# Isotope Effect Studies of the Chemical Mechanism of Nicotinamide Adenine Dinucleotide Malic Enzyme from *Crassula*†

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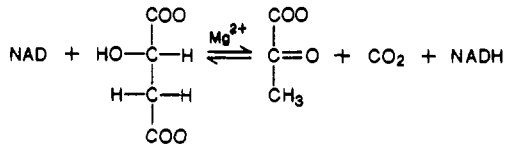
ABSTRACT: The $^{13}C$ primary kinetic isotope effect on the decarboxylation of malate by nicotinamide adenine dinucleotide malic enzyme from *Crassula argentea* is 1.0199 ± 0.0006 with proteo L-malate-*2-H* and 1.0162 ± 0.0003 with malate-*2-d*. The primary deuterium isotope effect is 1.45 ± 0.10 on $V/K$ and 1.93 ± 0.13 on $V_{max}$. This indicates a stepwise conversion of malate to pyruvate and $CO_2$ with hydride transfer preceding decarboxylation, thereby suggesting a discrete oxaloacetate intermediate. This is in agreement with the stepwise nature of the chemical mechanism of other malic enzymes despite the *Crassula* enzyme's inability to reduce or decarboxylate oxaloacetate. Differences in morphology and allosteric regulation between enzymes suggest specialization of the *Crassula* malic enzyme for the physiology of crassulacean acid metabolism while maintaining the catalytic events found in malic enzymes from animal sources.

The nicotinamide adenine dinucleotide (NAD)[1] malic enzyme [L-malate:NAD oxidoreductase (decarboxylating), EC 1.1.1.39] catalyzes the oxidative decarboxylation of L-malate to pyruvate and $CO_2$ with concomitant reduction of NAD to NADH:




$Mg^{2+}$ can be replaced by a variety of divalent metal cations, and NADP can replace NAD, but with a lower affinity (Grover et al., 1981). The kinetic mechanism is sequential, with equilibrium random binding of substrates (Wedding & Black, 1983).

The enzyme from plants does not decarboxylate or reduce oxaloacetate (OAA). This distinguishes it from other malic enzymes [EC 1.1.1.38 and 1.1.1.40; see Viega Salles and Ochoa (1950) and Park et al. (1986), respectively] which will reduce OAA to malate in the presence of reduced pyridine nucleotide or decarboxylate it to pyruvate in the presence of either oxidized or reduced nucleotide. This suggested the possibility of a fundamental difference in the chemical mechanism of hydride transfer and decarboxylation. The chicken liver NADP malic enzyme (Hermes et al., 1982) and the *Ascaris suum* NAD malic enzyme (Dr. P. F. Cook, personal communication) proceed through a stepwise mechanism with hydride transfer occurring first to generate an enzyme-bound OAA–metal–reduced nucleotide complex which undergoes decarboxylation to yield pyruvate, $CO_2$, metal, and reduced nucleotide as indicated in Figure 1. The intrinsic $^{13}C$ isotope effect ($^{13}k$) for decarboxylation by chicken liver NADP malic enzyme is 1.0493 with $Mg^{2+}$ as cofactor (Grissom & Cleland, 1985; Grissom, 1985). This is close to the $Mg^{2+}$-catalyzed chemical decarboxylation of OAA in solution at 25 °C (Grissom & Cleland, 1986) of 1.0489, which suggests a similarity of transition states for the chemical and enzyme-catalyzed processes. This study was undertaken to determine whether the *Crassula* NAD malic enzyme also produces OAA as a discrete reaction intermediate (despite its inability to decarboxylate exogenous OAA) or whether decarboxylation and hydride transfer are concerted in a single molecular event.

## Materials and Methods

*Enzyme Purification.* The NAD malic enzyme was purified to homogeneity from *Crassula argentea*. The procedure of Wedding and Black (1983) was used with the addition of two affinity columns, Reactive Red 120 and agarose–hexane–CoA (Willeford, 1986). The enzyme had a final specific activity of 88.2 IU/mg as assayed in the direction of malate decarboxylation.

*Chemicals.* L-Malate-*2-d* was provided by Dr. Paul F. Cook. It was purified by anion-exchange chromatography on an AG-1–HCOOH column with elution by HCOOH. L-Malate-*2-H* was from Matheson Coleman and Bell. NAD, 3-Acetylpyridine–NADP, chicken liver NADP malic enzyme, glutathione reductase, and glutamate–pyruvate transaminase were obtained from Sigma Chemical Co. $Cl^-$ is a potent inhibitor of NAD malic enzyme (Canellas et al., 1983) and must be avoided. To fulfill the divalent metal ion requirement, $Mg^{2+}$ was delivered as the Mg–TES complex according to Canellas et al. (1983). The plant NAD malic enzyme uses only free malate(2–) and $Mg^{2+}$ as substrates rather than the Mg–malate complex (Canellas & Wedding, 1980). Hence, the amount of complex was corrected for with the following dissociation constants: Mg–malate, $K_d$ = 28.2 mM (Sillen & Martell, 1964); Mg–NAD, $K_d$ = 20.6 mM (Apps, 1973). The concentration of the malate solutions was determined by

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[1] Abbreviations: OAA, oxaloacetate; TES, *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid; pyr, pyruvate; NAD, nicotinamide adenine dinucleotide; NADH, reduced form of nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; CoA, coenzyme A.

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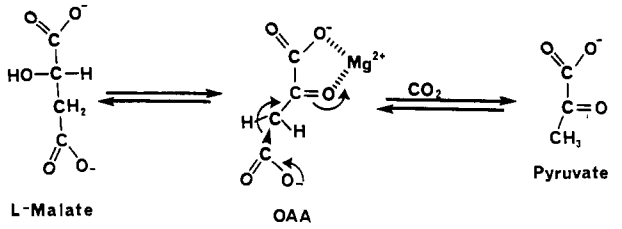


FIGURE 1: Stepwise mechanism for malic enzyme. Hydride transfer to NAD generates a discrete OAA intermediate which is subsequently decarboxylated to pyruvate and $CO_2$.

end-point analysis with chicken liver NADP malic enzyme and 3-acetylpyridine–NADP. The more positive redox potential of 3-acetylpyridine affords complete conversion of the malate to pyruvate and $CO_2$ with an observable spectral change at 363 nm ($\epsilon$ = 9100 $M^{-1}$ $cm^{-1}$).

*$^{13}C$ Isotope Effects.* $^{13}C$ isotope effects on NAD malic enzyme were determined by the general method of O'Leary (1980) by using L-malate with 1.1% natural-abundance $^{13}C$. The isotopic composition of the starting malate was determined by completely decarboxylating an aliquot of malate with chicken liver NADP malic enzyme. To ensure complete conversion of the starting malate, the pyruvate produced was converted to alanine with glutamate–pyruvate transaminase in the presence of excess L-glutamate. The reduced nucleotide produced was recycled with glutathione reductase in the presence of oxidized glutathione. The low-conversion reactions contained saturating NAD at a much higher level and were allowed to proceed to less than 5% reaction so that recycling of the nucleotide or coupling of the pyruvate produced was unnecessary. The concentration of reagents in a 20-mL reaction volume was as follows: complete conversion, 100 mM TES, pH 7.2, 1.5 mM L-malate (proteo or deuterio), 0.4 mM NADP, 40 mM oxidized glutathione, 100 mM L-glutamate, and 5 mM $MgCl_2$; incomplete conversion, 100 mM TES, pH 7.2, 15.4 mM L-malate (proteo or deuterio), 18.65 mM NAD, and 11.0 mM Mg–TES. One hundred units of the transaminase, 120 units of glutathione reductase, and 30 units of chicken liver NADP malic enzyme (complete conversion only) were typically added to each reaction vessel. Enough NAD malic enzyme was added to obtain the desired fraction of reaction in 1 h. The reaction vessels were maintained at 25 °C during turnover. For each assay, the amount of $CO_2$ produced (measured manometrically following isolation) corresponded to the extent of reaction indicated spectrophotometrically (determined by withdrawing aliquots of the reaction solution prior to acid quenching and determining the amount of NADH produced). The $^{12}C/^{13}C$ ratio of the $CO_2$ was analyzed on a Finnigan delta-E isotope ratio mass spectrometer equipped with a dual inlet system.

*Initial Velocity Studies.* All kinetic studies were performed by monitoring changes in NADH concentration at 340 nm with a Beckman Model 25 spectrophotometer. The cuvette compartment was maintained at 25 °C with thermospacers. Routine assay conditions were 50 mM TES, pH 7.2, 15 mM free NAD (18.6 mM total NAD), and 5 mM free $Mg^{2+}$.

*Isotope Effect Nomenclature.* 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 $^{13}C$, respectively) is used. 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.

*Data Analysis.* The deuterium isotope effect on $V$ and $V/K$ was determined by fitting the data to eq 1 by the method of

$$\text{rate} = V^*A/[K(1 + I^*VKI) + A(1 + I + VI)] \quad (1)$$

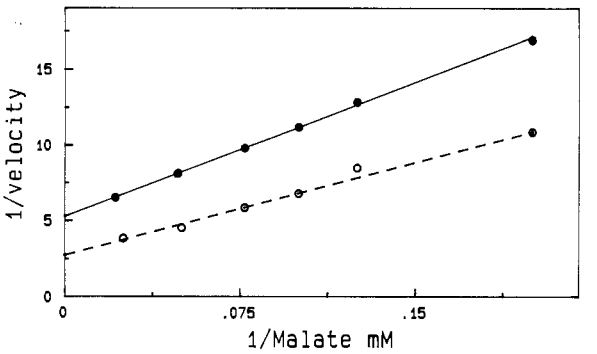


FIGURE 2: Deuterium isotope effect at 50 mM Tes, pH 7.2, 5 mM free $Mg^{2+}$, 15 mM free NAD, and 25 °C. Open circles are unlabeled L-malate, and closed circles are L-malate-*2-d*.

Table I: $^{13}C$ Isotope Effects for NAD Malic Enzyme[a]

| substrate | fraction reaction (%) | partial conversion[b] | $^{13}(V/K)$ |
|---|---|---|---|
| L-malate-*2-H*[c] | 4.2 | 10697.89 | 1.01974 |
| | 4.0 | 10689.40 | 1.02055 |
| | 4.7 | 10701.39 | 1.01945 |
| | | | av 1.0199 ± 0.0006[e] |
| L-malate-*2-d*[d] | 2.2 | 10924.44 | 1.01643 |
| | 2.2 | 10929.12 | 1.01599 |
| | | | av 1.0162 ± 0.0003[f] |

[a] Reaction conditions were pH 7.2 and 25 °C in the presence of 15 mM free NAD and 5 mM free $Mg^{2+}$. [b] Isotopic ratios of $^{12}C/^{13}C$ have been multiplied by $10^6$ and corrected for $^{17}O$ content according to the geochemical ratio of $^{17}O/^{18}O$. [c] The $^{12}C/^{13}C$ ratio of the complete conversion of the unlabeled malate sample was determined to be 10904.6 ± 1.6 (three determinations). [d] The $^{12}C/^{13}C$ ratio of the complete conversion of the deuteriated malate sample was determined to be 11101.9 ± 0.7 (three determinations). [e] For $^{13}(V/K)_H$. [f] For $^{13}(V/K)_D$.

Cleland (1979). $^{13}C$ isotope effects determined by the internal competition method were calculated by use of eq 2. In the

$$^{13}(V/K) = \log (1 - f)/\log [1 - f(R_f/R_0)] \quad (2)$$

above expression, $R_f$ is the $^{13}C/^{12}C$ ratio at fractional reaction $f$ and $R_0$ is the $^{13}C/^{12}C$ ratio of the starting material.

## Results and Discussion

*Deuterium Isotope Effect.* The primary deuterium isotope effect for *Crassula* NAD malic enzyme was determined by comparing reaction rates with unlabeled and 2-deuteriomalate (Figure 2). $^{D}V$ and $^{D}(V/K)$ are 1.93 ± 0.13 and 1.45 ± 0.12.

*Carbon Isotope Effect.* $^{13}(V/K)$ values for NAD malic enzyme with unlabeled and L-malate-*2-d* are shown in Table I. NAD and $Mg^{2+}$ are at least 15-fold greater than their respective $K_m$'s. Hence, the species combining with malate is enzyme–NAD–$Mg^{2+}$. An average value of 1.0199 ± 0.0006 is seen for $^{13}(V/K)_H$ and decreases to 1.0162 ± 0.0003 for $^{13}(V/K)_D$ with L-malate-*2-d*. The diminution of the $^{13}C$ isotope effect on $V/K$ with deuteriated malate is indicative of a stepwise reaction mechanism in which decarboxylation and hydride transfer are separate events (Hermes et al., 1982).

Slower hydride transfer with deuteriated malate increases the commitment for decarboxylation (the $^{13}C$-sensitive step). This is consistent with the stepwise nature of the chicken liver NADP malic enzyme (Hermes et al., 1982) and the *Ascaris suum* NAD malic enzyme (Dr. P. F. Cook, personal communication). Since the deuterium isotope effect on $V/K$ is known, this allows the determination of the order of the deuterium and $^{13}C$-sensitive steps. If hydride transfer precedes decarboxylation, then eq 3 will be true. By substituting in our

results, we obtain expression 4 (assuming a value of 1.17 ± 0.01 for $^{D}K_{eq}$ of malate (Cook et al., 1980).

$$\frac{^{13}(V/K)_h - 1}{^{13}(V/K)_d - 1} = \frac{^{D}(V/K)}{^{D}K_{eq}} \quad (3)$$

$$1.23 \pm 0.04 = 1.24 \pm 0.10 \quad (4)$$

If decarboxylation precedes hydride transfer, then eq 5 holds. With our values, we obtain relationship 6 [assuming a value of 1.0027 ± 0.0005 for $^{13}K_{eq}$ (O'Leary & Yapp, 1978)].

$$\frac{^{13}(V/K)_h - ^{13}K_{eq}}{^{13}(V/K)_d - ^{13}K_{eq}} = ^{D}(V/K) \quad (5)$$

$$1.27 \pm 0.001 = 1.45 \pm 0.12 \quad (6)$$

Clearly, expression 4 is a closer fit than expression 6, which indicates that oxidation occurs first to produce an OAA intermediate which is decarboxylated in a subsequent step.

Although the apparent chemical mechanism of stepwise hydride transfer and decarboxylation is the same as for other malic enzymes, the enzyme's inability to reduce or decarboxylate OAA must be reconciled. $^{13}C$ kinetic isotope effect studies of the chemical decarboxylation of OAA suggest the chicken liver NADP malic enzyme has only a minor role in the decarboxylation of the intermediate E–Mg–NADPH–OAA complex (Grissom & Cleland, 1986; Grissom, 1985). It is suggested that C-4 of OAA is bent out of the plane defined by C-1 and C-2 to facilitate decarboxylation. If *Crassula* NAD malic enzyme were to promote the binding of exogenous OAA with the C-4 carboxyl in the same plane as C-1 and C-2, then decarboxylation would be inhibited, regardless of the presence of an electron sink at C-2. This would explain the enzyme's inability to catalyze the decarboxylation of exogenous OAA. Perhaps the out-of-plane configuration of C-4 can only be generated by hydride transfer in the E–Mg–NAD–malate complex or carboxylation in the E–Mg–pyr–$CO_2$ complex.[2] An alternative explanation is that the enzyme does not bind OAA and hence never forms a productive E–Mg–OAA or E–Mg–OAA–NAD(H) complex. This latter explanation is supported by the inability of exogenous OAA to inhibit either the oxidative decarboxylation of malate or the reductive carboxylation of pyruvate. It is possible free enzyme can adopt a conformation which will bind OAA but does not exist at significant concentrations because it is energetically unfavorable.

In view of the morphological differences between the *Crassula* and other malic enzymes, it is not surprising that the molecular events which poise the substrate and active site for catalysis might be different. The *Crassula* enzyme exists in an inactive dimer which associates to an active tetramer of dissimilar subunits when diluted in the presence of substrates. It has a pronounced lag of over 1 h which cannot be overcome by incubation with single substrates. It is susceptible to activation and inhibition by both organic and inorganic anions. These characteristics, unique to plant malic enzymes, suggest molecular events different than those which occur in malic enzymes from animal sources. The stepwise nature of the chemical events necessary to transform enzyme-bound malate to enzyme-bound pyruvate and $CO_2$ is similar, however, to the processes which occur in other malic enzymes. Although the morphological properties of the plant enzyme have been changed to allow for regulation consistent with the physiology of crassulacean acid metabolism, the basic tenet of a stepwise chemical transformation has been conserved.

[2] The enzyme must obey the principle of microscopic reversibility.

## ACKNOWLEDGMENTS

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**Registry No.** EC 1.1.1.39, 9028-46-0; L-malic acid, 97-67-6; L-malic acid-*2-d*, 71655-88-4; carbon-13, 14762-74-4; deuterium, 7782-39-0.

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