Metal Ion Activator Effects on Intrinsic Isotope Effects for Hydride Transfer and Decarboxylation in the Reaction Catalyzed by the NAD−Malic Enzyme from *Ascaris suum*†

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ABSTRACT: The mechanism of the oxidative decarboxylation reaction catalyzed by the NAD−malic enzyme from *Ascaris suum* has been examined with several different divalent metal ion activators and dinucleotide substrates. Primary deuterium and tritium isotope effects have been obtained and, in combination with the partitioning ratios of the oxalacetate intermediate to malate and pyruvate, have been used to calculate commitment factors, intrinsic deuterium isotope effects on the hydride transfer step, and intrinsic ^{13}C isotope effects for the decarboxylation step. A survey of malate analogs has been undertaken to define the geometry of the active site and to identify functional groups on malate important for substrate binding. With NAD as dinucleotide substrate, a direct correlation between the size of the divalent metal ion activator and the intrinsic deuterium isotope effect is observed. An isotope effect significantly greater than the semiclassical limit is seen when Cd^{2+} is the metal ion activator, indicating a substantial tunneling contribution. The primary intrinsic ^{13}C isotope effect on the decarboxylation step increases over the series $Mg^{2+} < Mn^{2+} < Cd^{2+}$, which is in contrast to the equal isotope effects measured for these metal ions for the nonenzymatic decarboxylation of oxalacetate [Grissom, C. B., & Cleland, W. W. (1986) *J. Am. Chem. Soc. 108*, 5582]. With Mn^{2+} or Cd^{2+} as the divalent metal ion activator, the data support a stepwise mechanism for the enzymatic oxidative decarboxylation with NAD as the dinucleotide substrate, but a change to a concerted mechanism is indicated with more redox-positive dinucleotide substrates as suggested previously with Mg^{2+} as activator [Karsten, W. E., & Cook, P. F. (1994) *Biochemistry 33*, 2096]. A survey of malate inhibitory analogs suggests the enzyme is quite forgiving with respect to functional group substitutions at the β-carbon and also indicates that the metal ion provides a major determinant for substrate binding.

The mitochondrial NAD[1]−malic enzyme (EC 1.1.1.39) from *Ascaris suum* catalyzes the divalent metal ion dependent oxidative decarboxylation of L-malate utilizing NAD to yield pyruvate, CO_2, and NADH. Several different divalent metal ions including Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, and Zn^{2+} (Schimerlik et al., 1977; Grissom & Cleland, 1988) have been shown to support catalysis in the similar NADP−malic enzyme from chicken liver. On the basis of the observation that the NADP−malic enzyme will, in the presence of NADPH, catalyze the reduction of oxalacetate to malate and the decarboxylation of oxalacetate to pyruvate and CO_2 (Viega Salles & Ochoa, 1950; Hsu, 1970; Tang & Hsu, 1973), a stepwise oxidative decarboxylation of malate has been proposed with hydride transfer from malate preceding decarboxylation of the resulting oxalacetate intermediate. The nonenzymatic decarboxylation of oxalacetate catalyzed by several different divalent metal ions has been shown to proceed with nearly equal ^{13}C isotope effects of about 1.05, suggesting similar transition states for this process (Grissom & Cleland, 1986). The ^{13}C isotope effect of 1.05 is equal to the intrinsic ^{13}C isotope effect estimated for the NADP−malic enzyme catalyzed oxidative decarboxylation of malate with Mg^{2+} as the metal ion activator (Hermes et al., 1986; Grissom & Cleland, 1985). In the malic enzyme reaction the metal ion has been proposed to act as a Lewis acid positioned near the carbonyl of the oxalacetate intermediate to facilitate decarboxylation (Grissom & Cleland, 1988). If the role proposed for the divalent metal ion in the decarboxylation reaction is correct, then the metal ion is likely positioned in the vicinity of the hydroxyl group of enzyme-bound malate where it could also influence the hydride transfer step. Little is known of the effect of the metal ion on the hydride transfer step.

The aforementioned stepwise oxidative decarboxylation mechanism is supported by the multip isotope effect results of Hermes et al. (1982) and Weiss et al. (1991) with NADP or NAD as the dinucleotide substrate. A determination of intrinsic deuterium and ^{13}C isotope effects in the NAD−malic enzyme reaction with Mg^{2+} as the divalent metal ion has strongly suggested a switch from a stepwise oxidative decarboxylation of malate with NAD(P) to a concerted

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[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; MDH, malate dehydrogenase; NAD, nicotinamide adenine dinucleotide (the + charge is omitted for convenience); NADP, nicotinamide adenine dinucleotide 2′-phosphate; APAD, 3-acetylpyridine adenine dinucleotide; PAAD, 3-pyridinealdehyde adenine dinucleotide.

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reaction with the more redox-positive dinucleotide substrates. The most likely reason for the change in mechanism was suggested to be a result of differences in the binding interactions of malate with the alternative dinucleotide substrates (Karsten & Cook, 1994).

To further investigate the mechanism of the NAD−malic enzyme reaction, we have used alternative dinucleotide substrates and divalent metal ions that have the potential to alter the rate of the chemical interconversion steps. A survey of malate inhibitory analogs has been carried out with either Mg^{2+} or Mn^{2+} as the divalent metal ion activator in order to determine what features of the substrate are important for binding and to potentially map out the geometry of the malate binding site. In addition, deuterium, tritium, and ^{13}C primary kinetic isotope effects are reported using several different divalent metal ions to support catalysis in the NAD−malic enzyme reaction. The combination of the isotope effect values with the results from the enzyme-catalyzed partitioning of oxalacetate to pyruvate and malate allows an estimation of commitment factors and intrinsic isotope effects. Data are discussed in terms of the role of the metal ion on the hydride transfer step and the switch from a stepwise to concerted mechanism as the dinucleotide substrate and metal ion activator are changed.

MATERIALS AND METHODS

Chemicals. All chemicals and reagents were obtained from sources provided or prepared according to methods described previously (Karsten & Cook, 1994). The NAD−malic enzyme from *A. suum* was purified by either the method of Allen and Harris (1981) or that of Karsten and Cook (1994).

Initial Velocity Studies. Malic enzyme was assayed spectrophotometrically in the direction of oxidative decarboxylation using a Gilford 250 spectrophotometer equipped with a Brinkman Servogor 210 chart recorder with multispeed drive. All assays were run at 25 °C with the temperature maintained using a circulating water bath to heat or cool the thermospacers of the Gilford. Reaction cuvettes were 1 cm in path length and 1 mL in volume. The reaction was initiated by addition of malic enzyme, and production of the appropriate reduced nucleotide was monitored at the corresponding λ_{max} of the reduced dinucleotide. Initial velocity studies of malate oxidative decarboxylation in the presence of malate analogs were run in a typical assay using the following: 100 mM Mes, pH 6.5, 1 mM DTT, 2 mM NAD, variable concentrations of malate, metal, and the malate analog, and 2.2−6.6 nM *A. suum* malic enzyme.

Deuterium Isotope Effects. Deuterium isotope effect measurements were performed as described previously utilizing L-malate-*2-d* (Karsten & Cook, 1994) with the exception that metal ions other than Mg^{2+} were used and that thio-NAD was also used as an alternative dinucleotide substrate. An ϵ_{395} of 11.3 mM^{-1} cm^{-1} was used for thio-NAD. The *A. suum* malic enzyme utilizes the unliganded form of the divalent metal ion and reactants, and thus correction for the metal−chelate complex was made according to Park et al. (1984). The following dissociation constants were used: Mn−malate, 5.8 mM; Cd−malate, 4.4 mM; Co−malate, 1.4 mM; Ni−malate, 0.68 mM; Zn−malate, 1.2 mM; and Mn−NAD, 12.9 mM (Martell & Smith, 1979; Park et al., 1984). The dissociation constants for the chelate complexes formed with the alternative dinucleotides were assumed to be the same as for metal−NAD (Karsten & Cook, 1994). Inactivation of the NAD−malic enzyme by Cd^{2+} has been reported (Gavva et al., 1991). Consequently, in all the studies reported here low levels of Cd^{2+} (0.2 mM) were used to avoid enzyme inactivation over the time scale of these experiments. At the metal ion concentrations employed in these studies, neither Cd^{2+} or any other metal ion was observed to induce enzyme inactivation over the time scale used.

Tritium and ^{13}C Isotope Effects. Tritium isotope effects were performed as described in Karsten and Cook (1994). Briefly, assays were performed in a 1-mL total volume containing 100 mM Hepes, pH 7.0, 10 mM dinucleotide substrate, 2 mM L-malate-*2-t* (1.35×10^6 cpm/μmol), and 0.2 mM $CdSO_4$ or 5 mM $MnSO_4$. The reaction was monitored at the appropriate wavelength for the dinucleotide used and typically run to 10% of reaction completion. The reactions were stopped by adding 100 μL of CCl_4 followed immediately by vigorous vortexing. The tritiated reduced dinucleotide was separated from the other reaction components on a DEAE-Sephadex A25 column, and those fractions containing constant specific radioactivities were used to determine the tritium isotope effects.

The ^{13}C isotope effects are determined using the natural abundance of ^{13}C in the C-4 position of L-malate. High-conversion (100% reaction) and low-conversion (~10% reaction) samples were collected and the $^{12}C/^{13}C$ isotope ratios in the CO_2 produced determined by isotope ratio mass spectrometry. From these ratios the relative rates of reaction for ^{12}C versus ^{13}C are determined to give the isotope effects (Weiss et al., 1991).

Oxalacetate Partitioning. Oxalacetate partitioning experiments were performed according to the method of Grissom and Cleland (1985) as modified by Karsten and Cook (1994). In these partitioning experiments and those reported previously by Karsten and Cook (1994), glutamate−pyruvate transaminase (GPT) was not included in the partitioning experiments. This is in contrast to the method of Grissom and Cleland (1985) where GPT was included to remove pyruvate as it was produced during the partitioning experiment to avoid an increase in absorbance at 282 nm due to the pyruvate produced. We have determined empirically that under the partitioning conditions employed in these studies the maximum amount of pyruvate produced would give a total absorbance at 282 nm of less than 0.002, an insufficient change to interfere with the results. Assays were carried out using a Hewlett-Packard 8452A diode array spectrophotometer in a total volume of 1 mL in 0.5-cm path-length cuvettes. An effective extinction coefficient for oxalacetate was determined at 282 nm (ϵ_{282} 1.78 mM^{-1} cm^{-1} with 4 mM oxalacetate and 0.5 mM Mn^{2+} and ϵ_{282} 1.88 mM^{-1} cm^{-1} with 4 mM oxalacetate and 0.2 mM Cd^{2+}) under the exact partitioning conditions for each experiment. Partitioning reaction mixtures contained in a 1-mL total volume 100 mM Hepes, pH 7.0, 0.4 mM reduced dinucleotide (NADH or APADH), 4 mM oxalacetate, 0.5 mM $MnSO_4$ or 0.2 mM $CdSO_4$, and approximately 1 unit of the NAD−malic enzyme. Lower enzyme concentrations were used in experiments with Cd^{2+} or Mn^{2+} than with Mg^{2+} as the divalent metal ion activator, and it was unnecessary to correct for a background rate resulting from a small amount of contaminating malate dehydrogenase activity present in the malic

Scheme 1

$$\text{EMA} + \text{B} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \text{EMAB} \underset{k_8}{\overset{k_7}{\rightleftharpoons}} (\text{EMAB}) \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} (\text{EMXR}) \xrightarrow{k_{11}}$$

enzyme preparations. Partitioning experiments using NADD or APADD were conducted in an identical manner.

Data Processing. Reciprocal initial velocities were plotted as a function of reciprocal substrate concentrations, and all plots were linear. Data were fitted using the appropriate rate equation and Fortran programs developed by Cleland (1979). Data conforming to a sequential initial velocity pattern were fitted using eq 1, while data for linear competitive and noncompetitive inhibition were fitted using eqs 2 and 3, respectively. In eqs 1−3, K_a and K_b are the apparent

$$v = (VAB)/(K_{ia}K_b + K_aB + K_bA + AB) \quad (1)$$

$$v = (VA)/(K_a(1 + I/K_{is}) + A) \quad (2)$$

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

Michaelis constants for A and B, where A, B, and I are reactant and inhibitor concentrations. Inhibition constants for A, slope, and intercept are K_{ia}, K_{is}, and K_{ii}, respectively. V is the maximum velocity. The isotope effect data were fitted using BASIC versions of the Fortran computer programs of Cleland (1979), as described in Karsten and Cook (1994). The isotope effect nomenclature of Northrop (1972) as modified by Cook and Cleland (1981) and Hermes et al. (1982) is used in this paper where a leading superscript (D,T,13) indicating the type of isotope effect precedes the kinetic parameter [$^D(V/K)$, $^T(V/K)$, $^{13}(V/K)$, etc.] and a following subscript refers to the constant isotopic species in a multiple isotope effect experiment; for example, a $^{13}(V/K)_H$ reflects a ^{13}C isotope effect on V/K with malate-*2-h*.

Calculation of Intrinsic Isotope Effects and Commitment Factors. Theory for calculation of intrinsic isotope effects and commitment factors assuming a stepwise and concerted oxidative decarboxylation of L-malate, respectively, is as given in Karsten and Cook (1994). The equations for stepwise (Scheme 1) and concerted (Scheme 2) mechanisms are provided for convenience. In Scheme 1 M is metal ion, A is oxidized dinucleotide, B is malate, X is the enzyme-bound oxalacetate intermediate, and R is reduced dinucleotide. The equations for the isotope effects and partitioning ratios are

$$^D(V/K) = [^Dk_9 + c_f + {}^DK_{eq}(c_r)]/(1 + c_f + c_r) \quad (4)$$

$$^T(V/K) = [(^Dk_9)^{1.44} + c_f + (^DK_{eq})^{1.44}(c_r)]/(1 + c_f + c_r) \quad (5)$$

$$^{13}(V/K)_H = [^{13}k_{11} + (1 + c_f)/c_r]/[1 + (1 + c_f)/c_r] \quad (6)$$

$$^{13}(V/K)_D = [^{13}k_{11} + (^Dk_9/{}^DK_{eq}(c_r))(1 + c_f/{}^Dk_9)]/[1 + (^Dk_9/{}^DK_{eq}(c_r))(1 + c_f/{}^Dk_9)] \quad (7)$$

$$r_H = (1 + c_f)/c_r \quad (8)$$

$$r_D = (^Dk_9 + c_f/{}^DK_{eq})/c_r \quad (9)$$

where the forward commitment to catalysis, c_f, is $(k_9/k_8)(1 + k_7/k_6)$, the reverse commitment to catalysis, c_r, is k_{10}/k_{11}, $^Dk_9$ is the intrinsic primary deuterium isotope effect, $^{13}k_{11}$ is the intrinsic primary ^{13}C isotope effect, r_H is the partition ratio with nondeuteriated dinucleotide, r_D is the partition ratio with deuteriated dinucleotide, and $^DK_{eq}$ is the deuterium isotope effect on the equilibrium constant which has been determined by Cook et al. (1980) to be 1.18. There are six equations and only four unknowns (c_f, c_r, ^{13}k, and ^Dk), and consequently it is possible to calculate an exact solution. A concerted mechanism for oxidative decarboxylation may be described as in Scheme 2. In Scheme 2, M, A, B, and R are as defined above, Y is enolpyruvate, and P is CO_2. For a concerted mechanism the equations for the isotope effects are

$$^D(V/K) = (^Dk_9 + c_f)/(1 + c_f) \quad (10)$$

$$^T(V/K) = [(^Dk_9{}^{1.44} + c_f)]/[1 + c_f] \quad (11)$$

$$^{13}(V/K)_H = (^{13}k_9 + c_f)/(1 + c_f) \quad (12)$$

$$^{13}(V/K)_D = (^{13}k_9 + c_f/{}^Dk_9)/(1 + c_f/{}^Dk_9) \quad (13)$$

where c_f is $(k_9/k_8)(1 + k_7/k_6)$, $^Dk_9$ is the intrinsic primary deuterium isotope effect, and $^{13}k_9$ is the intrinsic primary ^{13}C isotope effect. A computer program has been written to make these calculations.

RESULTS

Inhibition Studies by Malate Analogs. Inhibition studies were carried out with malate as the varied substrate at different fixed levels of a number of malate analogs. Values of K_i are reported with the exception of cases where the metal−ligand stability constants were not known. In these cases, an apparent K_i value is reported using a fixed concentration of metal and correcting only for Mg−NAD and Mg−malate. The results are summarized in Table 1.

Kinetic Survey of Divalent Metal Ion Activators. A number of different divalent metal ions will support catalysis in the reaction catalyzed by the NAD−malic enzyme (Table 2). With NAD as the dinucleotide substrate, the K_m value for malate varies by over an order of magnitude from a low value of 0.1−0.2 mM with Co^{2+}, Ni^{2+}, and Cd^{2+} to about 1 mM with Mg^{2+} as the divalent metal ion activator. Values of K_{malate} are consistently lower whatever the metal ion activator when APAD and PAAD are the dinucleotide substrates. In contrast, the identity of the divalent metal ion generally has less effect on the K_m for the dinucleotide substrate, with an average observed value of about 35 μM for NAD. The V_{max} with NAD as substrate varies about 3-fold with the lowest value seen in the presence of Cd^{2+} and the highest with Mn^{2+}, while a 5-fold and 2.5-fold variation is observed with APAD and PAAD.

Isotope Effects and Partitioning of Oxalacetate. Deuterium, tritium, and ^{13}C isotope effects on the NAD−malic enzyme reaction measured with different divalent metal ions are presented in Table 3. With NAD as the dinucleotide

Scheme 2

$$\text{EMA} + \text{B} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \text{EMAB} \underset{k_8}{\overset{k_7}{\rightleftharpoons}} (\text{EMAB}) \xrightarrow{k_9} (\text{EMYPR})$$

substrate ^{D}V is generally greater than $^{D}(V/K)$, whatever the divalent metal ion activator (also with Cd^{2+} and APAD or thio-NAD). Similar results are obtained with Mg^{2+} as the divalent metal ion (Karsten & Cook, 1994). An exception to the generalization is observed with Ni^{2+}, where it is unclear that there are significant isotope effects on V and V/K. Within error, when the dinucleotide substrate has a redox potential more positive than NAD, the observed $^{D}(V/K)$ values are equal to ^{D}V for both Mn^{2+} and Co^{2+} (also with NADP and Mn^{2+}). The only case in which $^{D}(V/K)$ is greater than ^{D}V is when Zn^{2+} is the divalent metal ion activator and APAD is the dinucleotide substrate. Similar to what has been reported with Mg^{2+} as the divalent metal ion activator (Weiss et al., 1991), $^{13}(V/K)_H$ is greater than $^{13}(V/K)_D$ with NAD and either Mn^{2+} or Cd^{2+}. However, significantly smaller values of $^{13}(V/K)_H$ are observed when APAD is the dinucleotide substrate, and they are lower than $^{13}(V/K)_D$.

The oxalacetate partitioning data obtained with NADH(D) or APADH(D) are summarized in Table 4. The partitioning ratios (pyruvate/malate) are larger with Cd^{2+} than with Mn^{2+} regardless of the dinucleotide substrate, and all are larger than those reported with Mg^{2+} (Karsten & Cook, 1994). The partition ratios increase when a deuterated dinucleotide is used due to the slowing down of the reverse hydride transfer step.

Hermes et al. (1982) have shown that in a two-step chemical mechanism where hydride transfer precedes decarboxylation the following equality should be satisfied.

$$(^{13}(V/K)_H - 1)/(^{13}(V/K)_D - 1) = {}^{D}(V/K)/{}^{D}K_{eq} \quad (14)$$

With the ^{13}C and the deuterium isotope effect values reported in Table 3 and 1.18 for $^{D}K_{eq}$, the equality gives 1.50 = 1.51 for Mn^{2+} and 1.31 = 1.39 for Cd^{2+} with NAD as the dinucleotide substrate. These results are consistent with a two-step chemical mechanism with hydride transfer preceding decarboxylation. The isotope effect values for APAD with either Mn^{2+} or Cd^{2+} do not conform to the equality expressed in eq 14 and, therefore, are not consistent with a two-step chemical mechanism.

The primary intrinsic ^{13}C isotope effect may be calculated using the partitioning ratios (Grissom & Cleland, 1985) and eqs 15 and 16 to obtain two separate values. With the values

$$^{13}k_{11} = {}^{13}(V/K)_H + r_H(^{13}(V/K)_H - 1) \quad (15)$$

$$^{13}k_{11} = {}^{13}(V/K)_D + r_D(^{13}(V/K)_D - 1) \quad (16)$$

reported in Tables 3 and 4, eqs 15 and 16 give respectively $^{13}k_{11}$ = 1.0685 and $^{13}k_{11}$ = 1.0594 for NAD and Mn^{2+} and $^{13}k_{11}$ = 1.0842 and $^{13}k_{11}$ = 1.0866 with NAD and Cd^{2+}. There is reasonable agreement between these calculated values for $^{13}k_{11}$ with an average value equal to 1.064 for Mn^{2+} and 1.085 for Cd^{2+}. The value with Mn^{2+} as the divalent metal ion activator is essentially identical to the value of 1.065 reported by Grissom and Cleland (1985) with the NADP−malic enzyme from chicken liver. Using eqs 15 and 16 with APAD as the dinucleotide substrate, the calculated values are $^{13}k_{11}$ = 1.0435 and $^{13}k_{11}$ = 1.132 with Mn^{2+} and $^{13}k_{11}$ = 1.0466 and $^{13}k_{11}$ = 1.185 with Cd^{2+}.

Using the data presented in Tables 3 and 4 and eqs 4−9, values for the commitment factors and the intrinsic isotope effects may be calculated on the basis of a stepwise mechanism and are shown in Table 5. The calculated value for the intrinsic deuterium isotope effect with Mn^{2+} and NAD is 3.9. In contrast, the calculated value for the intrinsic deuterium isotope effect with Cd^{2+} is quite large with NAD. A primary intrinsic ^{13}C isotope effect of 1.069 is calculated with Mn^{2+} and NAD, which increases to about 1.087 with Cd^{2+} and NAD. With APAD as the dinucleotide substrate, the data are fitted using a model that assumes a concerted oxidative decarboxylation mechanism, eqs 10−13, and the calculated values for the forward commitments and intrinsic isotope effects are presented in Table 6.

DISCUSSION

Effects of Metal Ions on Hydride Transfer. With NAD, the intrinsic deuterium isotope effect is small (3.9) with Mn^{2+} as the divalent metal ion. The small intrinsic deuterium effect could reflect an early or late transition state (Westheimer, 1961), but the intrinsic effect becomes smaller as the redox potential becomes more oxidizing (Table 6; $^{D}k_9$ = 2.7 for APAD), indicating the transition state with NAD may be early (see below). The value of $^{D}k_9$ increases to about 11 with Mg^{2+} (Karsten & Cook, 1994), 13[2] with Zn^{2+}, and up to 23[3] with Cd^{2+}. The large values for Mg^{2+}, Zn^{2+}, and Cd^{2+} suggest that a significant tunneling contribution to hydride transfer is present in these cases. In a stepwise mechanism for oxidative decarboxylation there are two discrete transition states, one for hydride transfer and a second for decarboxylation of the oxalacetate intermediate. The intrinsic deuterium isotope effect correlates very well with the size of the metal ion if two assumptions are made; first, the metal ions must be six coordinate when bound to enzyme, and second, Mn^{2+} must be low spin. If these two conditions are met, the ionic radii of the metal ions are Mn^{2+} = 81 pm, Mg^{2+} = 86 pm, Zn^{2+} = 88 pm, and Cd^{2+} = 109 pm (Douglas et al., 1983). The correlation between the size of the metal ion and the intrinsic deuterium isotope effect is presented in Figure 1 for both NAD and APAD. The observation of a direct correlation between the ionic radius of the metal ion and the intrinsic deuterium isotope effect may be rationalized as follows. The K_m value for NAD is similar for each of these four metal ions, suggesting that NAD binds to enzyme in an essentially identical fashion and is likely in the same relative position on enzyme regardless of which metal ion is also bound. Malate must bind to enzyme with metal already bound in order to form a catalytically productive complex (Park et al., 1984; Chen et al., 1988; Mallick et al., 1991). If the metal ion when bound to enzyme is in the vicinity of the α-hydroxyl group of malate, then the position of malate relative to NAD could be expected to be a function of the size of the metal ion. A

[2] The intrinsic deuterium isotope effect with Zn^{2+} is calculated from $(^{D}(V/K) - 1)/(^{T}(V/K) - 1) = {}^{D}k/{}^{D}k^{1.44}$ (Northrop, 1982).

[3] A better value of ^{D}k with Cd^{2+} is based on a calculated value of $^{D}(V/K)$ = 1.54 using eq 14 (Hermes et al., 1982). The ^{13}C isotope effect values have small associated errors, and consequently the value of $^{D}(V/K)$ with Cd^{2+} calculated from the ^{13}C isotope effects is likely more accurately determined than the experimental value of 1.6.

Table 1: Inhibition Constants for L-Malate Analogs with either Mg^{2+} or Mn^{2+} as the Divalent Metal Ion Activator

			K_i (mM)		metal−ligand dissociation constant	
	R_1[a]	R_2	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
oxalate[b]			0.08	0.007	1.7	0.6
tartronate	−OH	$-COO^-$	1.4	0.05	6.8	1.7
L-lactate	−OH	$-CH_3$	3.2	0.7	118	65
D-lactate	−OH	$-CH_3$	32.9	14.9	118	65
thioglycolate	−SH	−H	7.7[c]	0.02		0.042
glycolate	−OH	−H	55.3	6.2	120	26
thiomalate	−SH	$-CH_2COO^-$	2.0	0.007[d]		
isoserine	−OH	$-CH_2NH_3^+$	no inhibition	75		
β-chloroalanine	$-NH_3^+$	$-CH_2Cl$	1.8[e]	0.8	10.9	3.4
p-hydroxyphenylpyruvate	=O	$-CH_2-C_6H_4-OH$	2.9	0.3		

[a] The analog structures are based on L-malate $R_2CH(R_1)COO^-$, where R_1 is −OH and R_2 is $^-OOCCH_2-$ for L-malate. Errors in the reported values are ≤20%. [b] Oxalate is considered an analog of enolpyruvate as follows: $O{=}C(O^-)COO^-$, $H_2C{=}C(O^-)COO^-$. [c] The substrates for malic enzyme are unliganded form, not the metal−substrate complex (Kiick & Cook, 1984), and therefore, the inhibition constants reported in the table are based on the concentration of the metal ion free form of the substrate analog. However, in some cases the metal−analog dissociation constants are unavailable; consequently the inhibition constants are based on total analog concentrations. [d] The K_i value in the table is the calculated value for thiomalate assuming a similar metal−thiomalate dissociation constant for Mn^{2+}−thioglycolate. [e] The metal−ligand dissociation constants reported in the table are for alanine and assumed to be the same for β-chloroalanine.

Table 2: Kinetic Parameters for Malic Enzyme with Different Dinucleotide Substrates and Divalent Metal Ion Activators

	NAD			APAD			PAAD	
	V/E_t[a]	K_{malate}[b]	K_{NAD}	V/E_t	K_{malate}	K_{APAD}	V/E_t	K_{malate}	K_{PAAD}
Mg^{2+}[c]	1.0	1.2 ± 0.1	11 ± 1	2.4	0.48 ± 0.03	8 ± 0.5	0.05	0.29 ± 0.03	9 ± 1
Mn^{2+}	1.6	0.3 ± 0.05	30 ± 5	1.76	0.23 ± 0.07	26 ± 4	0.03	0.12 ± 0.02	20 ± 2
Co^{2+}	1.45	0.1 ± 0.02	8.0 ± 0.2		ND[d]			ND	
Ni^{2+}	0.94	0.11 ± 0.03	40 ± 8		ND			ND	
Zn^{2+}	0.73	0.47 ± 0.13	36 ± 7		ND			ND	
Cd^{2+}	0.45	0.17 ± 0.03	65 ± 6	0.5	0.15 ± 0.01	100 ± 6	0.02	0.040 ± 0.003	46 ± 4

[a] V/E_t are relative values compared to values of V/E_t determined with Mg^{2+} and NAD. [b] The K_m values are in millimolar for malate and in micromolar for the dinucleotide substrate. [c] Values for Mg^{2+} are from Weiss et al. (1991). [d] ND = not determined.

Table 3: Isotope Effects with the NAD−Malic Enzyme from *A. suum* with Different Divalent Metal Ions and Dinucleotide Substrates

metal ion	dinucleotide[a]	$^D(V/K)$[b]	^DV	$^T(V/K)$[c]	$^{13}(V/K)_H$	$^{13}(V/K)_D$
Mn^{2+}	NAD	1.8 ± 0.1	2.1 ± 0.2	2.6 ± 0.4	1.0353 (0.0005)[d]	1.02348 (0.00008)
	NADP	2.1 ± 0.2	1.8 ± 0.2	ND	1.037 (0.002)	ND
	thio-NAD	3.3 ± 0.4	3.5 ± 0.4	ND	ND	ND
	PAAD	3.5 ± 0.5	2.9 ± 0.2	ND	ND	ND
	APAD	2.7 ± 0.5	2.8 ± 0.3	4.1 ± 0.2	1.0068 (0.00005)	1.0089 (0.0002)
Cd^{2+}	NAD	1.6 ± 0.2	2.2 ± 0.2	3.5 ± 0.2	1.032 (0.001)	1.02449 (0.00003)
	APAD	2.5 ± 0.5	3.4 ± 0.3	5.1 ± 0.3	1.00627 (0.00004)	1.0081 (0.0002)
	thio-NAD	2.4 ± 0.1	3.34 ± 0.03	ND	ND	ND
Zn^{2+}	NAD	1.5 ± 0.2	1.8 ± 0.1	2.6 ± 0.4	ND	ND
	APAD	3.2 ± 0.3	1.4 ± 0.2	ND	ND	ND
Co^{2+}	NAD	1.27 ± 0.04	1.7 ± 0.2	ND	ND	ND
	APAD	2.03 ± 0.08	1.9 ± 0.3	ND	ND	ND
	PAAD	1.92 ± 0.08	2.1 ± 0.44	ND	ND	ND
Ni^{2+}	NAD	1.20 ± 0.16	1.18 ± 0.14	ND	ND	ND

[a] The redox potentials [E_0' (volts)] are as follows: NAD, NADP = −0.32; thio-NAD = −0.285; PAAD = −0.262; and APAD = −0.258. [b] The deuterium isotope effects are a mean average of at least six separate determinations. [c] The tritium isotope effects are a mean average of at least five separate determinations. [d] Values in parentheses are the standard errors for the ^{13}C isotope effects.

Table 4: Pyruvate/Malate Partition Ratios for the Oxalacetate Intermediate in the NAD−Malic Enzyme Reaction[a]

metal ion	dinucleotide	r_H	r_D	r_H/r_D
Mn^{2+}	NADH(D)	0.9 ± 0.3	1.7 ± 0.1	0.5 ± 0.1
	APAD(D)	5.4 ± 0.4	13.8 ± 0.1	0.39 ± 0.03
Cd^{2+}	NADH(D)	1.6 ± 0.1	2.52 ± 0.04	0.63 ± 0.04
	APAD(D)	6.4 ± 0.2	22 ± 1	0.29 ± 0.02

[a] Partition ratios are the mean average of at least three separate determinations.

Table 6: Commitment Factors and Intrinsic Isotope Effects for NAD−Malic Enzyme Assuming a Concerted Chemical Mechanism with APAD

metal ion[a]	c_f	$^Dk_9$	$^{13}k_9$
Mg^{2+}	1.5	4.9	1.0175
Mn^{2+}	0.0	2.7	1.0078
Cd^{2+}	3.6	7.9	1.033

[a] Values for Mg^{2+} are taken from Karsten and Cook (1994).

relatively small metal ion such as Mn^{2+} would lead to a relatively large distance between malate and the pyridinium ring of NAD, which would become smaller as the ionic radius of the metal ion increases. Increasingly smaller distances between malate and NAD would lead to compression of the reaction coordinate, conditions that favor an increased tendency for a tunneling contribution to hydride transfer (Cha et al., 1989; Klinman, 1991). This appears to

Table 5: Commitment Factors and Intrinsic Isotope Effects for NAD−Malic Enzyme with NAD and Different Divalent Metal Ion Activators Assuming a Stepwise Mechanism

metal ion[a]	c_f	c_r	$^Dk_9$	$^{13}k_{11}$
Mg^{2+}	6.6	14.3	10.8	1.052
Mn^{2+}	1	2.15	3.9	1.069
Cd^{2+}	22.8	14.6	23.0	1.087

[a] The values for Mg^{2+} are from Karsten and Cook (1994).

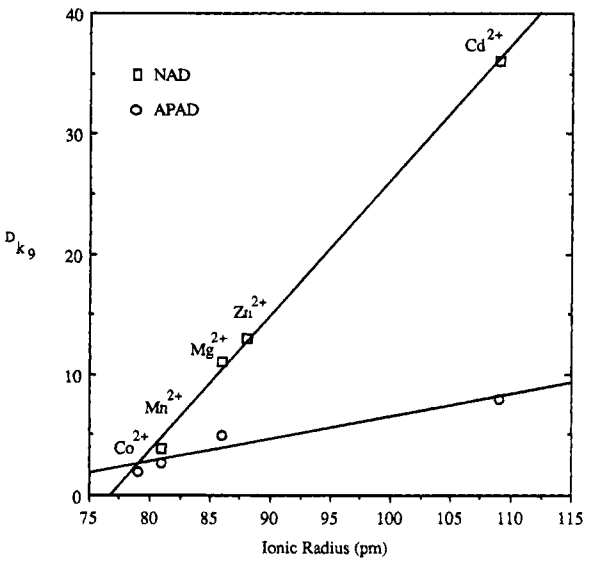

FIGURE 1: Plot of intrinsic primary deuterium isotope effect ($^Dk_9$) vs the ionic radius of the divalent metal ion activator. The data are plotted assuming the divalent metal ion has a coordination number of 6 when bound in the E·NAD·metal·malate complex and Mn^{2+} is low spin.

be the situation that occurs with NAD as the dinucleotide substrate.

Mechanism with NAD. A switch from a stepwise mechanism with NAD or NADP to a concerted mechanism with Mg^{2+} and APAD was considered most likely by Karsten and Cook (1994) on the basis of several considerations. A fit of the isotope effect data obtained with APAD to a model that assumes a stepwise mechanism results in a calculated value for the primary intrinsic ^{13}C isotope effect of about 1.0. This value is considerably different from the value of 1.052 calculated for the intrinsic ^{13}C isotope effect with NAD and suggests that the redox potential of the dinucleotide substrate could influence the transition state for the decarboxylation step, and this did not seem very likely. The data obtained with Cd^{2+} as the divalent metal ion and APAD in the present study do provide a reasonable value for the intrinsic primary ^{13}C isotope effect when fitted using a model for a stepwise mechanism, but a value of slightly less than 1.0 is obtained with Mn^{2+} and APAD (data not shown). Taken together, these results again suggest that a concerted mechanism is the more likely possibility with these metal ions when APAD is the dinucleotide substrate. As is the case when NAD is the dinucleotide substrate and Mg^{2+} is the divalent metal ion activator (Karsten & Cook, 1994), a stepwise chemical mechanism is supported by the data with Mn^{2+} and Cd^{2+} in the present study when NAD is the dinucleotide substrate. For both Mn^{2+} and Cd^{2+} the equality expressed by eq 14 is satisfied by the data in Table 3 with NAD as the dinucleotide substrate, but not with APAD. Additionally, for Mn^{2+} or Cd^{2+}, realistic values for the intrinsic primary ^{13}C isotope effect are obtained as calculated by either eq 15 or eq 16 or presented in Table 5. Thus, the change from a stepwise mechanism with NAD to a concerted mechanism with APAD appears to occur whatever the identity of the divalent metal ion activator.

For the nonenzymatic decarboxylation of oxalacetate the ^{13}C isotope effect is about 5% independent of the metal ion activator. For both the chicken liver NADP−malic enzyme (Grissom & Cleland, 1988) and the *A. suum* NAD−malic enzyme (Karsten & Cook, 1994) an intrinsic primary ^{13}C isotope effect of about 5% is obtained with Mg^{2+} as the divalent metal ion. With the NADP−malic enzyme and Mn^{2+} an intrinsic ^{13}C isotope effect of about 6.5% was determined (Grissom & Cleland, 1985), very similar to the intrinsic ^{13}C isotope effect of about 6.9% calculated for the NAD−malic enzyme (Table 5) with Mn^{2+} as divalent metal ion activator. There is a trend toward an even larger value of the intrinsic ^{13}C isotope effect with Cd^{2+} and the NAD−malic enzyme. The enzymatic decarboxylation appears to differ from the nonenzymatic case with increasing bond breaking present between C-3 and C-4 in the transition state for decarboxylation of oxalacetate as the metal is varied from Mg^{2+} to Cd^{2+}. Mg^{2+} is less polarizable and a poorer Lewis acid than the other metal ions utilized and tends to make ionic type interactions with ligands. At the other extreme is Cd^{2+}, which is highly polarizable and tends to form more covalent-like bonds with coordinate ligands. The trend in the intrinsic primary ^{13}C isotope effects follows the trend in polarizability of the metal ion and suggests that the better the Lewis acidity of the metal ion, the more covalent-like the interaction with the carbonyl oxygen of oxalacetate and the more product-like the transition state for decarboxylation of the oxalacetate intermediate. However, this relationship is opposite that expected, since a better Lewis acid should delocalize more of the electron density from the carbonyl oxygen (and thus the C-3−C-4 bond) of oxalacetate. A greater degree of delocalization should lead to an earlier, not later, transition state for decarboxylation. In the nonenzymatic metal ion assisted decarboxylations, the Lewis acidity of the metal appears not to matter since the ^{13}C isotope effects are all essentially equal regardless of the metal (Grissom & Cleland, 1986). Since the Lewis acidity of the metal does not influence the transition-state structure in the nonenzymatic case, this suggests that the Lewis acidity of the metal ion alone is not what is responsible for the relationship observed in the enzyme-catalyzed decarboxylations. A possible explanation for the observed relationship between the metal ion activator and the ^{13}C isotope effects could be differences in the mode of binding of the oxalacetate intermediate with the different metal ions, leading to a decrease in the zero-point energy at the β-carboxyl; this aspect will require further study.

Mechanism with More Redox-Positive Dinucleotide Substrates. In the case of APAD the results presented in Table 6 indicate that the forward commitment value is equal to zero with Mn^{2+}. A comparison of the calculated intrinsic deuterium isotope effect of 2.7 with the measured value of ^DV reveals that within error they are equal and is consistent with the suggestion that the catalytic step limits the overall reaction. A comparison of the $^D(V/K)$ and ^DV values with Mn^{2+} and thio-NAD or PAAD reveals that, within error, $^D(V/K)$ is equal to ^DV. Although equal off rates for substrates and products will result in equal deuterium isotope effects

on V and V/K, given the similar trends in the isotope effect values for all the alternative dinucleotide substrates, these results suggest that the commitment values are equal to zero for thio-NAD and PAAD and suggest that these values are intrinsic ones. Taking an average of $^{D}(V/K)$ and ^{D}V as intrinsic values for thio-NAD and PAAD and the calculated intrinsic deuterium values for NAD and APAD, the following set of intrinsic deuterium isotope effect values are obtained: NAD (E_0' (V) = −320) = 3.9, thio-NAD (E_0' (V) = −285) = 3.4, PAAD (E_0' (V) = −262) = 3.2, and APAD (E_0' (V) = −258) = 2.7. A change to a dinucleotide substrate with a more positive redox potential and thus more thermodynamic driving force should lead to an earlier transition state for hydride transfer (Hermes et al., 1984; Scharschmidt et al., 1984; Grissom & Cleland, 1988). The trend in these intrinsic isotope effect values follows the change in redox potential for these dinucleotides. These results suggest that with Mn^{2+} the transition state for hydride transfer is early with NAD and becomes progressively earlier through the series of dinucleotide substrates. However, if a significant tunneling correction is also present with Mn^{2+} as the divalent metal ion activator, which is likely, little can be stated with certainty concerning the transition-state structure with Mn^{2+} and these alternative dinucleotide substrates.

None of the criteria that could be employed to support a stepwise mechanism with Mg^{2+} and NAD(P) as the dinucleotide substrate were satisfied when the dinucleotide substrate was changed to APAD (Karsten & Cook, 1994). The same result is observed with either Mn^{2+} or Cd^{2+} as the divalent metal ion activator where a switch from a stepwise mechanism with NAD to a concerted mechanism with APAD is also indicated. Assuming a concerted chemical mechanism with APAD, the calculated values for the intrinsic deuterium and intrinsic primary ^{13}C isotope effects with Mg^{2+} are $^{D}k_9$ = 4.9 and $^{13}k_9$ = 1.0175 (Karsten & Cook, 1994). Both of these values fall between those values calculated with Mn^{2+} or Cd^{2+} (Table 6). There is a consistent trend toward both larger intrinsic deuterium and ^{13}C isotope effects as the metal ion is varied from Mn^{2+} to Mg^{2+} to Cd^{2+}. The trend in these values follows the same correlation with size of the metal ion as seen with the intrinsic primary deuterium isotope effects and NAD. The most straightforward explanation for the intrinsic deuterium isotope effect values is that the early transition state observed with Mn^{2+} becomes later and tends toward a nearly symmetrical structure with Cd^{2+} (Jencks, 1969). This suggestion assumes that there is little contribution to these calculated values from coupled motion or hydrogen tunneling (Klinman, 1991), an assumption that almost certainly does not hold as suggested above for data obtained with NAD as the dinucleotide substrate. The early transition state for hydride transfer with Mn^{2+} is associated with a small intrinsic ^{13}C isotope effect, suggestive of a reactant-like structure to the transition state in a concerted mechanism and indicative of C−C bond breaking lagging behind C−H bond cleavage as previously suggested with Mg^{2+} as the metal ion activator (Karsten & Cook, 1994). The data suggest that as hydride transfer becomes later from Mg^{2+} through Cd^{2+}, there is a concomitant increase in bond breaking between C-3 and C-4 indicated by the larger primary intrinsic ^{13}C isotope effects.

Substrate Analog Studies. The malate analog survey was undertaken to determine which substrate functional groups are responsible for malate binding, to determine the stereochemistry involved in substrate/inhibitor binding, and to potentially define the geometry of the malate binding site. Oxalate is the tightest binding inhibitor discovered to date. Oxalate is an analog of enolpyruvate thought to be the product of the oxidative decarboxylation reaction catalyzed by malic enzyme which must then tautomerize to pyruvate (Schimerlik & Cleland, 1977; Kiick et al., 1986). Oxalate is a noncompetitive inhibitor vs malate as a result of binding to two forms of the E·NAD complex, only one of which binds malate (Park et al., 1989; Rajapaksa et al., 1993).

L-Lactate binds with greater affinity than D-lactate, indicating a strong preference for binding the L-isomer. Pyruvate binds with greater affinity to the enzyme form in which the general base, which accepts the proton from the hydroxyl group of malate during the course of oxidative decarboxylation, is protonated. When the protonation state of the enzyme is taken into account, the pH-independent K_m value for pyruvate[4] (Park et al., 1986) is about 30 μM, less than the K_i value for L-lactate. This observation suggests that the enzyme binds with greater affinity a planar carbonyl group compared to a hydroxyl group and suggests that differences resulting from specific hydrogen-bonding interactions to group(s) on enzyme between a hydroxyl and a carbonyl or differences in the relative orientation of the planar carbonyl within the active site relative to a hydroxyl group are significant.

A negative charge at C-3 or C-4 increases binding affinity (compare tartronate to L-lactate or glycolate) but is not essential since L-lactate binds with an affinity only 2−3 times less than malate (Table 1). The enzyme is quite forgiving with regard to changes in the region of C-3 of malate since the bulky analog *p*-hydroxyphenylpyruvate binds fairly well, indicating there is little steric hindrance around C-3. The β-carboxyl group of L-malate must be fairly loosely or flexibly bound to enzyme, allowing for movement of this functional group as the oxidative decarboxylation reaction proceeds. A flexibly bound β-carboxyl group could allow alternative binding orientations for the β-carboxyl group, consistent with the suggestion made by Karsten and Cook (1994) that a change from a stepwise mechanism with NAD to a concerted mechanism with APAD is the result of differences in substrate binding interactions with enzyme. The enzyme is also reasonably forgiving for substituents at C-2 since the enzyme will bind β-chloro-L-alanine with reasonable affinity, an analog which contains a positively charged amine functional group at C-2.

With Mn^{2+} as the divalent metal ion, thioglycolate binds greater than 2 orders of magnitude more tightly than glycolate. The apparent K_i value for thiomalate is similar to the K_m value for malate. If the metal−ligand dissociation constant with Mn^{2+} and thioglycolate can be used as a reasonable estimate of the thiomalate−Mn^{2+} dissociation constant, then the true K_i value for thiomalate would be considerably less than the apparent K_i value and of the same

[4] It is normally more appropriate to compare K_i values for inhibitors to K_d values for substrates rather than K_m values. In the case of pyruvate as substrate, this is the slow direction for the malic enzyme reaction, and the pyruvate K_m value is likely equal to K_d. For malate, the K_d values may be calculated from $[^{D}(V/K - 1)/(^{D}V - 1)]K_m$ according to Klinman and Mathews (1985). Since the $^{D}(V/K)$ values are nearly equal to the ^{D}V values, the difference in the calculated K_d values compared to the K_m values will be relatively small (about 40%), and the same general trend in the values will be retained.

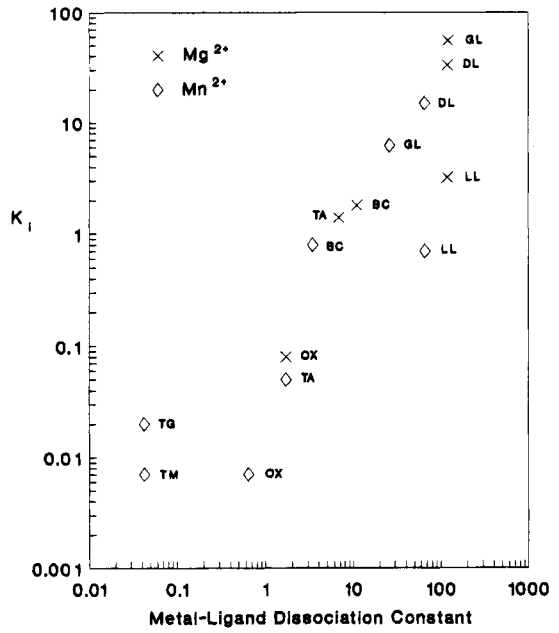

FIGURE 2: Comparison of enzyme inhibition constants (K_i) for various malate analogs vs the metal−ligand dissociation constants with either Mg^{2+} or Mn^{2+} as the divalent metal ion activator. The abbreviations used for the various malate analogs are as follows: TM, thiomalate; TG, thioglycolate; OX, oxalate; TA, tartronate; BC, β-chloroalanine; LL, L-lactate; GL, glycolate; and DL, D-lactate.

order of magnitude as thioglycolate (Table 1). The results with the thio analogs indicate that the enzyme prefers a thiol group over a hydroxyl functional group at C-2 despite the bulkier size of the sulfur. The above suggests that the metal ion may play a significant role in inhibitor/substrate binding. This trend is further substantiated by studying groups of analogs with Mg^{2+} or Mn^{2+}. There is a correlation in the magnitude of the metal−ligand dissociation constant and the K_i value for the inhibitor. Furthermore, each malate analog tested forms a more stable complex with Mn^{2+} than with Mg^{2+} and in turn gives a lower K_i value with Mn^{2+} as the divalent metal ion activator compared to Mg^{2+}. These trends are depicted in Figure 2. This same trend is generally observed for the malate K_m values through the series of different divalent metal ions used in Table 2 and is consistently seen with each dinucleotide substrate. These results all serve to strongly suggest that the metal ion provides a major determinant for malate binding. Data are entirely consistent with the observation made from kinetic studies that have shown that the divalent metal ion must bind to enzyme prior to malate or pyruvate for productive formation of the Michaelis complex (Park et al., 1984; Chen et al., 1988; Mallick et al., 1991).

Conclusions. These results indicate that the identity of the metal ion can effect both the transition state for hydride transfer and decarboxylation in the reaction catalyzed by the NAD−malic enzyme. For its effect on hydride transfer the most important characteristic of the metal ion appears to be the variation in size of the metal ion. In cases where the chemical mechanism is concerted, the transition-state structure correlates most closely with the size of the metal ion, which is similar to the effect seen on hydride transfer in the stepwise mechanism. Although the identity of the metal ion can influence the size of the intrinsic deuterium isotope effect, it does not appear to have an effect on the mechanism of oxidative decarboxylation. It is only a change from NAD(P) to a more redox-positive dinucleotide substrate that can elicit the change in mechanism from stepwise to concerted.

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