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Multiple isotope effects as a probe of the tartrate dehydrogenase-catalyzed oxidative decarboxylation of D-malate

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Abstract A change in the dinucleotide reactant from NAD^+ to the more oxidizing $APAD^+$ in the malic enzyme reaction results in a change in the mechanism of oxidative decarboxylation of malate from stepwise to concerted [7]. In order to determine whether this is a phenomenon general to metal ion dependent β-hydroxyacid oxidative decarboxylases, tartrate dehydrogenase, which catalyzes a reaction diastereotopic to malic enzyme, was studied using the technique of multiple isotope effects. A primary deuterium isotope effect of 1.41 on V/K_{malate} was measured, as well as a primary ^{13}C-isotope effect of 1.0096. A decrease in the measured value of the ^{13}C-isotope effect to 1.0078 is consistent with a stepwise mechanism, as observed for malic enzyme. The ^{13}C-isotope effect with thioNAD^+ also decreases from a value of 1.0053 using D-malate to 1.0009 using D-malate-2-D, consistent with stepwise oxidative decarboxylation with this alternative dinucleotide substrate. The data suggest that the change from a stepwise to a concerted mechanism with malic enzyme may be a unique phenomenon.

Key words isotope effects • malic enzyme • oxidative decarboxylation • tartrate dehydrogenase

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Pyridine nucleotide linked β-hydroxyacid oxidative decarboxylases are known to catalyze their reaction in three steps [2, 8, 10, 11]. The proposed reaction suggests that a β-hydroxyacid (L-malate for the malic enzyme reaction and D-malate for the tartrate dehydrogenase reaction) is first oxidized to a β-ketoacid, which is decarboxylated to give an enol or enediol intermediate that is tautomerized to the final ketone product. A depiction of the tartrate dehydrogenase-catalyzed oxidative decarboxylation of D-malate is shown in Scheme 1. Multiple primary deuterium-primary ^{13}C kinetic isotope effects have been instrumental in establishing the proposed mechanism [5–7, 15]. If one observes a finite primary deuterium isotope effect on the hydride transfer step and a finite primary ^{13}C isotope effect on the decarboxylation step, data are consistent with both steps contributing to rate limitation of the overall reaction. The ^{13}C effect is then repeated with the position for hydride transfer deuterated. A decrease in the observed ^{13}C isotope effect with deuterated *vs.* protium-substituted substrate signals a stepwise oxidative decarboxylation reaction, while an increase or no change in the ^{13}C isotope effect indicates a concerted reaction (no change would indicate the oxidative decarboxylation step is solely rate-determining).

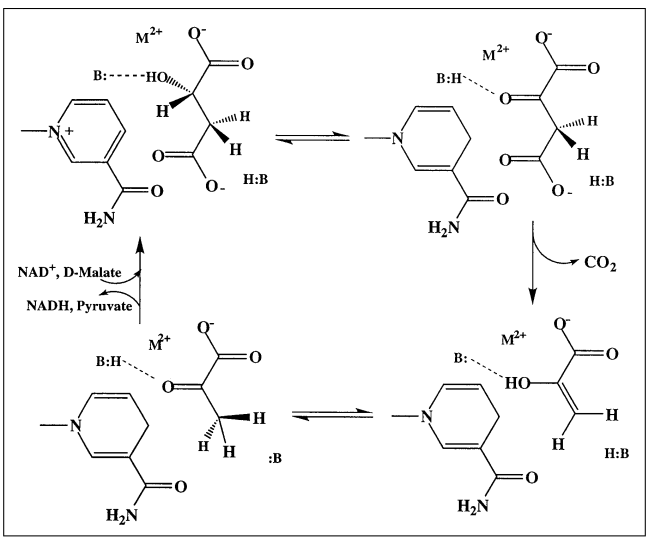
Multiple isotope effects have been used to probe the metal ion dependent malic enzyme reaction with a number of dinucleotide reactants that vary in their redox potential [7, 15]. With the natural substrate, NAD^+, a stepwise oxidative decarboxylation is indicated, while using the more oxidizing 3-acetylpyridine adenine dinucleotide ($APAD^+$) the mechanism changes to asynchronous concerted with C-C bond cleavage lagging behind C-H bond cleavage [7]. The

change in mechanism was further corroborated by Edens *et al.* [3], who measured the ^{13}C-isotope effect at C-2 and C-3 with protium and deuterium substituted malates. The change in mechanism was attributed to an increase in rate-limitation of the hydride transfer step, such that the barrier for this step is higher than that of the decarboxylation step, which effectively eliminates the intermediate well for the oxalacetate intermediate. This coupled to an increase in the equilibrium constant for hydride transfer results in the asynchronous concerted reaction.

When similar experiments are carried out with the metal ion-independent 6-phosphogluconate dehydrogenase reaction, no such change in reaction mechanism was observed [6]. It is important to determine whether the change in mechanism observed for malic enzyme is specific for this enzyme, or whether it is a general phenomenon for metal ion dependent β-hydroxyacid oxidative decarboxylases. Does the presence of the metal ion contribute to a decrease in the stability of the β-ketoacid intermediate? Toward this end, we have extended studies to include the tartrate dehydrogenase-catalyzed oxidative decarboxylation of D-malate, a reaction diastereotopic to the malic enzyme reaction [14].

Methods

- *Chemicals and enzymes.* The D-malate-2-D was prepared by $NaBD_4$ reduction of oxalacetate to give racemic D,L-malate-2-D, which was then resolved kinetically using L-malic enzyme, and the D-malate-2-D purified by ion exchange chromatography. All other chemicals were obtained from commercial sources, were the highest quality available, and were used without further treatment.
- *Purification of TDH.* The BL21 *E. coli* strain containing the plasmid is grown in 2 liters of LB media at 32°C and enzyme expression is induced by the addition of IPTG to the cell culture. After harvesting the cells by centifugation, the cells are resuspended in triethanolamine buffer, sonicated, and centrifuged again to recover the crude extract. The enzyme is about 85–90% pure after the final blue A-agarose step [12].



Scheme 1. Proposed chemical mechanism for oxidative decarboxylation of D-malate by tartrate dehydrogenase.

- *Isotope effects.* All isotope effects were carried out as described previously [7].
- *Oxalacetate partitioning.* Partitioning of the oxalacetate intermediate was carried out as described previously [13], with the exception that the experiments were carried out as a function of pH. Buffers used and their respective pH range were as discussed by Tipton [13].

Results and discussion

NAD^+ as the dinucleotide substrate

D-Malate is oxidatively decarboxylated to pyruvate and CO_2 using NAD^+ as the oxidant at pH 7.5 in the presence of 1 mM Mn^{2+} and 30 mM KCl [14]. Kinetic parameters obtained under these conditions are V/E_t, 13 s^{-1}, $V/K_{malate}/E_t$, 2.6×10^5 $M^{-1}s^{-1}$, with K_{malate} equal to 50 μM.

Primary deuterium isotope effects on V and V/K_{malate} were measured with D-malate-2-(H,D) via the direct comparison of initial rates. Carbon-13 isotope effects were obtained using the natural abundance of ^{13}C in D-malate as reported previously [7]. A deuterium isotope effect of 1.41±0.05 was obtained for $^{D}(V/K_{malate})$. The primary deuterium kinetic isotope effect on $V/K_{D\text{-}malate}$ suggests the hydride transfer step is only partially rate-limiting, since the magnitude of intrinsic deuterium isotope effects is 6–8 on the hydride transfer step. The primary ^{13}C kinetic isotope effect on $V/K_{D\text{-}malate}$ with protium-labeled D-malate is 1.0096±0.0004, further suggesting the decarboxylation step is only partially rate-limiting. The measured value of $^{13}(V/K_{D\text{-}malate})_D$ is 1.0078±0.0001. Data satisfy the equality for a mechanism in which oxidation precedes decarboxylation [5], suggesting a mechanism qualitatively identical to the malic enzyme reaction with NAD as the dinucleotide substrate [7]

$$[^{13}(V/K_{D\text{-}malate})_H - 1]/[^{13}(V/K_{D\text{-}malate})_D - 1] = {}^{D}(V/K_{D\text{-}malate})/{}^{D}K_{eq}$$

$$1.23\pm0.01 = 1.20\pm0.04$$

A quantitative analysis was carried out according to Karsten and Cook [7] for malic enzyme using the above data, a partition ratio (r_d = 2.1) of the oxalacetate intermediate using NADD as the reduced dinucleotide [4] and the previously determined $^{T}(V/K_{malate})$ of 2.08±0.06 data [13]. Solution of the above simultaneous equations give the following estimated values; $^{D}k_{hydride}$ = 9.2; $^{13}k_{decarboxylation}$ = 1.025; c_f = 14; and c_r = 9.2. These parameters can be compared with those obtained for the malic enzyme: $^{D}k_{hydride}$ = 10.8; $^{13}k_{decarboxylation}$ = 1.052; c_f = 6.6; and c_r = 14 [7]. Data suggest very similar overall reaction pathways, but a somewhat earlier transition state for decarboxylation of the oxalacetate intermediate ($^{13}k_{decarboxylation} = 1.052 > {}^{13}k_{decarboxylation} = 1.025$).

The values of the intrinsic deuterium and ^{13}C isotope effects provide information on the transition state structures for hydride transfer and decarboxylation. The value of 9.3 for ^{D}k is greater than the range of values expected for strictly semiclassical behavior, 6–8 [9]. Data suggest the presence of hydrogen tunneling in the transition-state for the hydride transfer step. The large observed ^{13}C isotope effect for the malic enzyme reaction suggests a late transi-

tion state for decarboxylation, while the much smaller value observed for tartrate dehydrogenase is suggestive of an earlier transition state for C-C bond cleavage. Data may be indicative of the reaction tending toward a concerted oxidative decarboxylation.

ThioNAD as the dinucleotide substrate

Tartrate dehydrogenase is known to utilize thioNAD$^+$ (NAD$^+$ in which the carbonyl oxygen of the carboxamide side chain of the nicotinamide ring is replaced with sulfur) as a substrate with an efficiency nearly identical to NAD$^+$ [1]. The V/E_t for thioNAD$^+$ is 9.3 s^{-1}, $V/K_{malate}/E_t$, 1.9×10^4 $M^{-1}s^{-1}$, $V/K_{NAD}/E_t$, 9.3×10^4 $M^{-1}s^{-1}$, K_{malate} 0.5 mM, and $K_{thioNAD}$ 0.1 mM. The value of the ^{13}C primary kinetic isotope effect with D-malate is 1.0053, while the value decreases to 1.0009 with D-malate-2-D. The ^{13}C isotope effect data suggest there is no change in the mechanism of the tartrate dehydrogenase-catalyzed oxidative decarboxylation of D-malate to a concerted reaction with the more oxidizing thioNAD$^+$. These results are in contrast to those obtained with malic enzyme and suggest the change in mechanism discussed above for the malic enzyme are a unique property of this enzyme.

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