Alternative Substrates for Malic Enzyme: Oxidative Decarboxylation of L-Aspartate†

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ABSTRACT: The NAD-malic enzyme from *Ascaris suum* will utilize L-aspartate, (2*S*,3*R*)-tartrate, and *meso*-tartrate as substrates with V/K values $10^{-4}-10^{-5}$ with respect to malate. There is a strict requirement for the 2*S* stereochemistry for all of these reactants. Since aspartate is unique as an amino acid reactant for malic enzyme, it was informative to determine the details of its mechanism of oxidative decarboxylation. The initial rate of NADH appearance is directly proportional to the concentration of aspartate, and saturation is difficult to achieve. The pH dependence of $V/K_{aspartate}E_t$ shows a decrease at low pH, giving a pK of 5.7. The pH-independent value of $V/K_{aspartate}E_t$ is 3 M^{-1} s^{-1}, 12500-fold lower than that obtained with L-malate. The dissociation constant for aspartate as a competitive inhibitor of malate is 60 mM at neutral pH, allowing an estimate of about 0.18 s^{-1} for V/E_t with L-aspartate compared to a value of 39 s^{-1} obtained with L-malate. The deuterium isotope effect on $V/K_{aspartate}$ is pH independent over the range 5.1−6.9 with an average value of 3.3. Data suggest that the monoanion of L-aspartate binds to enzyme and that the same general base, general acid mechanism that is responsible for the oxidative decarboxylation of malate to pyruvate applies to the oxidative decarboxylation of aspartate to iminopyruvate. In addition, the oxidation step appears to be largely rate determining with aspartate as the substrate.

Malic enzyme catalyzes the oxidative decarboxylation of the β-hydroxy acid, L-malate, to pyruvate and CO_2 using NAD(P)[1] as an oxidant. The enzyme's substrate specificity has been considered absolute for L-malate until the recent report by Urbauer et al. (*1*), which documented the ability of the chicken liver malic enzyme to catalyze the NADP-dependent conversion of (2*R*,3*R*)-*erythro*-fluoromalate to 3-fluoropyruvate and CO_2. The enzyme apparently does not utilize the 3*S*-diastereomer. The overall rate of the reaction with fluoromalate is decreased by about 3000-fold compared to the reaction with malate.

The reaction catalyzed by the malic enzyme occurs in three distinct steps, with oxidation of the 2-hydroxyl preceding elimination of the β-carboxylate, which is followed by the tautomerization of enolpyruvate to the final product, pyruvate (*2*). Availability of the alternative substrate for the malic enzyme has allowed a probe of the sequencing of reaction steps within the active site. However, the thermodynamics of the overall reaction with fluoromalate are not as favorable as they are with malate (*1*), limiting the utility of fluoromalate as a probe. As a result, it would be advantageous to have substrates for the malic enzyme reaction that allow one to probe the individual steps along the reaction pathway.

In this paper, we report the identity of three new substrates for the malic enzyme reaction, L-aspartate, (2*S*,3*R*)-tartrate, and *meso*-tartrate. All of the substrates are characterized with respect to the reaction catalyzed and the kinetics of the reaction as compared to those observed with L-malate. In addition, the mechanism of oxidative decarboxylation of L-aspartate to the imino acid is characterized in detail. The substrates have aided in defining the details of the overall mechanism of the malic enzyme reaction.

MATERIALS AND METHODS

Chemicals. Aspartate aminotransferase from porcine heart and L-aspartate were from Sigma.

Preparation of L-*Aspartate-2-d*. Deuterated aspartate was prepared from unlabeled L-aspartate via the aspartate aminotransferase-catalyzed exchange of the α-proton in D_2O (*3*, *4*). L-Aspartate and potassium phosphate were dissolved in D_2O and brought to pD 7.4 with KOD, lyophilized, and redissolved in D_2O to remove trace H_2O, while aspartate aminotransferase (2000 units, 4.5 mg) was dialyzed against 50 mL of 10 mM potassium phosphate, pD 7.4. To a 100 mL solution of L-aspartate (10 g, 75.2 mmol), oxalacetate (52.8 mg, 0.4 mmol), and 10 mM phosphate, pD 7.4, the aspartate aminotransferase and a drop of toluene were added. The progress of deuterium incorporation into C-2 of L-

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[1] Abbreviations: KOD, potassium deuterioxide; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD^+; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Taps, 3-[tris(hydroxymethyl)amino]propanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Caps, 3-(cyclohexylamino)-1-propanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

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aspartate was monitored via the disappearance of the signal at 3.6 ppm via a Bruker 400 MHz ^{1}H NMR. A deuterium incorporation at C-2 of L-aspartate of 98 atom % was attained by ^{1}H NMR. The spectrum for L-aspartate gave the following δ values, C-2 (q, 3.57 ppm) and C-3 (q, 2.3 ppm; q, 2.65 ppm), while L-aspartate-2-D gave the following δ values, C-3 (d, 2.3 ppm; d, 2.65 ppm). The solution was then heated to denature the protein, which was removed by filtration. The solution was then titrated to pH 1.5, followed by addition of ethanol, and L-aspartate (potassium salt) was obtained as a white solid (90%, 9.0 g).

Initial Rate Studies. The malic enzyme-catalyzed oxidative decarboxylation of L-aspartate was monitored by the formation of NADH spectrophotometrically at 340 nm. All kinetic assays were carried out at 25 °C, and the pH of the assay solution was maintained with 100 mM quantities of the following buffers over the range indicated: Mes, 5.4−6.8; Hepes, 6.8−7.8; Taps, 7.8−8.7; Ches, 8.7−9.4; Caps, 9.4−10. Initial rates were measured at varied concentrations of unlabeled or deuterated L-aspartate with 100 mM Mg^{2+}_f [where f reflects correction for chelate complexes with NAD^+ and aspartate (*5*)] and 0.2 mM NAD^+_f. The concentration of L-aspartate was determined by enzymatic end-point assay, monitoring the change in absorbance at 340 nm, using aspartate aminotransferase and malate dehydrogenase in the presence of 6 mM α-ketoglutarate and 0.15 mM NADH.

The concentration of aspartate was corrected for the metal−chelate complex using a value of 4 mM for the dissociation constant for the Mg−aspartate complex, where aspartate is the dianion with its α-amino group unprotonated. Since the p*K* for the aspartate amine is 9.8, the stability constant was corrected for the protonation state of the α-amine using the modified equation:

$$K_{Mg-Asp} = [Mg_f][aspartate_f]/[Mg-Asp][1 + H/K_{Asp}] \quad (1)$$

In eq 1 K_{Mg-Asp} is the dissociation constant for the Mg−aspartate chelate complex (Mg−Asp), Mg_f is the uncomplexed form of Mg^{2+} (*5*), Asp_f is the uncomplexed form of the aspartate dianion, *H* is the hydrogen ion concentration, and K_{Asp} is the acid dissociation constant for the α-amino group of L-aspartate.

Data Processing. Initial rates were obtained at fixed and saturating concentrations of NAD and Mg^{2+} and varied concentrations of L-aspartate, *meso*-tartrate, or D-tartrate. All reciprocal plots were linear. In the case of L-aspartate, reciprocal plots passed through the origin, indicating a strictly second-order process. All data were fitted using BASIC versions of programs written by Cleland (*6*). Data for tartrate saturation curves were fitted using eq 2. Data for aspartate saturation curves were fitted using the equation for a straight line. Data for inhibition patterns by aspartate vs malate were fitted using eq 3, while data for primary deuterium isotope effects were fitted using eq 4. Data for the pH dependence of $V/K_{aspartate}$ were fitted using eq 5.

$$v = VA/(K_a + A) \quad (2)$$

$$v = VA/[K_a(1 + I/K_{is}) + A] \quad (3)$$

$$v = VA/K_a(1 + F_iE_{V/K}) \quad (4)$$

$$\log V/K_{aspartate} = \log[C/(1 + H/K_1)] \quad (5)$$

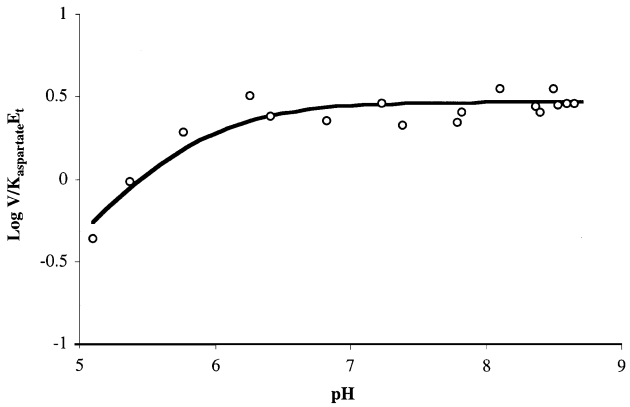


FIGURE 1: pH−rate profile for $V/K_{aspartate}$. The curve was fitted using eq 5. Points are experimental, while the curve is theoretical based on the fit.

In eqs 2−4, *v* is the initial rate, *V* is the maximum rate, K_a is the K_m for *A*, F_i is the fraction of deuterium in the labeled aspartate, and $E_{V/K}$ is the isotope effect minus 1 on *V*/*K*. In eq 5, $V/K_{aspartate}$ is the observed value of *V*/*K* at any pH, *C* is the pH independent value of *V*/*K*, *H* is the hydrogen ion concentration, and K_1 is the acid dissociation constant for the enzyme or reactant functional group.

RESULTS

Addition of NAD-malic enzyme to a solution containing Mg^{2+}, NAD^+, and L-aspartate results in an increase in absorbance at 340 nm. The initial rate of increase is directly proportional to the malic enzyme added. Addition of lactate dehydrogenase to an assay in progress results in an immediate bleaching in A_{340} as a result of conversion of NADH and pyruvate to NAD^+ and lactate. The rate is directly proportional to the concentration of aspartate up to 200 mM at any pH. The failure to saturate with aspartate allows one to obtain estimates of only $V/K_{L-aspartate}E_t$, which is 0.4 M^{-1} s^{-1} at pH 7.3.

D-Aspartate was also tested as a potential substrate for the malic enzyme. No increase in absorbance was observed with 6.4 μg of malic enzyme at 200 mM D-aspartate, which gives a rate of 0.5 μM/min with L-aspartate. If D-aspartate is a substrate, it is utilized at a rate $<10^5$-fold compared to L-malate.

pH Dependence of $K_{i\ L-aspartate}$. Since L-aspartate is such a poor substrate, it can be used as a competitive inhibitor vs L-malate. Competitive inhibition by L-aspartate was observed on the basis of initial velocity patterns at pH 7 and 8.4, giving K_i values of 80 ± 20 mM and 58 ± 16 mM, respectively. Attempts to obtain inhibition constants at pH values lower than 6 resulted in only slight inhibition at the highest aspartate concentration used, consistent with an increase in the K_i for aspartate.

pH Dependence of $V/K_{L-aspartate}$. $V/K_{L-aspartate}E_t$ was measured as a function of pH. The pH−rate profile decreases at low pH with a limiting slope of +1, giving a p*K* value of 5.7 ± 0.2 (Figure 1). Data could not be collected above pH 8.8 as a result of the high concentrations of L-aspartate required once correction for the Mg−aspartate chelate complex was effected. The pH-independent value of $V/K_{L-aspartate}E_t$ is 3.0 ± 0.3 M^{-1} s^{-1}. Data can be compared to the p*K* value of 4.7 obtained with L-malate and the pH-

Table 1: Deuterium Kinetic Isotope Effects[a]

pH	$^{D}(V/K_{aspartate})$
6.9	2.9 ± 0.2
	3.2 ± 0.2
	3.4 ± 0.4
5.8	2.7 ± 0.1
	2.6 ± 0.2
5.1	3.4 ± 0.3
	4.2 ± 1.2
	3.8 ± 0.9
	av: 3.3 ± 0.4

[a] Multiple determinations were carried out at each pH in order to decrease the overall error of the value for $^{D}(V/K)$.

Table 2: Kinetic Parameters Measured with *meso*- and D-Tartrates for Malic Enzyme

	V/E_t (s^{-1})	V/KE_t ($M^{-1}\,s^{-1}$)	K_m (mM)
(2*S*)-malate	39	3.8×10^4	1.0
(2*R*,3*R*)-*erythro*-fluoromalate	0.013 (2) (3000)	6.3 (0.8) (6000)	2.0 (0.5) (2)
(2*S*,3*R*)-tartrate	0.0083 (2) (4800)[a]	0.40 (3) ($\sim 10^5$)	20 (2) (20)
meso-tartrate	0.0062 (2) (6500)	0.16 (1) ($\sim 2 \times 10^5$)	40 (4) (40)
(2*S*)-aspartate	0.09[b] (430)	1.5 (0.1) (25000)	60[b] (60)

[a] Values in parentheses on the same line as the value represent the SE in the last digit of the parameter, while those under the value represent the fold decrease in *V* and *V*/*K*, and fold increase in K_m, compared to data obtained with (2*S*)-malate. [b] $K_{i\,L-aspartate}$ is assumed equal to $K_{L-aspartate}$ on the basis of the low overall rate of the reaction. Using the value of 60 mM for $K_{L-aspartate}$, a value of 0.09 s^{-1} is estimated for V/E_t.

independent value of 4×10^4 M^{-1} s^{-1} for $V/K_{L-malate}E_t$ (*2*). On the basis of the pH-independent values, aspartate is used at a rate 12500 times slower than that obtained with L-malate. Using the value of 60 mM for $K_{aspartate}$ (assuming $K_{i\,aspartate} = K_{aspartate}$, reasonable given the low rate of the reaction), a value of 0.18 s^{-1} is estimated for V/E_t.

Primary Kinetic Deuterium Isotope Effects. The primary kinetic deuterium isotope effect on $V/K_{L-aspartate}$ was obtained using the direct comparison of initial velocities measured with L-aspartate and L-aspartate-2-*d*. The isotope effect is pH independent with values given in Table 1.

Tartrates as Alternative Substrates. D-Tartrate, L-tartrate, and *meso*-tartrate were also tested as substrates for the NAD-malic enzyme. Kinetic parameters were measured in the presence of 100 mM $Mg^{2+}{}_f$ and 0.2 mM $NAD^{+}{}_f$ at pH 7.8 (Table 2). Note that the rate obtained with D-tartrate is slightly greater than that obtained with *meso*-tartrate. No measurable rate was observed with L-tartrate.

DISCUSSION

Dicarboxylic Acid Substrate Specificity. The NAD-malic enzyme tolerates substitution at the 3-position of malate but will allow only small substituents such as hydroxyl or fluorine (Figure 2) and has an absolute requirement for the correct stereochemistry at C-2. The exception is L-aspartate, which will be considered separately below. Substitution by hydroxyl does, however, give a decrease in binding energy. The K_d's (Table 2) for the tartrates are increased by 20−40-fold compared to that obtained for malate, reflecting a loss of about 2 kcal/mol in binding energy. The exception

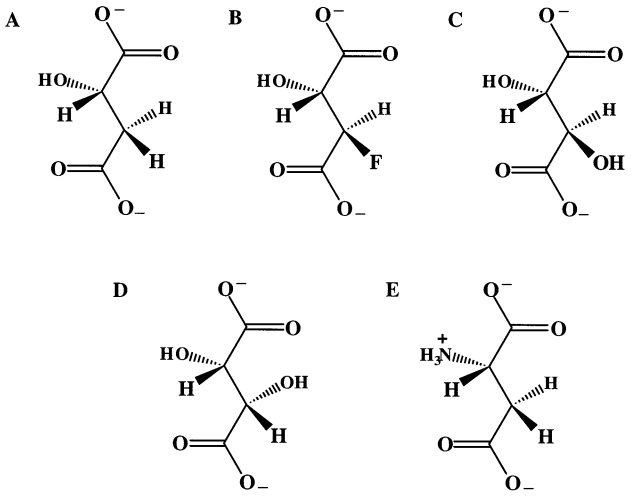


FIGURE 2: Structures of L-malate and substrate analogues. The compounds are (A) (2*S*)-malate, (B) (2*R*,3*R*)-*erythro*-fluoromalate, (C) (2*S*,3*R*)-tartrate, (D) *meso*-tartrate, and (E) (2*S*)-aspartate.

is substitution with fluorine (about the same size as a proton) which gives little, if any, change in K_d.

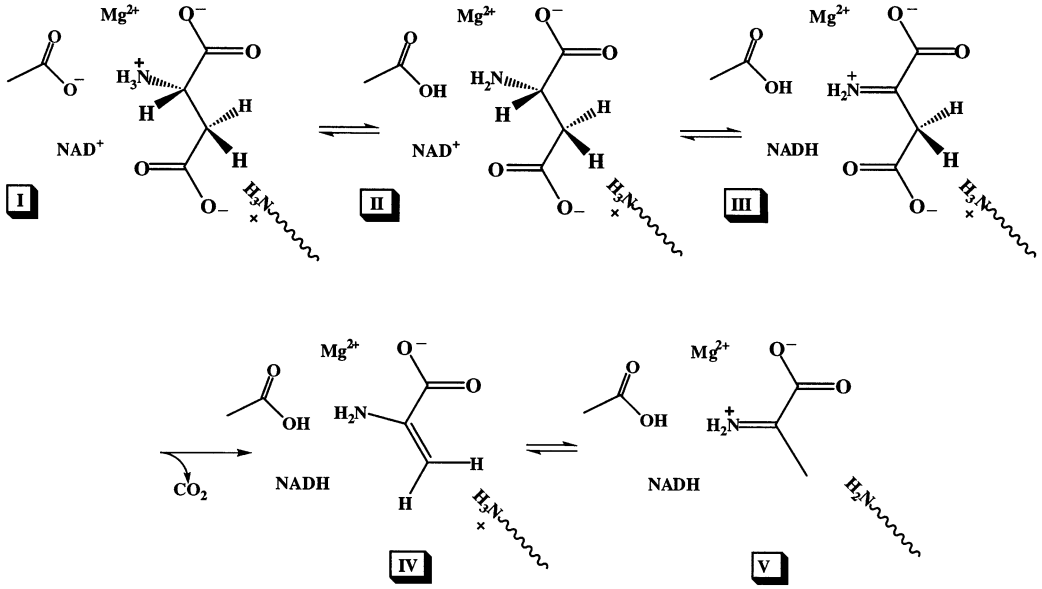
Interpretation of the $V/K_{aspartate}$ pH−Rate Profile. The pH−rate profile for $V/K_{aspartate}$ decreases at low pH, giving a p*K* of about 6. The pH−rate profile for V/K_{malate} decreases at low and high pH, giving p*K* values of about 5−5.1 and 9 (*2*).[2] The two ionizations have been assigned to a general base that accepts a proton from the 2-hydroxyl of L-malate and shuttles it between itself and the 2-oxo group in all steps of the reaction and a general acid that protonates C-3 as enolpyruvate tautomerizes to pyruvate (*2*). The group with a p*K* of 5.7 is likely the same general base observed with L-malate as a substrate. In both cases the p*K* reflects the ionization of a group in the E·NAD·Mg complex, and the difference must therefore result from error in measurement. The p*K* of 9 observed in the V/K_{malate} profile was not observed in the $V/K_{aspartate}$ profile because data cannot be collected above pH 8.8.

Thus, the mechanism of oxidative decarboxylation of L-aspartate likely mimics that of L-malate (Scheme 1). Aspartate binds as the monoanion, and its protonated α-amine is stabilized via an ionic interaction with the general base, I. The general base is thought to be D295 based on site-directed mutagenesis studies (*7*). The general base then likely accepts a proton from the α-amine prior to hydride transfer, II, to decrease electron withdrawing by the α-amine and facilitate hydride transfer. Hydride transfer then gives protonated α-iminoaspartate, III, which would facilitate decarboxylation to give α-eneaminopropionate, IV. Protonation of C-3 of the enamine by the general acid (K199; *8*) would give iminopyruvate, V, which dissociates and decomposes nonenzymatically to give pyruvate and ammonia. Alternatively, the enamine could dissociate since we cannot document a requirement for the general acid over the pH range accessible to us.

Deuterium Isotope Effects. The pH dependence of the primary deuterium isotope effect on $V/K_{aspartate}$ provides further information on the mechanism of oxidative decarboxylation of aspartate. The large primary deuterium isotope

[2] Ionization of the *β*-carboxylate of L-malate is also observed in the V/K_{malate} pH−rate profile (*2*).

Scheme 1: Proposed Mechanism for Oxidative Decarboxylation of l-Aspartate



effect on $V/K_{aspartate}$ suggests the hydride transfer step limits the overall reaction. However, one cannot rule out some contribution by decarboxylation or other steps to rate limitation. This can be compared to the rather small effect of 1.5 observed with L-malate (*2*). The overall reaction is limited by decarboxylation with L-malate, corroborated by the large primary ^{13}C kinetic isotope effect of 3.5% (*9*, *10*). (Because of the very low value of V/K with aspartate, ^{13}C effects could not be carried out in this case.)

Large primary deuterium kinetic isotope effects of about 3 have also been observed with the alternative dinucleotide substrates 3-acetylpyridine adenine dinucleotide and 3-pyridinealdehyde adenine dinucleotide and L-malate, and hydride transfer was shown to be the rate-limiting step (*9*). However, in this case the oxidative decarboxylation of malate occurred in a single step, due to the more oxidizing nature of the dinucleotide (increase in the equilibrium constant for hydride transfer) and a shallow well for the oxaloacetate intermediate (*10*, *11*). In addition, an enzyme modified at a thiol sensitive to oxidation to SCN gave large primary deuterium kinetic isotope effects (*12*). The thiol is now known to be outside the active site, but its modification increases the K_m for malate 30-fold. As a result, isotope effects of about 3 are measured as a result of eliminating the forward commitment factor and unmasking the hydride transfer step, as well as increasing the overall rate limitation of the hydride transfer step compared to decarboxylation likely due to change in the geometry of bound malate. The latter effect is very similar to what is observed with aspartate, with a dissociation constant 60-fold greater than that of malate and an increase in the overall rate limitation of the hydride transfer step.

Aspartate as an alternative substrate can thus be used to study the hydride transfer step in the malic reaction. In addition, aspartate as a substrate for the malic enzyme reaction will arguably serve as a model for hydride transfer in $NAD(P)^+$-dependent oxidative deamination reactions such as those catalyzed by glutamate dehydrogenase.

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