Intermediate Partitioning in the Tartrate Dehydrogenase-Catalyzed Oxidative Decarboxylation of D-Malate†

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ABSTRACT: The oxidative decarboxylation of D-malate catalyzed by tartrate dehydrogenase has been examined in detail. Enzyme-catalyzed partitioning of oxalacetate has been determined to proceed with formation of pyruvate and D-malate in a ratio of 3.7 to 1. These data, along with the deuterium and tritium kinetic isotope effects on hydride transfer, allow exact calculation of the intrinsic isotope effect and the forward and reverse commitments to catalysis, which have values of 5.1 ± 0.8, 6.3 ± 1.0, and 2.0 ± 0.3, respectively. The viscosity dependence of the tritium isotope effect was measured, which allowed determination of the internal and external components of the commitment factors. The reverse commitment has no external portion, but the forward commitment can be divided into external and internal portions of 3.7 ± 1.2 and 2.6 ± 1.6, respectively. These data indicate that the reaction becomes committed to catalysis in the forward direction by formation of the Michaelis complex; reverse hydride transfer from NADH to OAA is twice as fast as decarboxylation of OAA, and recarboxylation of pyruvate occurs at a negligible rate. The rate constant for dissociation of OAA from the enzyme active site was estimated to be approximately 4 orders of magnitude slower than that for dissociation of oxaloglycolate, which is the product of the enzyme-catalyzed oxidation of (+)-tartrate.

Tartrate dehydrogenase (TDH) is isolated from *Pseudomonas putida* grown on (+)-tartrate[1] as the sole carbon source and catalyzes the first step in the metabolism of (+)-tartrate, which is the NAD-dependent oxidation to oxaloglycolate (Kohn et al., 1968). The purified enzyme has been found to utilize *meso*-tartrate and D-malate as substrates as well (Tipton & Peisach, 1990). Surprisingly, TDH catalyzes different chemical reactions with these substrates than it does with (+)-tartrate. D-Malate is oxidatively decarboxylated to yield pyruvate and CO_2, while *meso*-tartrate is converted to D-glycerate and CO_2 via a reaction in which no net oxidation or reduction occurs. All three reactions require Mn^{2+} and K^+.

An attractive hypothesis to explain these multiple catalytic activities is that all three substrates start down a common pathway, but that the intermediates dissociate at different stages. Thus, the initial step in all three reactions is the NAD-dependent oxidation of the carbon acid substrate. In the (+)-tartrate reaction (3*R*)-oxaloglycolate dissociates from the enzyme, but oxalacetate and (3*S*)-oxaloglycolate produced from D-malate and *meso*-tartrate, respectively, remain bound to the enzyme and undergo enzyme-catalyzed decarboxylation. Pyruvate, arising from OAA[2] decarboxylation, dissociates from TDH, but hydroxypyruvate generated by (3*S*)-oxaloglycolate decarboxylation remains in the active site and is reduced to D-glycerate by the NADH that was produced in the initial step of the reaction. The *meso*-tartrate reaction cycle is completed by dissociation of D-glycerate from the enzyme.

Determination of the partitioning of enzyme-bound species and the location of rate-determining steps along the catalytic pathways is clearly crucial for understanding the enzyme–substrate interactions which determine the products of the different reactions. We have undertaken a series of kinetics investigations of the D-malate reaction which have yielded the partition ratios for each enzyme–intermediate complex between D-malate addition and pyruvate release. This was accomplished by experiments in which OAA was added to the enzyme–NADH complex, and the partitioning of OAA forward to pyruvate and CO_2 and backward to D-malate was monitored directly and by measurement of ^2H and ^3H isotope effects on hydride transfer from D-malate to NAD^+ and determination of the viscosity dependence of the ^3H kinetic isotope effect. In addition, steady-state kinetics determinations of the dissociation constants of the substrates and products from TDH were used to estimate the rate constant for OAA dissociation from the enzyme–NADH complex.

MATERIALS AND METHODS

Tartrate dehydrogenase was isolated from *Pseudomonas putida* as described previously (Tipton & Peisach, 1991). Glutamate–pyruvate transaminase, malate dehydrogenase, and malic enzyme were purchased from Sigma Biochemicals, as were all common biochemicals. Sodium borodeuteride (96 atom % deuterium) was obtained from Aldrich Chemical Co., and sodium boro[^{3}H]hydride (5–20 Ci/mmol) was from Amersham.

Steady-State Kinetics Measurements. TDH-catalyzed reactions were monitored by using a Beckman DU monochromator interfaced to a personal computer for data collection or a Hewlett-Packard 8452A diode array spectrophotometer. The cuvette holders of both instruments were thermostated at 25 °C with a circulating water bath. All assays were performed in 1-cm path length quartz cuvettes. One unit of TDH is defined as that amount of enzyme which catalyzes the formation of 1 μmol of NADH/min, detected at 340 nm (ϵ = 6220 M^{-1} cm^{-1}) in a solution of 1.5 mM NAD^+, 10 mM (+)-tartrate, and 0.4 mM Mn^{2+} (Kohn et al., 1968). All assays and steady-state kinetics studies were conducted in 100 mM K^+ HEPES, pH 8.0, containing 1 mM DTT. Stock solutions of D-malate and NAD^+ were calibrated by end-

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[1] (+)-Tartrate, (+)-(2*R*,3*R*)-tartrate; *meso*-tartrate, (2*R*,3*S*)-tartrate; D-malate, (2*R*)-malate.

[2] Abbreviations: OAA, oxalacetate; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; HPLC, high-performance liquid chromatography.

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point assay with TDH, and OAA stock solutions were calibrated by end-point assay with malate dehydrogenase.

Initial velocity kinetics measurements were fitted to the appropriate equations using BASIC versions of the programs of Cleland (1979) provided by Dr. Charles B. Grissom. Saturation curves with a single variable substrate were fitted to eq 1.

$$v = VA/(K + A) \quad (1)$$

Synthesis of Isotopically-Labeled Malates. [2-^{3}H]Malate was prepared by reduction of OAA with NaB^3H_4. Four milligrams of OAA was dissolved in 10 mL of H_2O and chilled on ice. The solution was quickly titrated to pH 5, and 50 μCi of NaB^3H_4 in 0.1 M NaOH was added. The pH of the solution was readjusted to approximately 6, and the solution was stirred on ice for 1.5 h. Three milligrams of $NaBH_4$ was added, and the solution was stirred for an additional hour. Excess $NaBH_4$ was destroyed by addition of 100 μL of 1 N HCl. Quantitative production of malate was confirmed by assaying an aliquot of the reaction mixture for L-malate using malic enzyme. L-Malate was removed from the product by oxidative decarboxylation catalyzed by malic enzyme. The reaction mixture contained 1 mmol of K^+ HEPES, pH 8.0, 10 μmol of DTT, 5 μmol of $Mn(OAc)_2$, and 22 μmol of $NADP^+$. Malic enzyme (2.3 units) was added, and the reaction mixture was incubated at room temperature overnight. The D-malate remaining in the reaction mixture was isolated by anion-exchange chromatography. The reaction mixture was chromatographed on a 1.5 × 15 cm column of Dowex AG1-x8 equilibrated in H_2O. The column was eluted with a linear gradient from 0 to 2 N formic acid over 200 mL and was then further washed with 50 mL of 2 N formic acid. Fractions containing D-malate were pooled and concentrated by rotary evaporation. [2-^{2}H]Malate was prepared in a similar manner, using NaB^2H_4 to reduce OAA. [2-^{2}H,3-^{3}H]Malate was prepared by dissolving 10 mg of OAA in 0.1 mL of ^3H_2O (0.5 mCi) and incubating the solution at room temperature for 1.5 h. The solution was neutralized, and the OAA was reduced with NaB^2H_4; the resulting D-[2-^{2}H,3-^{3}H]malate was isolated as described above.

^{2}H Kinetic Isotope Effects. $^D(V/K)_{D\text{-malate}}$ and ^DV were determined by the direct comparison method in solutions containing 0.6 mM NAD^+ and 0.5 mM $Mn(OAc)_2$, and D-malate or D-[2-^{2}H]malate concentrations varied between 20 and 500 μM. Data were fitted to eq 2, which assumes equal isotope effects on V/K and V. $E_{V/K}$ is $^D(V/K) - 1$, E_V is $^DV - 1$, and F_i is the fractional enrichment of the substrate with the isotopic label.

$$v = \frac{VA}{K(1 + F_iE_{V/K}) + A(1 + F_iE_V)} \quad (2)$$

To obtain a more precise value for $^D(V/K)_{D\text{-malate}}$ D-[2-^{2}H,3-^{3}H]malate was utilized in internal competition experiments where the ^{3}H served as a remote label for the ^{2}H undergoing hydride transfer. These experiments were conducted in a manner similar to those described below for determining $^T(V/K)_{D\text{-malate}}$, except that residual D-[2-^{2}H,3-^{3}H]malate was isolated from the reaction mixtures by chromatography on Dowex AG1-x8. Malate was eluted from the columns with 0.01 N HCl. $^D(V/K)_{D\text{-malate}}$ was calculated according to eq 3, where f is the fractional completion of the reaction, R_s is the specific activity of the residual D-malate, and R_o is the specific activity of the D-malate before initiation of the reaction.

$$^D(V/K)_{D\text{-malate}} = \frac{\log(1-f)}{\log[(1-f)(R_s/R_o)]} \quad (3)$$

^{3}H Kinetic Isotope Effects. The value of $^T(V/K)_{D\text{-malate}}$ was determined by internal competition experiments. Low-conversion samples contained 0.31 mM D-[2-^{3}H]malate, 60 μM NAD^+, and 0.5 mM $Mn(OAc)_2$ in a total volume of 1 mL. The samples were contained in 1-cm path length plastic cuvettes; reactions were initiated by the addition of TDH, and the progress of the reaction was monitored at 340 nm. When the reaction approached a fractional conversion of D-malate to pyruvate of 0.2, it was terminated by the addition of EDTA to a final concentration of 5 mM. High-conversion samples contained 31 μM D-[2-^{3}H]malate, 0.6 mM NAD^+, and 0.5 mM $Mn(OAc)_2$ in a volume of 1 mL. Reactions were monitored for complete conversion of D-malate to pyruvate, after which EDTA was added.

Immediately after termination of the reactions, the samples were chromatographed to separate the [^{3}H]NADH from the residual D-[2-^{3}H]malate. Two different chromatographic methods were used, which gave equivalent results. The first method used benzyl DEAE cellulose equilibrated in 10 mM potassium phosphate, pH 8.2. Samples were loaded onto 1-mL columns, which were washed with 15 mL of 10 mM potassium phosphate, pH 8.2, to elute D-malate. NADH was eluted with 9 mL of 100 mM potassium phosphate, pH 8.2. One-milliliter fractions were collected directly into scintillation vials; 9 mL of scintillation fluid was added to each vial, and the vials were counted. Alternatively, NADH and D-malate were separated by HPLC on a 4.6 × 150 mm Alltech C18 column equilibrated in 0.4 M potassium phosphate, pH 7.05, and eluted isocratically at 1.0 mL/min. Fractions were collected directly into scintillation vials, and 14 mL of scintillation fluid was added to each vial prior to counting.

The value of $^T(V/K)_{D\text{-malate}}$ was calculated according to eq 4, where f refers to the fraction of D-malate which reacted prior to termination of the reaction, R_p is the specific activity of the NADH formed in the low-conversion samples, and R_o is the specific activity of the D-malate before the initiation of the reaction. The value of R_o is determined by measuring the specific activity of NADH formed in the high-conversion samples.

$$^T(V/K)_{D\text{-malate}} = \frac{\log(1-f)}{\log[1 - f(R_p/R_o)]} \quad (4)$$

Viscosity Measurements. Solution viscosities were determined using an Ostwald viscometer thermostatted at 25 °C by immersion in a water bath. Glycerol, sucrose, and polyethylene glycol (8000 av mol wt) were used as viscosogens.

OAA Partitioning. Conversion of OAA to pyruvate and D-malate was monitored directly by simultaneously measuring the disappearance of OAA at 282 nm and the oxidation of NADH at 340 nm using a Hewlett-Packard 8452A diode array spectrophotometer. The isosbestic point for the NAD–NADH interconversion is 281.5 nm, so OAA disappearance was monitored directly at 282 nm without interference from the changing absorbance due to NADH oxidation. OAA decarboxylation was made irreversible by coupling pyruvate formation to α-ketoglutarate production via the action of glutamate–pyruvate transaminase in the presence of a high concentration of L-glutamate.

OAA partitioning was measured in solutions containing 0.5 mM OAA, 0.5 mM Mn^{2+}, 0.1 M L-glutamate, 0.1 mM NADH, and 2 units of glutamate–pyruvate transaminase.

Samples were allowed to reach thermal equilibrium at 25 °C and then were monitored spectrophotometrically for 5 min in order to determine the rate of nonenzymatic decarboxylation of OAA. Reactions were initiated by addition of TDH and monitored for at least an additional 5 min. The rate of nonenzymatic decarboxylation of OAA was approximately 10% of the rate of enzyme-catalyzed OAA turnover and was subtracted from the enzymatic rate before the rate of D-malate production was calculated.

The rate of conversion of OAA to D-malate was calculated directly from the rate of disappearance of NADH; the rate of OAA decarboxylation was calculated by subtracting the rate of D-malate production from the rate of OAA disappearance. The extinction coefficient for OAA at 282 nm under the experimental conditions was calculated by preparing a solution containing 0.5 mM OAA, 0.15 mM NADH, and 0.5 mM Mn^{2+} in the same buffer as was used in the partitioning experiments. The absorbance at 282 nm was measured, and the OAA concentration was determined by end-point assay with malate dehydrogenase. By these means a value of ϵ_{282} = 1760 M^{-1} cm^{-1} was determined. The ratio of pyruvate to D-malate formed from OAA is equal to the ratio of the rates of pyruvate and D-malate formation.

RESULTS

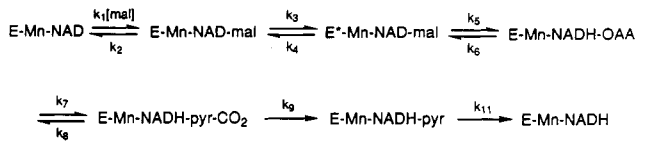
Kinetic Isotope Effects. The deuterium isotope effects on V/K and V were equal for the TDH-catalyzed oxidation of D-malate; $^{D}(V/K)$ was 1.36 ± 0.08, and ^{D}V was 1.39 ± 0.04. A more precise determination of $^{D}(V/K)$ was obtained by using doubly labeled D-malate; this experiment yielded a value of $^{D}(V/K)$ of 1.48 ± 0.03. In these experiments, ^{3}H was used as a remote label for ^{2}H; since the hybridization of the carbon to which the ^{3}H is attached does not change during the hydride-transfer step, we assumed that secondary isotope effects introduced by the ^{3}H would be negligible. The close agreement between the value of $^{D}(V/K)$ determined in this experiment and the value determined by direct comparison bears out this expectation. On the basis of three measurements of the tritium isotope effect, $^{T}(V/K)$ was 2.08 ± 0.03. The observed deuterium and tritium isotope effects on V/K clearly do not satisfy the Swain–Schaad relationship (Swain et al., 1958): $^{D}(V/K)^{1.442} = 1.76 \neq {}^{T}(V/K)$. Thus, the intrinsic isotope effect on hydride transfer is attenuated by finite commitment factors (Northrop, 1981).

OAA Partitioning. The ratio of pyruvate to D-malate formed from OAA was 3.67 ± 0.02. If the OAA concentration was increased above 0.5 mM, the time courses for NADH oxidation exhibited some curvature. Therefore, the experiment was conducted at the lowest practical concentration of OAA, where the deviation from linearity was negligible. When higher concentrations of OAA were used, the pyruvate to D-malate ratio derived from the data was not different from that obtained at lower OAA concentrations as long as the rate of D-malate formation was calculated using the initial portion of the time course.

The relationships between the observed V/K isotope effects and the intrinsic isotope effect on hydride transfer are shown in eqs 5 and 6. The microscopic rate constants used in these and subsequent equations are those from Scheme I, which describes the kinetic mechanism for TDH (Tipton & Peisach, 1990).

In these equations the isotope-sensitive step is k_5, and the commitment factors refer to the hydride-transfer step. The forward commitment factor is the ratio of k_5 to the net rate constant for dissociation of D-malate from the enzyme; the reverse commitment is the ratio of k_6 to the net rate constant for dissociation of CO_2 from the enzyme. Thus, $c_f = k_5/k_4(1 + k_3/k_2)$ and $c_r = k_6/k_7(1 + k_8/k_9)$. The equilibrium isotope effect has been determined to be 1.18 for oxidation of L-malate and should be the same for oxidation of D-malate (Cook et al., 1980).

Scheme I



$$^{D}(V/K) = \frac{(^{D}k_5 + c_f + c_r{}^{D}K_{eq})}{(1 + c_f + c_r)} \quad (5)$$

$$^{T}(V/K) = \frac{[(^{D}k_5)^{1.442} + c_f + c_r(^{D}K_{eq})^{1.442}]}{(1 + c_f + c_r)} \quad (6)$$

The ratio of pyruvate to malate produced from OAA, r, is given by the ratio of the net rate constants for their formation from E·Mn·NADH·OAA. The net rate constant for the formation of pyruvate is $k_7/(1 + k_8/k_9)$; the net rate constant for formation of malate is $k_6/[1 + k_5/k_4(1 + k_3/k_2)]$. Thus, r can be expressed in terms of the commitment factors defined above.

$$r = (1 + c_f)/c_r \quad (7)$$

Equations 5, 6, and 7 constitute three independent equations which relate the experimentally determined OAA partition ratio and the deuterium and tritium isotope effects to three unknown parameters: c_f, c_r, and $^{D}k_5$, the intrinsic isotope effect on hydride transfer. These three equations were solved simultaneously using the SolvEq software package from Biosoft, which yielded the following results: c_f = 6.3 ± 1.0; c_r = 2.0 ± 0.3; and $^{D}k_5$ = 5.1 ± 0.8. The errors associated with this analysis were evaluated in the following way. Simulations were conducted in which one experimental parameter was allowed to vary while the other two were held constant, and the resulting effect on the values of ^{D}k, c_f, and c_r was determined. From this exercise it was determined that the final solution is very sensitive to errors in $^{D}(V/K)$, less sensitive to errors in $^{T}(V/K)$, and almost completely insensitive to errors in r (data not shown). Thus, a reasonable estimate of the errors associated with these calculations can be obtained by examining the uncertainty in the determination of ^{D}k calculated from experimental measurements of $^{D}(V/K)$ and $^{T}(V/K)$ (Northrop, 1975). This analysis relies on the relationship between ^{D}k and ^{T}k derived by Swain et al. (1958), and the uncertainty associated with this analysis has long been recognized (Albery & Knowles, 1977). The variance of ^{D}k resulting from errors associated with the experimental determination of $^{D}(V/K)$ and $^{T}(V/K)$ can be calculated as described by Northrop and Duggleby (1990). With our data the standard error associated with the determination of ^{D}k is ±0.8, which is approximately 15% of the value itself. Thus, we consider our standard error to be on the order of 15% of the calculated parameter, which leads to the results presented above.

Viscosity Dependence of V/K. Increased solution viscosity slows the rate of association of substrates with the enzyme and also the rate of dissociation of substrates and products from the enzyme (Brouwer & Kirsch, 1982). In terms of Scheme I, k_1, k_2, k_9, and k_{11} should be decreased in proportion

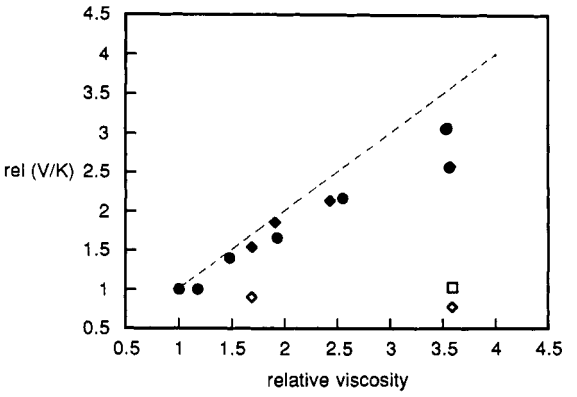


FIGURE 1: Viscosity dependence of TDH-catalyzed reactions. The dashed line shows the expected behavior for reactions which are completely rate-limited by diffusion of substrates to the enzyme. The closed circles are for the D-malate reaction in glycerol; the closed diamonds, the D-malate reaction in sucrose; the open diamonds, the (+)-tartrate reaction in sucrose; and the open square, the D-malate reaction in polyethylene glycol.

to increased solution viscosity. The viscosity dependence of the D-malate and (+)-tartrate reactions of TDH is shown in Figure 1. The value of $V/K_{\text{D-malate}}$ is sensitive to solution microviscosity, but the identity of the viscosogen does not appear to be critical. When the solution viscosity is increased by the addition of the macroviscosogen polyethylene glycol, $V/K_{\text{D-malate}}$ is unaffected. The value of $V/K_{(+)\text{-tartrate}}$ is independent of solution viscosity.

Viscosity Dependence of the Tritium Isotope Effect. The observed tritium isotope effect may also be dependent on the solution viscosity through its effect on the commitment factors. The forward and reverse commitments can be separated into internal and external components (Cleland, 1986): $c_{\text{f-ex}}$ is k_5k_3/k_4k_2, $c_{\text{f-in}}$ is k_5/k_4, $c_{\text{r-ex}}$ is k_6k_8/k_7k_9, and $c_{\text{r-in}}$ is k_6/k_7. The external forward and reverse commitment factors contain k_2 and k_9, respectively, which are potentially viscosity-dependent. Thus, the solution viscosity will manifest its effect on the observed isotope effect by increasing the magnitude of the external commitment factors. The equation for the observed tritium isotope effect on V/K can be written to include the viscosity dependence of the external commitments, where η is the relative viscosity of the solution.

$$^{T}(V/K)_{\eta} = \frac{^{T}k_5 + c_{\text{f-in}} + \eta c_{\text{f-ex}} + (c_{\text{r-in}} + \eta c_{\text{r-ex}})^{T}K_{\text{eq}}}{1 + c_{\text{f-in}} + c_{\text{r-in}} + \eta(c_{\text{f-ex}} + c_{\text{r-ex}})} \quad (8)$$

The results of several determination of $^{T}(V/K)$ as a function of viscosity are shown in Figure 2. These data were obtained using glycerol as the viscosogen, and experiments conducted using sucrose as the viscosogen yielded similar results. The data in Figure 2 were fitted to eq 8 by nonlinear least squares fitting methods using the MINSQ software package from MicroMath, Inc. This analysis yielded values of 3.7 ± 1.2 for $c_{\text{f-ex}}$ and 0 ± 2 for $c_{\text{r-ex}}$. The internal forward commitment can be calculated to be 2.6 ± 1.6 by subtracting the external forward commitment from the total forward commitment derived from the partitioning and isotope effect experiments. Table I lists the values of the ratios of rate constants that were derived from these analyses.

The TDH·NADH·OAA complex can be formed by two different pathways: direct addition of OAA to the TDH·NADH complex, as in the OAA partitioning experiments, or via oxidation of D-malate by NAD^+ in the enzyme active site, as occurs in the course of the catalytic reaction. These two pathways are related thermodynamically in the manner shown

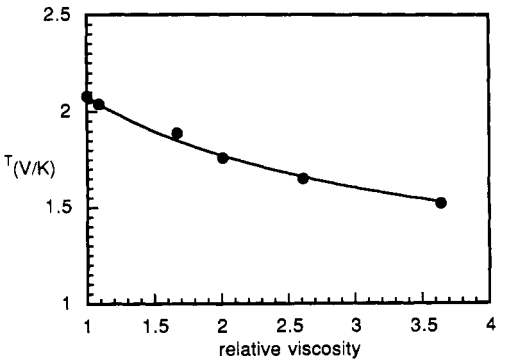


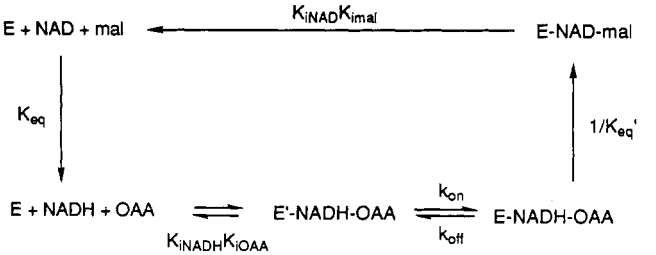
FIGURE 2: Viscosity dependence of the tritium isotope effect on hydride transfer. Reactions were conducted using glycerol as the viscosogen. Data shown are averages of three independent determinations of the isotope effect. The standard errors on these values are not greater than ±0.03. The line shows the fit of the data to eq 8.

Table I: Partition Ratios for TDH-Catalyzed D-Malate Oxidative Decarboxylation[a]

parameter	value
k_3/k_2	1.4 ± 1.9
k_5/k_4	2.6 ± 1.6
k_6/k_7	2.0 ± 0.3
k_8/k_9	0 ± 2

[a] Microscopic rate constants refer to those defined in Scheme I.

Scheme II



in Scheme II, and the product of the equilibrium constants for each side of the box must equal 1 (Cleland, 1990). This model can be used to evaluate the rate of dissociation of OAA from the active site of TDH. In eq 9 k_{on} and k_{off} refer to

$$\frac{K_{\text{eq}}k_{\text{on}}K_{i_{\text{NAD}}}K_{i_{\text{mal}}}}{K_{i_{\text{NADH}}}K_{i_{\text{OAA}}}k_{\text{off}}K_{\text{eq}}'} = 1 \quad (9)$$

first-order rate constants for the interconversion of the initially formed collision complex between the enzyme and OAA, and the Michaelis complex from which reaction occurs. The equilibrium constant for oxidation of malate at pH 8 is 2×10^{-5} (Cook et al., 1980). The turnover number for OAA reaction in the partitioning experiment, 0.5 s^{-1}, was used as an estimate of k_{on}. $K_{i_{\text{NADH}}}$ and $K_{i_{\text{OAA}}}$ were determined from initial velocity kinetics experiments in which NADH and OAA were used as inhibitors of the (+)-tartrate and D-malate reactions and are 0.03 and 0.08 mM, respectively (Tipton & Peisach, 1990). $K_{i_{\text{NAD}}}$ is 0.24 mM, as determined from initial velocity kinetics experiments in which the concentrations of D-malate and NAD^+ were varied. Klinman and Matthews (1985) have noted that for the situation where the isotope effects on V and V/K are the same, the Michaelis constant for a substrate equals its true dissociation constant. Since ^{D}V equals $^{D}(V/K)_{\text{malate}}$, the K_m for D-malate, 0.06 mM (Tipton & Peisach, 1990), is also its true dissociation constant, $K_{i_{\text{mal}}}$. In eq 11 K_{eq}' is the equilibrium constant for the reaction on

the surface of the enzyme and is unknown. However, as discussed below, a value of unity for K_{eq}' is probably not an unreasonable estimate. The above values were used to solve eq 9 for k_{off}; the calculated value of k_{off} was 5×10^{-5} s^{-1}.

DISCUSSION

One of the most intriguing features of tartrate dehydrogenase is the multiplicity of catalytic activities it displays. Although its substrates (+)-tartrate, D-malate, and *meso*-tartrate are strikingly similar, TDH distinguishes between them such that it catalyzes their oxidation, oxidative decarboxylation, and net nonoxidative decarboxylation, respectively. We have previously demonstrated that all three of these substrates bind to the same active site by determining that they are mutually competitive inhibitors with respect to one another (Tipton & Peisach, 1990). As described above, we propose that the different products arise from a common catalytic pathway and that they result from release of the intermediates from the enzyme at different steps along the pathway. Presumably, each intermediate follows the lowest energy pathway available to it at each step in the pathway, whether it is further chemical reaction or dissociation. A quantitative understanding of the catalytic mechanism therefore requires knowledge of the energy barriers surrounding each intermediate. As a step in this direction, we have determined the partition ratios for intermediates along the D-malate reaction pathway.

These data were obtained from kinetics experiments which took advantage of the fact that OAA partitioning and observed deuterium and tritium isotope effects are defined by common groups of rate constants, so that one is able to generate the same number of independent experimental measurements as there are unknown parameters in the equations describing these phenomena. Because of the extreme sensitivity of the equations to errors associated with the determination of $^D(V/K)$, we resorted to an internal competition experiment using a ^3H remote label to measure the isotope effect associated with the transfer of ^2H from C2 to NAD^+. The kinetics analysis was further refined by examining the viscosity dependence of the observed isotope effect on hydride transfer in order to separate the contributions of the internal and external components of the commitment factors. Although this analysis allows calculation of the intrinsic isotope effect on hydride transfer, it is difficult to draw meaningful mechanistic conclusions concerning the transition state in the absence of experiments addressing the possible contributions of quantum mechanical tunneling and coupled motion.

The attenuation of the isotope effect on hydride transfer arises from both the forward and reverse commitment factors. Addition of D-malate to the enzyme does not commit the reaction to catalysis; k_3/k_2 is 1.4, which indicates that dissociation of D-malate is almost as likely as formation of the Michaelis complex. Once the Michaelis complex is formed, however, hydride transfer occurs 2.6 times faster than reversion back to the quaternary collision complex. Thus, it is largely at this stage that the reaction becomes committed to product formation. The partition analysis revealed that the reaction has a finite reverse commitment, but the viscosity dependence of the isotope effects showed that there is no external portion to the reverse commitment. Thus, the reverse commitment is k_6/k_7, and its calculated value indicates that hydride transfer from NADH to OAA is twice as fast as decarboxylation of OAA. It is not surprising that k_8 is negligible, because it represents carboxylation of pyruvate, a step that one would expect to be unfavorable.

TDH and malic enzyme catalyze the same reaction, although they act on different enantiomers of malate, and it is interesting to compare their reaction profiles. Chicken liver malic enzyme is characterized by values of k_7/k_6, k_5/k_4, and k_3/k_2 of 0.1, 2.2, and 0.5, respectively, and the intrinsic isotope effect on hydride transfer is 5.6 (Grissom & Cleland, 1988). These partition ratios are qualitatively similar to those observed for TDH.

It is worthwhile to comment on the viscosity experiments. Measuring the viscosity dependence of kinetics parameters is a useful technique for determining the extent to which diffusion of the substrate to the enzyme limits the rate of the reaction (Brouwer & Kirsch, 1982), and the viscosity dependence of isotope effects can be utilized to reveal the magnitudes of external commitment factors. However, it has been noted that viscosity-variation experiments require proper controls (Blacklow et al., 1988; Sweet & Blanchard, 1990), and interpretation of results is not always straightforward (Grissom & Cleland, 1988; Gates & Northrop, 1988). Therefore, it is important to demonstrate that the viscosogen is benign with regard to the protein structure and stability and that its only effect is on the rate of diffusion of small molecules to and from the enzyme. Two controls are particularly useful. The first is to examine the effect of viscosity on the kinetic parameters of a slow catalytic reaction in which diffusion of the substrate to the enzyme is not likely to be rate-determining. For TDH the turnover number of the (+)-tartrate reaction is 30-fold lower than for the D-malate reaction, and Figure 1 shows that $V/K_{(+)\text{-tartrate}}$ is indeed viscosity-independent. This result argues that increased viscosity does not adversely affect the structure or stability of TDH or any rate-determining conformation changes that may occur. It is also important to examine the effect of polymeric viscosogens on V/K. Such compounds increase the viscosity of the solution, but do not affect the rate of diffusion of small molecules. The value of $V/K_{D\text{-malate}}$ was unaffected by polyethylene glycol at concentrations that increased the viscosity of the solution 3.5-fold. It is also worth noting that sucrose and glycerol had similar effects on $V/K_{D\text{-malate}}$, consistent with the conclusion that the observed effects arose from changes in solution viscosity and not from inhibition of the enzyme by either compound.

The (+)-tartrate and D-malate reaction pathways diverge after hydride transfer to NAD^+ when oxaloglycolate dissociates from the enzyme, but OAA remains bound in the active site. To determine how closely poised between dissociation and further reaction the enzyme–OAA complex is, the rate constant for dissociation of OAA from TDH was calculated. The rate constant for oxaloglycolate dissociation is not known, but the turnover number for the (+)-tartrate reaction, 0.5 s^{-1}, can be taken as a lower limit. Comparison of this number to the calculated rate constant for OAA dissociation of 5×10^{-5} s^{-1} reveals a difference of 4 orders of magnitude. Clearly the calculation of the OAA dissociation rate constant is not an exact one; for example, the true value of K_{eq}' is not known. However, on the basis of experimental measurements of internal equilibrium constants with several enzymes, our estimate of unity for K_{eq}' is probably not unreasonable. Lactate dehydrogenase and yeast and liver alcohol dehydrogenases have been found to have internal equilibrium constants close to 1 (Nambiar et al., 1983). The accuracy of the dissociation constants used in the calculation of the OAA dissociation rate constant can be checked by comparing the thermodynamic Haldane relationship, eq 10, which contains many of the dissociation constants used in eq 9, with the experimentally

$$K_{eq} = \frac{K_{i_{CO2}} K_{i_{pyr}} K_{i_{NADH}}}{K_{i_{NAD}} K_{i_{mal}}} \quad (10)$$

determined equilibrium constant for L-malic enzyme of 13 mM at pH 8 (Barman, 1969). The thermodynamic Haldane relationship yields a calculated value of 2.2 mM for K_{eq}; although a likely source of error in this calculation is the value for the dissociation constant of CO_2, which is ill-defined experimentally and does not appear in eq 9, this comparison suggests that the value of k_{off} calculated from eq 9 should be considered accurate within an order of magnitude. Even if we allow for considerable error in our value of k_{off} for OAA, there is clearly a significant difference between the rates of OAA and oxaloglycolate dissociation, and the enzyme appears to be firmly committed to catalyzing the decarboxylation of OAA rather than letting it escape into solution. Further experimentation and structural information will be required to determine whether the differences in the interactions between the enzyme and the two β-keto acids arise from repulsive interactions between the hydroxyl group on oxaloglycolate and the protein, from differences in conformations of the bound β-keto acids, or from different conformations of the protein itself.

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