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Use of Intermediate Partitioning To Calculate Intrinsic Isotope Effects for the Reaction Catalyzed by Malic Enzyme†

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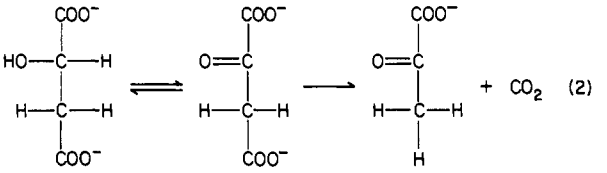
ABSTRACT: For those enzymes that proceed via a stepwise reaction mechanism with a discrete chemical intermediate and where deuterium and ^{13}C isotope effects are on separate steps, a new method has been developed to solve for the intrinsic deuterium and ^{13}C kinetic isotope effects that relies on directly observing the partitioning of the intermediate between the forward and reverse directions. This observed partitioning ratio, along with the values of the primary deuterium, tritium, and ^{13}C kinetic isotope effects on V/K for the substrate with the label being followed, allows an exact solution for the intrinsic deuterium and ^{13}C isotope effects, the forward commitment for the deuterium-sensitive step, and the partition ratio for the intermediate in the reaction. This method allows portions of the reaction coordinate diagram to be defined precisely and the relative energy levels of certain activation barriers to be assigned exactly. With chicken liver triphosphopyridine nucleotide (TPN) malic enzyme activated by Mg^{2+}, the partitioning of oxalacetate to pyruvate vs. malate in the presence of TPNH, 0.47, plus previously determined isotope effects gives an intrinsic deuterium isotope effect of 5.7 on hydride transfer and a ^{13}C isotope effect of 1.044 on decarboxylation. Reverse hydride transfer is 10 times faster than decarboxylation, and the forward commitment for hydride transfer is 3.3. The ^{13}C isotope effect is not significantly different with reduced acetylpyridine adenine dinucleotide phosphate replacing TPNH (although the pyruvate/malate partitioning ratio for oxalactate is now 9.9), but replacement of Mg^{2+} by Mn^{2+} raises the value to 1.065 (partition ratio 0.99).

TPN malic enzyme catlayzes the oxidative decarboxylation of L-malate with concomitant reduction of triphosphopyridine nucleotide (TPN):[1]

$$\text{L-malate} + \text{TPN}^+ \overset{M^{2+}}{\rightleftharpoons} \text{pyruvate} + CO_2 + \text{TPNH} \quad (1)$$

The enzyme will use several divalent metal cations, most commonly Mg^{2+} and Mn^{2+}. The enzyme will also utilize the alternate nucleotide 3-acetylpyridine adenine dinucleotide phosphate. The kinetic mechanism of pigeon liver TPN malic enzyme was shown by Hsu et al. (1967) to be ordered sequential with TPN adding before malate, followed by release of CO_2, pyruvate, and then TPNH.

In addition to the oxidative decarboxylation of malate, the enzyme will also catalyze the TPNH-dependent reduction of oxalacetate to malate and the decarboxylation of oxalacetate to pyruvate and CO_2 (Viega Salles & Ochoa, 1950). Because of this, it has long been believed that dehydrogenation of malate in the central complex occurs first to yield an oxalacetate intermediate of finite lifetime, which is then decarboxylated to yield CO_2 and pyruvate:



(2)

Hermes et al. (1982) used the multiple isotope effect method of examining the primary ^{13}C kinetic isotope effect with deuterated and unlabeled substrates to show that this mechanism was correct but were only able to establish limits on the intrinsic deuterium and ^{13}C isotope effects for the reaction.

Any intermediate generated in a chemical reaction will partition between reaction to give products and reversion to starting material according to the relative heights of the energy

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[1] Abbreviations: TPN, triphosphopyridine nucleotide; TPNH, triphosphopyridine nucleotide, reduced; TPND, triphosphopyridine nucleotide, reduced, A-side deuterated; Acpyr-TPNH, 3-acetylpyridine adenine dinucleotide phosphate, reduced; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; mal, L-malate; pyr, pyruvate; EDTA, ethylenediaminetetraacetic acid.

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barriers surrounding the intermediate, and enzymatically generated intermediates are no exception. In this paper we will show that by directly generating the central E–TPNH–oxalacetate complex and measuring how it partitions between the production of malate and pyruvate under initial velocity conditions it is possible to determine the heights of some of these barriers, as well as the intrinsic isotope effects on the bond-breaking processes. This method should be applicable to any stepwise reaction in which the intermediate is stable enough to prepare and use in partitioning experiments.

Materials and Methods

Instrumentation. A Cary 118 double-beam spectrophotometer with repetitive scan attachment was used to measure the change in absorbance alternately at 340 and 281 nm. The spectrophotometer was fitted with an external 10-mV output of absorbance to bypass the built-in strip chart recorder, since the latter pauses while changing wavelengths, thus distorting the apparent change in absorbance with time. An external 10-mV recorder with chart drive independent of spectrophotometer control was used with the external output to circumvent this problem. All assays were in 0.5-cm path-length quartz cuvettes of total volume 1.5 mL. Reference and sample cell temperature was maintained at 27 ± 0.1 °C with thermospacers.

Chemicals. An oxalacetate solution was prepared daily by dissolving the solid free acid in H_2O and immediately adjusting the solution to pH 10 with KOH and chilling to 0 °C. The solution was allowed to remain at this temperature for at least 1 h to allow the various forms of oxalacetate to equilibrate (Pogson & Wolfe, 1972). Spontaneously formed pyruvate was usually about 5% on the basis of assay with lactate dehydrogenase and DPNH. Metal solutions were exhaustively extracted with dilute dithizone in CCl_4 (Johnson, 1964).

Reduced Nucleotide Preparation. A-side deuterated TPND was prepared by reaction of 1 mmol of TPN (Boehringer-Mannheim) with 1 mmol of *threo*-DL-isocitrate-*2-d* (prepared by the method of O'Leary & Limburg, 1977) in 100-mL volume in the presence of 100 units of porcine heart TPN isocitrate dehydrogenase. The α-ketoglutarate generated was removed by reaction with 30 mmol of L-alanine in the presence of 250 units of glutamate-pyruvate transaminase. The pH was maintained between 7.8 and 8.0 with dilute KOH. After 3.5 h, the pH no longer changed, and spectrophotometric assay at 340 nm showed the reaction (uncorrected for TPNH decomposition) to be 82.4% complete. The enzymes were removed by ultrafiltration at 4 °C with an Amicon UM-10 membrane, and the solution was reduced to 8 mL by rotary evaporation and chromatographed on a 1.7 × 25 cm column of Dowex AG MP-1 equilibrated with 0.2 M LiCl at pH 10 according to the procedure of Newton et al. (1983). Those reduced nucleotide fractions with a 260/340-nm absorbance ratio of 2.50 or less were concentrated and desalted on a 2.5 × 25 cm column of Bio-Rad P-2 equilibrated with 7.5 mM Ches, pH 10.

Reduced 3-acetylpyridine adenine dinucleotide phosphate (Acpyr-TPNH) was prepared by reduction of 244 μmol of 3-acetylpyridine adenine dinucleotide phosphate (Sigma) by 40 units of TPN malic enzyme in the presence of 5 mmol of L-malate and 20 mM $MgCl_2$ in 200-mL volume. The pH was maintained at 7.8–8.0 with dilute KOH. After 1 h, the pH no longer changed, and spectrophotometric assay at 363 nm showed the reaction to be 80% complete, with no correction made for decomposition of Acpyr-TPNH. The chromatographic purification scheme was the same as described for deuterated TPNH above, except that the 260/363-nm absorbance ratio was used as the criterion of purity (the final preparation had a ratio of 1.67 or lower).

Enzymes. A stock solution of glutamate-pyruvate transaminase was prepared by dissolving 2000 units of the lyophyllized powder (Sigma) in 2 mL of 50 mM Pipes and 50% glycerol. Chicken liver TPN malic enzyme (Sigma) was supplied in 2.9 M ammonium sulfate, 10 mM potassium phosphate, 0.5 mM 2-mercaptoethanol, 10 mM $MnCl_2$, and 3 mM EDTA. A total of 0.22 mL (50 units) was dissolved in 0.5 mL of 50 mM Pipes, pH 7.0, 1 mM dithiothreitol, and 50% glycerol and dialyzed against the same plus 10 mM EDTA. The enzyme solution was then chromatographed on a 1 × 10 cm column of Sephadex G-10 in 50 mM Pipes, 50% glycerol, and 0.5 mM dithiothreitol.

Assay Procedure. All solutions (except enzymes) were separately sparged with CO_2-free nitrogen for 8 h and kept tightly capped until used. Two cuvettes (one to be used as a blank) were prepared with the following components: 100 mM buffer (KOAc below pH 5.5, Mes at pH 5.5–6.5, and Pipes at pH 6.5–7.5), 100 mM L-glutamate, 5 mM oxalacetate, 0.445 mM reduced nucleotide (TPNH, TPND, or Acpyr-TPNH), and 10 mM $MgCl_2$ or 2 mM $Mn(OAc)_2$. The assay components were mixed and allowed to stand for twice the amount of time required for glutamate-pyruvate transaminase to convert any contaminating pyruvate to α-ketoglutarate. The time necessary for this conversion was determined by following α-ketoglutarate production in a separate assay with glutamate dehydrogenase and NH_3; typically, this was 3–4 min. This also allowed sufficient time for the oxalacetate to come to equilibrium between its keto and enol forms.

To initiate the reaction, 1–10 μL of TPN malic enzyme solution was added to the sample cuvette, and both cuvettes were capped to avoid an uptake of CO_2. The rate of change of absorbance at 340 (363 nm for AcPyr-TPNH) and 281.5 nm was alternately monitored in 75-s cycles for 20–30 min. In most cases, the observed rate at the two wavelengths remained linear over this time, but the rate observed during the initial 2–3 min of the reaction was discarded, as the temperature was not stable during this time.

Isosbestic Point Determination. The exact isosbestic point for the conversion of DPNH to DPN and Acpyr-DPNH to Acpyr-DPN in our Cary 118 was determined in 100 mM Tricine, pH 8.0, to be 281.5 nm. We assume that the isosbestic point for TPNH to TPN conversion is also 281.5 nm.

Determination of Oxalacetate Extinction Coefficient. The effective extinction coefficient for oxalacetate was determined for each metal ion concentration and pH used by adding a known amount of oxalacetate (concentration enzymatically calibrated with malate dehydrogenase and DPNH) to a cuvette containing the same concentration of buffer and metal ion and pH to be encountered in the actual partitioning assay. The change in absorbance at 281.5 nm was noted and the effective extinction coefficient calculated. The extinction coefficient of α-ketoglutarate at 281.5 nm was found to be 18 M^{-1} cm^{-1} and did not vary with pH. Extinction coefficients for TPNH and Acpyr-TPNH were taken as 6220 M^{-1} cm^{-1} at 340 nm and 9100 M^{-1} cm^{-1} at 363 nm, respectively.

Isotope Effect Nomenclature. We use the nomenclature of Northrop (1977) in which isotope effects on a kinetic or thermodynamic parameter are indicated by a leading superscript (D, T, or 13 for deuterium, tritium, or ^{13}C). Thus, $^{D}K_{eq}$ is $K_{eq\,H}/K_{eq\,D}$, while ^{13}k is the ratio of rate constants for ^{12}C- and ^{13}C-containing substrates.

Theory

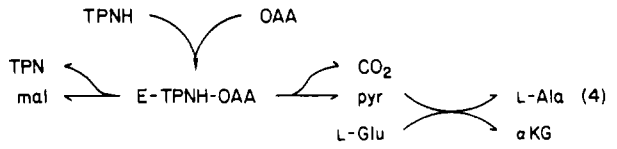
Hermes et al. (1982) showed that hydride transfer precedes

decarboxylation in the stepwise oxidative decarboxylation of malate by chicken liver TPN malic enzyme. The following mechanism was proposed:

$$EA \underset{k_2}{\overset{k_1B}{\rightleftharpoons}} EAB \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EAB^* \underset{k_6}{\overset{k_5}{\rightleftharpoons}} ERX \xrightarrow{k_7} ERPQ \xrightarrow{k_9} ERQ \xrightarrow{k_{11}} ER \quad (3)$$

where EA is enzyme–TPN, ER is enzyme–TPNH, B is malate, X is oxalacetate, P is CO_2, and Q is pyruvate. In this mechanism, k_5 and k_6 show a primary deuterium kinetic isotope effect with malate-*2-d*, and k_7 shows a primary ^{13}C kinetic isotope effect when the $^{13}C/^{12}C$ ratio of the CO_2 produced is compared with that of C-4 of malate. Hermes et al. (1982) used the ^{13}C isotope effect with deuterated and unlabeled malate and the deuterium and tritium isotope effects on V/K_{malate} reported by Schimerlik et al. (1977) to calculate that k_6/k_7 was 6–12, k_5/k_4 was 2.5–4.4, $^Dk_5$ was 5.3–6.0, and $^{13}k_7$ was 1.04–1.06. The pH independence of the $^D(V/K)$ value showed that $k_2 \gg k_3$.

When enzyme, TPNH, and oxalacetate are mixed together, the resulting E–TPNH–OAA complex can partition in two ways. Decarboxylation will yield pyruvate and CO_2, while reduction will give malate and convert TPNH to TPN. Each of these processes is irreversible when initial velocities are measured, and the changes in absorbance at 340 and 281.5 nm can be used to follow TPNH and oxalacetate disappearance. The former measures malate formation the the latter malate + pyruvate formation. Pyruvate is removed as it is produced with glutamate-pyruvate transaminase in the presence of excess glutamate:



Correction for the nonenyzmatic breakdown of oxalacetate and TPNH is made with a reference cuvette.

The observed ratio of [pyr]/[mal] formation is calculated in the following manner:

$$-d[OAA]/dt = d[mal]/dt + d[pyr]/dt \quad (5)$$

$$dA_{281}/dt = (d[Pyr]/dt)\epsilon_{281,\alpha KG} + (d[OAA]/dt)\epsilon_{281,OAA} \quad (6)$$

$$d[mal]/dt = -(dA_{340}/dt)/\epsilon_{340,TPNH} \quad (7)$$

Combining these three equations gives

$$\frac{d[pyr]}{dt} = \frac{(dA_{340}/dt)\epsilon_{281,OAA}/\epsilon_{340,TPNH} - dA_{281}/dt}{\epsilon_{281,OAA} - \epsilon_{281,\alpha KG}} \quad (8)$$

The observed ratio of [pyr]/[mal] is then given by

$$r_H = (d[pyr]/dt)/(d[mal]/dt) \quad (9)$$

In terms of the proposed mechanism, the observed ratio of [pyr]/[mal] is

$$r_H = (k_7/k_6)[1 + (k_5/k_4)(1 + k_3/k_2)] \quad (10)$$

which is the ratio of k_7 and the net rate constant for malate formation from E–TPNH–OAA:

$$k_6/[1 + (k_5/k_4)(1 + k_3/k_2)] \quad (11)$$

r_H is also the forward commitment for the decarboxylation step, so that if we assume there is no reverse commitment for the decarboxylation step (since the affinity for CO_2 is very low), it is simple to calculate the intrinsic ^{13}C isotope effect from the observed one. Since

$$^{13}(V/K)_H = (^{13}k_7 + r_H)/(1 + r_H) \quad (12)$$

we have

$$^{13}k_7 = {}^{13}(V/K)_H + r_H[{}^{13}(V/K)_H - 1] \quad (13)$$

The same value should result if $^{13}(V/K)_D$ (the ^{13}C isotope effect measured with malate-*2-d*) and r_D (the partition ratio measured with A-side deuterated TPNH and oxalacetate) are used:

$$^{13}k_7 = {}^{13}(V/K)_D + r_D[{}^{13}(V/K)_D - 1] \quad (14)$$

The value of r_H can be broken down into two parts, k_7/k_6 (the immediate partition ratio of E–TPNH–OAA and the reciprocal of the reverse commitment for hydride transfer) and $(k_5/k_4)(1 + k_3/k_2)$, which is the forward commitment for hydride transfer and can be designated c_{fH}. We then have

$$r_H = (k_7/k_6)(1 + c_{fH}) \quad (15)$$

$$r_D = (k_7/k_6)(^Dk_5 + c_{fH})/{}^DK_{eq} \quad (16)$$

where the more complex form of eq 16 reflects the fact that k_{5D} and k_{6D} rather than k_{5H} and k_{6H} are involved when deuterated TPNH is used. The values of r_H and r_D are not independent, but are related by

$$^D(V/K) = {}^DK_{eq}(r_D + 1)/(r_H + 1) \quad (17)$$

To determine the individual values of $^Dk_5$, k_7/k_6, and c_{fH}, one needs values for $^D(V/K)$, $^T(V/K)$, and r_H. The equation for $^D(V/K)$ is

$$^D(V/K) = \frac{^Dk_5 + c_{fH} + {}^DK_{eq}(k_6/k_7)}{1 + c_{fH} + (k_6/k_7)} \quad (18)$$

and the equation for $^T(V/K)$ is similar except that $^Dk_5$ and $^DK_{eq}$ are raised to the 1.442 power to reflect the relationship between the intrinsic deuterium and tritium isotope effects (Swain et al., 1958). If we define

$$T_r = \frac{^TK_{eq} - {}^T(V/K)}{r_H} \quad (19)$$

$$D_r = \frac{^DK_{eq} - {}^D(V/K)}{r_H} \quad (20)$$

we can combine the equations for $^D(V/K)$, $^T(V/K)$, and r_H to get

$$\frac{^T(V/K) - 1 - T_r}{^D(V/K) - 1 - D_r} = \frac{(^Dk_5)^{1.442} - {}^T(V/K) + T_r}{^Dk_5 - {}^D(V/K) + D_r} \quad (21)$$

This equation is readily solved by Newton's method of successive approximations to give $^Dk_5$, and then

$$c_{fH} = \frac{^Dk_5 - {}^D(V/K) + D_r}{^D(V/K) - 1 - D_r} \quad (22)$$

The internal partition ratio is given by

$$k_7/k_6 = r_H/(1 + c_{fH}) \quad (23)$$

A computer program has been written to make these calculations.

Results

Unlabeled and Deuterated TPNH. The observed rates, plus calculated partitioning ratios with TPNH and deuterated TPNH at 2 mM Mg^{2+} are presented in Table I. The observed pyr/mal partitioning ratio, r_H, does not vary with pH but remains constant at 0.47 ± 0.01 with TPNH. With deuterated

Table I: Observed Pyr/Mal Partitioning Ratios at 2 mM Mg^{2+}

pH	enzyme units[a]	nucleotide[b]	$\epsilon_{281,OAA}$	initial formation rate (μM/min) mal	pyr	pyr/mal ratio
6.83	0.5	TPNH	750	2.28	1.06	0.465
				2.22	1.02	0.459
				2.22	1.07	0.483
				2.30	1.09	0.472
						0.47 ± 0.01[c]
5.97	0.5	TPNH	853	2.45	1.16	0.472
				2.33	1.12	0.478
						0.475 ± 0.004[c]
4.95	1.0	TPNH	707	1.81	0.877	0.485
				1.81	0.869	0.479
				1.82	0.755	0.415
				1.72	0.860	0.500
				1.72	0.758	0.441
						0.464 ± 0.035[c]
6.85	0.6	TPND	750	2.03	1.70	0.840
				2.07	1.67	0.806
				2.06	1.63	0.794
						0.813 ± 0.024[c]
6.50	0.5	Acpyr-TPNH	811	0.417	4.08	9.79
				0.417	4.12	9.90
5.51	0.8	Acpyr-TPNH	918	0.710	7.03	9.90
				0.567	5.62	9.91
						9.88 ± 0.06[c]

[a] Units of TPN malic enzyme per assay volume of 1.5 mL. [b] Nucleotide concentration = 0.445 mM. [c] Average.

Table II: Intrinsic Isotope Effects with TPNH and 2 mM Mg^{2+}

parameter	eq	value
$^{13}k_7$	13[a]	1.044 ± 0.002
$^{13}k_7$	14[b]	1.045 ± 0.004
$^{D}k_5$	21	5.7 ± 0.3
c_{fH}	22[c]	3.3 ± 0.4
k_6/k_7	23	10 ± 1

[a] Calculated with r_H and $^{13}(V/K)_H$. [b] Calculated with r_D and $^{13}(V/K)_D$. [c] Forward commitment for malate.

nucleotide, the observed ratio, r_D, is 0.81 ± 0.02. These values, along with the observed primary deuterium, tritium, and carbon isotope effects of Hermes et al. (1982), allow solution of eq 13, 14, 21, 22, and 23 to give the intrinsic isotope effects, commitment, and internal partition ratio listed in Table II. Two independent values for the intrinsic carbon isotope effect, $^{13}k_7$, can be calculated with r_H and $^{13}(V/K)_H$ or r_D and $^{13}(V/K)_D$.

Acpyr-TPNH. The partitioning ratio, r_H, with Acpyr-TPNH as the reduced nucleotide and 2 mM Mg^{2+}, is 9.88 ± 0.06 (Table I). With the value of 1.0037 ± 0.0006 for $^{13}(V/K)_H$ with Acpyr-TPN from Hermes et al. (1982), the intrinsic carbon isotope effect, $^{13}k_7$, is calculated from eq 13 to be 1.040 ± 0.009. This is the only calculable parameter in this case, since the tritium isotope effect with this nucleotide has not been measured.

Manganese. The observed pyruvate/malate partitioning ratios with TPNH and 100 μM Mn^{2+} in Table III average 0.99 ± 0.08. Employing eq 13 and the observed $^{13}(V/K)_H$ value of 1.0324 ± 0.0006 (Hermes et al., 1982) for 0.5 mM Mn^{2+}, $^{13}k_7$ is calculated to be 1.065 ± 0.005. Once again, this is the only intrisic parameter calculable with the available data.

Discussion

Spectroscopically observing the partitioning of oxalacetate allows great precision in the determination of r_H. Since the ratio of two reaction rates in the same assay is being compared, pipetting errors and other variances that normally plague intial velocity studies are minimized. Assuming the extinction coefficient for TPNH is invariant under the conditions examined, the greatest source of error is probably the determination of the extinction coefficient for oxalacetate under the exact assay conditions. Hence, the error associated with this value must be propagated into the observed [pyr]/[mal] partition ratio. The observed enzymatic rates were linear for up to 30 min if TPNH and oxalacetate were not significantly depleted by enzymatic and nonenzymatic processes during this time.

Table III: Observed Pyr/Mal Partitioning Ratios at 0.1 mM Mn^{2+} with 0.445 mM TPNH

pH	enzyme units[a]	$\epsilon_{281,OAA}$	initial formation rates (μM/min) mal	pyr	pyr/mal ratio
7.95	0.5	505	0.747	0.679	0.910
			0.800	0.762	0.953
6.45	2.0	410	3.03	3.08	1.015
			2.85	3.13	1.096
					0.99 ± 0.08[b]

[a] Units of TPN malic enzyme per assay volume of 1.5 mL. [b] Average.

Partitioning with TPNH and Magnesium. The observed intrinsic deuterium isotope effect of 5.7 ± 0.3 is one of only a few enzymatic intrinsic isotope effects known with such precision. Similar in magnitude is the intrinsic deuterium isotope effect of 5.27 for glucose-6-phosphate dehydrogenase (Hermes et al., 1982).

Knowing only r_H and $^{13}(V/K)_H$, we are able to calculate the intrinsic ^{13}C isotope effect on decarboxylation to be 1.044 ± 0.002. A further determination of this value is obtained from r_D and $^{13}(V/K)_D$, giving 1.045 for the intrinsic ^{13}C isotope effect. The agreement of these two independent measurements of the same parameter is an indication of the power of this technique. It is now possible, with two simple experiments, to determine the intrinsic isotope effect on carbon–carbon bond scission in oxidative decarboxylations. This will enable a thorough study of the effect of various metal ions on the enzyme-mediated decarboxylation of β-ketocarboxylic acids.

Since the intrinsic carbon and deuterium isotope effects are known, it is possible to calculate the relative heights of the energy barriers that separate the metastable intermediates

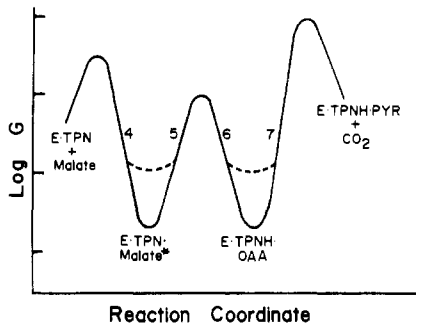


FIGURE 1: Reaction coordinate diagram for the reaction of malate and TPN catalyzed by malic enzyme. The numbers next to the activation barriers correspond to the rate constants in mechanism 3 (the reverse commitment for the decarboxylation step is assumed to be very small). Note that while the relative heights of the barriers are precisely known, the relative levels of the troughs corresponding to the intermediates are not (the dotted lines indicate this uncertainty). E–TPN–malate* and E–TPNH–OAA are shown as if $k_5 = k_6$, but there is no way to establish this without measuring the actual equilibrium constant on the enzyme.

along the reaction pathway. Knowing that the ratio of reverse hydride transfer (k_6) to decarboxylation (k_7) is 10 and the total commitment for malate is 3.3, it is possible to calculate a reaction coordinate diagram that precisely reflects the heights of the potential barriers surrounding the intermediates (Figure 1). It should be noted that, even though the ratios k_6/k_7 and k_4/k_5 are known precisely, the ratio of k_5/k_6 is *not* known [in a more general mechanism, k_4 would represent $k_4/(1 + k_3/k_2)$, but since the $^D(V/K)$ value is pH independent (Schimerlik et al., 1977), $k_2 \gg k_3$ and we can ignore the initial E–TPN–malate complex]. Hence, the rate of dehydrogenation relative to the rate of decarboxylation is still undetermined (that is, the relative levels of the E–TPN–malate* and E–TPNH–oxalacetate complexes in mechanism 3 and Figure 1 are not known).

Acpyr-TPNH and Magnesium. The observed partitioning ratio changes 20-fold with a change in nucleotide. Presumably, this reflects a change in the rate of hydride transfer from Acpyr-TPNH to oxalacetate because of the more positive redox potential of the nucleotide and not a significant change in the rate of decarboxylation of oxalacetate. The intrinsic ^{13}C isotope effect with Acpyr-TPNH of 1.040 ± 0.009 is not signifiantly different than the intrinsic ^{13}C isotope effect with normal TPNH, showing that the nature of the nucleotide does not greatly affect the transition state for decarboxylation.

Hermes et al. (1982) were able to set limits on k_6/k_7 and c_{fH} of 0.23–0.45 and 2.36–2.94, respectively, by assuming the intrinsic deuterium and ^{13}C isotope effects were the same for TPN and Acpyr-TPN. Although part of this assumption has been proved correct by this study, a value for the intrinsic deuterium isotope effect with Acpyr-TPN is not available. Knowing the partitioning ratio, r_H, with Acpyr-TPNH, it is possible to redefine these limits *without* making a priori assumptions as to the size of the intrinsic deuterium isotope effect. Given eq 20 and 23, the possible range of values for c_{mal} and k_6/k_7 is presented in Table IV.

Table IV: Calculated Values of Internal Partition Ratios and Forward Commitments for Acpyr-TPNH as Nucleotide[a]

c_{fH}	$^Dk_5$	k_6/k_7	c_{fH}	$^Dk_5$	k_6/k_7
0	2.28	0.101	3	6.12	0.405
1	3.56	0.202	4	7.40	0.506
2	4.84	0.304			

[a] Calculated from $^D(V/K) = 2.18$ (Hermes et al., 1982) and $r_H = 9.88$, with various assumed values of c_{fH}, the forward commitment for malate.

Manganese and TPNH. The calculated intrinsic ^{13}C isotope effect of 1.065 with manganese as the metal cofactor is significantly higher than the value of 1.044 with magnesium and is close to the intrinsic ^{13}C isotope effect of 1.06 at 10 °C on the nonenzymatic decarboxylation of oxalacetate by manganese ion (Seltzer et al., 1959). The agreement between the intrinsic ^{13}C isotope effects on the enzymatic and nonenzymatic decarboxylation of oxalacetate with manganese suggests a similarity of transition states. It would be of interest to see if this mechanistic agreement between metal ion catalysis in solution and at enzyme active sites holds true for other metal-activated decarboxylases or with other metal ions and malic enzyme.

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