}/k_{13})$. The latter two expressions can vary from 0 to 6.74 or 1.21, respectively (a high value of one corresponding to a low value of the other), but the value of k_9/k_8 can only vary between 0.124 (corresponding to a 0 value of the second expression) and 0.172 (a 0 value of the third expression). It is thus clear that at pH 7.8–8.0 hydride transfer in the reverse direction (k_8) is six to eight times faster than decarboxylation (k_9), so that decarboxylation, rather than hydride transfer, is largely rate limiting in the catalytic process.

Interpretation of Deuterium-Isotope Effect on V. For mechanism 12, the deuterium-isotope effect on V has a form similar to eq 13, with c_r given by eq 15, but with c_f replaced by a new constant involving the ratios of k_{7H} to k_5, and to the "net-rate constant" (Cleland, 1975) of each step following k_7:

$$\frac{V_H}{V_D} = \frac{k_{7H}/k_{7D} + c_{Vf} + c_r/\beta}{1 + c_{Vf} + c_r} \quad (20)$$

[3] For conversion of CO_2 to bicarbonate, the measured fractionation factor for ^{13}C is 1.007 (Thode et al., 1965), while for conversion of a carboxylic acid to bicarbonate (which involves replacement of the carbon chain by OH), an average value of 1.009 can be extracted from the calculations of Hartshorn and Shiner (1972) by assuming that fluorine and oxygen will have equivalent effects (V. J. Shiner, Jr., personal communication). The predicted β for conversion of [^{13}C]malate to $^{13}CO_2$ is thus about 1.002.

$$c_{Vf} = \left(\frac{k_{7H}}{1 + k_6/k_5}\right)\left(\frac{1}{k_5} + \frac{1}{k_9}\left(1 + \frac{k_{10}}{k_{11}}\left(1 + \frac{k_{12}}{k_{13}}\right)\right)\right) + \frac{1}{k_{11}}\left(1 + \frac{k_{12}}{k_{13}}\right) + \frac{1}{k_{13}} + \frac{1}{k_{15}} + \frac{1}{k_{17}}\right) \quad (21)$$

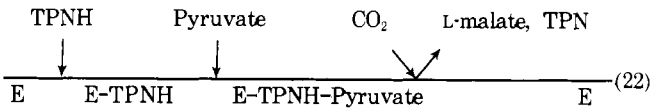
At pH 7-8, where no isotope effect on V is seen, c_{Vf} must be large, since our calculations above show that c_r can only have values in the range 5.8-17.8, and these are not high enough to eliminate the isotope effect (c_{Vf} would have to lie in the range 170-130, depending on the value of c_r, to reduce the observed isotope effect to 1.05, a value which could probably not be detected). Since the data in Table II suggest that TPNH release is rate limiting at pH 7 (see discussion below), the high value of c_{Vf} is probably the result of a high ratio of k_{7H}/k_{17}. At very low and very high pH, however, the catalytic reaction slows down as the result of the incorrect protonation of groups whose pK values are reported by Schimerlik and Cleland (1977b) in the following paper of this issue to be 6 and 8 in the E-TPN-Mg^{2+} complex. The resulting decrease in the apparent value of k_{7H} at low and high pH values decreases c_{Vf} eventually to zero, and leaves V_H/V_D determined only by c_r and k_{7H}/k_{7D}. If these had the same values they have at pH 8, V_H/V_D would reach a maximum value of 2.0 when c_{Vf} became zero; since values up to 3 were observed (Figure 3), it is likely that c_r becomes smaller at extreme pH values than at neutrality and the k_8/k_9 ratio is nearer to unity. This can not be checked, since it is not possible to measure the ^{13}C-isotope effect at these extreme pH values.

Deuterium-Isotope Effects with Metals Other than Mg^{2+}. The results in Table I show that the deuterium-isotope effects seen with Co^{2+}, Ni^{2+}, and Mn^{2+} are very similar to those seen with Mg^{2+} at pH 7. V/K effects of 1.3-1.9 are observed, but, despite considerable variation in V relative to that with Mg^{2+}, V_H/V_D values are either 1.0 or very close to it, with the exception of the high-concentration range for Mn^{2+}. Thus, the lower V values seen with Co^{2+}, Ni^{2+}, and Mn^{2+} in the low K_m range are apparently caused by slower release of TPNH, and not by an effect on the rate of hydride transfer or decarboxylation. Only with high Mn^{2+}, where the V exceeds that with Mg^{2+}, does catalysis become slightly rate limiting for V. In addition to having an effect on the rate of release of TPNH, the nature of the metal affects the affinity of the enzyme for L-malate, as shown by the 23-fold range in values seen for K_{malate}.

Evidence that TPNH Release Limits the Forward Reaction. The lack of a deuterium-isotope effect on V at neutral pH, together with the observation of an effect on V/K, shows that some step, other than catalysis or CO_2 release, is rate limiting under these conditions. The data in Table II show that, when L-malate and TPN are both saturating, the observed K_{ii} for pyruvate is the same as the dissociation constant measured in initial-velocity studies of the back-reaction, which requires that all of the enzyme be E-TPNH under these conditions. Thus, the release of TPNH, or a conformation change which permits TPNH release, is the rate-limiting step. At pH 8.5, however, the K_{ii} is twice the measured dissociation constant, showing that only half of the enzyme is E-TPNH when TPN and malate are saturating, and thus that other steps have become partly rate limiting.

Rate-Limiting Steps in the Reverse Reaction. As reported by Schimerlik and Cleland (1977a) in the previous paper of this issue, when a number of 2-hydroxy acids were used as inhibitors of the back-reaction at pH 7.2, the K_{ii} values vs. pyruvate were larger than the dissociation constants as competitive inhibitors vs. malate by factors of from 8 to 61. These experiments were carried out at saturating TPNH and at 7.5 times the K_m for CO_2, and thus, in the presence of near saturating substrates, E-TPN does not make up more than 2-12% of the enzyme in the steady state, and release of TPN is considerably faster than V in this direction.

Further evidence concerning the rate-limiting steps in the reverse reaction is provided by the initial-velocity patterns with pyruvate and CO_2 as variable reactants. At pH 7.1 the pattern is a normal intersecting one, but, at pH 8.5 and 9 the V appears infinite, and the data were fitted to eq 3. This equation corresponds to a mechanism of the type:



(22)

where reaction of CO_2 with E-TPNH-pyruvate results in such rapid catalysis and release of products that central complexes and E-TPN do not exist in appreciable concentration in the steady state. This mechanism is consistent with the conclusion that hydride transfer in the reverse direction is faster than decarboxylation.

Since V/K_{CO_2} and $V/K_{pyruvate}$ are the same at pH 7.1 and 8.5, and since the preponderance of evidence suggests that catalysis slows down, rather than increases at high pH, it seems unlikely that the rate-limiting step at pH 7.0 is the catalytic reaction. Release of malate is a possibility, but since its K_m is not that much greater at pH 8.5 than at 7.1, a more likely possibility is that the isomerization of E-TPNH to a form which can add pyruvate may be the slow step. The existence of such an isomerization is suggested by the fact that V/E_t in the forward direction (Schimerlik and Cleland, 1977a) is 20 times as great as the rate constant calculated for dissociation of TPNH from E-TPNH by the equations of Cleland (1963a). To get this inconsistency, the rate constant for isomerization of the initial E-TPNH complex to the form which can add pyruvate must be less than the rate constants for release of CO_2 and pyruvate, and less than the comparable rate constant for conversion of the initial E-TPN complex to the form which can add malate (see p 124 of Cleland (1963a) for the equations for this situation). These requirements are reasonable, and thus it is quite possible that this isomerization limits the reverse reaction at pH 7 and below where V appears finite. At higher pH, this isomerization apparently becomes fast enough not be to be rate limiting, and V becomes large enough that an accurate value can not be determined.

While the forward reaction at pH 7 appears limited by TPNH release, it may be that the actual slow step is not the release of TPNH itself, but the isomerization that permits it. In this case, this isomerization of the E-TPNH complex may limit V in both directions at this pH, while the comparable isomerization of E-TPN is much faster.

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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.