Stepwise versus Concerted Oxidative Decarboxylation Catalyzed by Malic Enzyme: A Reinvestigation†

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ABSTRACT: The NAD–malic enzyme catalyzes the divalent metal-ion-dependent oxidative decarboxylation of L-malate to yield CO_2, pyruvate, and the reduced dinucleotide. With Mg^{2+} as the divalent metal ion activator, primary deuterium and tritium isotope effects have been obtained with several different alternative dinucleotide substrates. The partitioning ratio of oxalacetate to malate and pyruvate has also been determined with either NAD or 3-acetylpyridine adenine dinucleotide (3-APAD). These data have been used to calculate estimates of commitment factors and intrinsic isotope effects for the NAD–malic enzyme reaction. The calculated values of the intrinsic ^{13}C and deuterium isotope effects with NADP are similar to the previously determined values for the chicken liver malic enzyme (Grissom, C. B., & Cleland, W. W. (1988) *Biochemistry 27,* 2927) and suggest that the transition-state structures are similar for the *Ascaris* NAD– and chicken liver NADP–malic enzymes. With NAD or NADP as the dinucleotide substrate, the data are all consistent with a stepwise chemical mechanism with oxidation of L-malate at C2 preceding decarboxylation of the bound oxalacetate intermediate. However, none of the data with the alternative dinucleotide substrates, 3-acetylpyridine adenine dinucleotide and 3-pyridine aldehyde adenine dinucleotide (PAAD), can be fit with satisfaction to the various criteria that support a stepwise mechanism with NAD(P). The mechanism with 3-APAD and PAAD is likely concerted. The most likely explanation for a change in the mechanism for oxidative decarboxylation from stepwise with NAD(P) to concerted with alternative dinucleotide substrates such as 3-APAD and PAAD is a difference in the configuration of bound malate when the different dinucleotide substrates are used.

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 to yield pyruvate, CO_2, and the reduced dinucleotide. The kinetic mechanism is steady-state random with the requirement that the divalent metal ion must bind prior to L-malate or pyruvate in order to form a catalytically competent complex (Park et al., 1984; Chen et al., 1988; Mallick et al., 1991). In the oxidative decarboxylation direction, pH-dependent changes in kinetic parameters indicate a requirement for enzymatic groups to be protonated and unprotonated for enzymatic activity (Kiick et al., 1986; Park et al., 1986). The unprotonated group is proposed to accept a proton from the hydroxyl group of L-malate, facilitating hydride transfer, the first step in a three-step chemical mechanism. Oxidation of L-malate would lead to an enzyme-bound oxalacetate intermediate which could then decarboxylate assisted by the divalent metal ion activator acting as a Lewis acid coordinated to the carbonyl oxygen. Decarboxylation of the oxalacetate intermediate would form the products CO_2 and enolpyruvate which would be protonated by an enzymatic general acid to form pyruvate.

The multiple isotope effect studies of Weiss et al. (1992) support a two-step mechanism for the oxidative decarboxylation of L-malate for the *A. suum* NAD–malic enzyme with the hydride-transfer step preceding decarboxylation with the dinucleotides NAD or NADP. Similar studies performed with alternative dinucleotide substrates yielded results consistent with a concerted chemical mechanism. However, with these alternative dinucleotide substrates, a two-step mechanism was supported by an increase in the observed deuterium isotope effect with a concomitant decrease in the observed ^{13}C isotope effect as the dinucleotide substrate is changed from NAD to 3-APAD. The deuterium and ^{13}C isotope effect data suggest that there is a concomitant loss of rate limitation of the decarboxylation step as hydride transfer becomes more rate limiting. In addition, the observation that oxalacetate partitions between malate and pyruvate with the alternative dinucleotides and that a substantial deuterium isotope effect is seen on the partition ratio (Grissom & Cleland, 1988) is also consistent with a two-step mechanism. The above apparently contradictory results, that is, concerted mechanism from multiple isotope effect studies but a stepwise mechanism from other considerations, were rationalized by Weiss et al. (1992) by proposing that there is expression of a β-secondary ^{13}C isotope effect on the hydride-transfer step. The hydride to be transferred was suggested to be trans to the β-carboxyl group with both out of the C1–C2–C3 plane of L-malate when bound to the enzyme. Since much of the electron density of the hydroxyl-group oxygen is delocalized by coordination to the divalent metal ion, electron density must be borrowed from the C3–C4 bond as the transition state for hydride transfer is approached. The enzyme enforces the out-

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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 positive charge is omitted for convenience); NADP, nicotinamide adenine dinucleotide 2′-phosphate; 3-APAD, 3-acetylpyridine adenine dinucleotide; PAAD, 3-pyridine aldehyde adenine dinucleotide.

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of-plane configuration at the C4 carboxyl group, leading to hyperconjugation with the concomitant bond-order decrease at C3–C4 and the β-secondary ^{13}C isotope effect on hydride transfer. If the measured ^{13}C isotope effect on V/K_{malate} observed with the alternative dinucleotides is predominantly an expression of a β-secondary isotope effect on hydride transfer rather than a primary effect, then the multiple isotope effect results could be explained since the observed ^{13}C isotope effect occurs in the same step as hydride transfer.

Values for the intrinsic isotope effects and commitment factors have been determined for the NADP–malic enzyme from chicken liver (Schimerlik et al., 1977; Hermes et al., 1984; Grissom & Cleland, 1985). With NADP and Mg^{2+} as the divalent metal ion activator, the intrinsic deuterium and ^{13}C isotope effects are reported to be 5.6 and 1.049, respectively (Grissom & Cleland, 1988).

In the present study, with Mg^{2+} as the divalent metal ion activator, the deuterium isotope effect on V and V/K_{malate} has been redetermined to within narrow limits by taking an average of multiple independent determinations. The tritium isotope effect on V/K_{malate} has been determined with NAD and several different alternative dinucleotide substrates. In addition, oxalacetate-partitioning experiments have been performed with NAD and 3-APAD. The combination of these data with the previously determined ^{13}C isotope effects (Weiss et al., 1991) has suggested that although the mechanism for oxidative decarboxylation *is* stepwise with NAD(P), it *changes* to concerted with alternative dinucleotides such as 3-APAD.

MATERIALS AND METHODS

Chemicals. NAD, NADP, and NADH were purchased from Sigma or Boehringer-Mannheim. Dinucleotide analogs, chicken liver malic enzyme, and horse liver alcohol dehydrogenase were purchased from Sigma. The dye ligand matrix gels were purchased from Amicon, and the ethanol-d_6 (99 atom % D) was from either Merck or ICN. Cyclohexanone was purchased from MCB, and the NaB^3H_4 (9.5 Ci/mmol) was purchased from Amersham. All other reagents were obtained commercially and were of the highest quality available.

Enzyme. Malic enzyme was purified from *A. suum* by the method of Allen and Harris (1981) or by a modification of this purification procedure. In the modified procedure, the initial two purification steps in the Allen and Harris procedure are retained but the remaining two steps are replaced by a tandem-column-affinity-chromatography step on Blue-B agarose and Orange-A agarose. An abbreviated purification protocol is as follows. The enzyme, which was previously taken through an ammonium-sulfate-fractionation step and a batch-DEAE-anion-exchange-purification step according to Allen and Harris (1981), was dialyzed overnight against two 1-L volumes of buffer containing 20 mM Mes, pH 6.8, 10 mM β-mercaptoethanol, and 0.5 mM EDTA. The enzyme was loaded onto a 2.5- × 16-cm Blue-B agarose column preequilibrated with buffer containing 20 mM Mes, pH 6.8, 5 mM β-mercaptoethanol, and 20 mM $MgSO_4$. The eluant from the Blue-B agarose column was directly applied to a 2.5- × 11-cm Orange-A agarose column. The tandem columns were washed with equilibration buffer until the absorbance at 280 nm of the column eluant was zero. The columns were then separated, and the malic enzyme was eluted from the Orange-A agarose column with equilibration buffer containing 500 mM NaCl. The active fractions were pooled and solid ammonium sulfate was added to 80% saturation. The precipitated protein was pelleted by centrifugation at 10000*g* for 25 min and, after removing the supernatant, was dissolved in a minimal volume of buffer containing 20 mM Hepes, pH 7.0, 2% glycerol (v/v), 5 mM β-mercaptoethanol, and 0.5 mM EDTA. The enzyme was dialyzed overnight against the same buffer and stored at −20 °C.

Preparation of Isotopically Labeled Substrates. L-Malate-2-*d* and A-side NADD or 3-APADD were prepared by the method of Viola et al. (1979). L-Malate-2-*t* was synthesized enzymatically from tritiated cyclohexanol in a 10-mL total volume containing 50 units of liver alcohol dehydrogenase, 50 units of malate dehydrogenase, 10 mM tritiated cyclohexanol, 1 mM NAD, 10 mM oxalacetate, and 10 mM Hepes, pH 8.0. After incubation at 25 °C for 7 h, additional oxalacetate was added to the reaction mixture to a final concentration of 5 mM. After storing overnight, the proteins were precipitated with CCl_4 and removed by filtration. The reaction mixture was applied to a 3- × 27-cm Dowex AG1-X8 column in the formate form. Malate-2-*t* was eluted from the column with a 300-mL linear gradient of formic acid from 0 to 4 N. Fractions containing L-malate-2-*t* were determined enzymatically. Malate-containing fractions were concentrated by rotary evaporation.

The tritiated cyclohexanol was prepared by NaB^3H_4 reduction of cyclohexanone. In a fume hood, 2 mL of cyclohexanone was added to 8 mL of H_2O on ice followed by slow addition of 1.5 g of $NaBH_4$ and 0.4 mg of NaB^3H_4 until the reaction was complete as indicated by the mixture becoming cloudy. The solution was allowed to stand for 15 min at room temperature. The remaining NaB^3H_4 was removed by adding 1 N HCl, and the cyclohexanol was then distilled on a condensing column.

Deuterium Isotope Effects. Enzyme assays were performed on a Gilford 260 spectrophotometer equipped with thermospacers connected to a circulating water bath to maintain a constant temperature of 25 °C. Assays were conducted in 1-cm light path cuvettes in a total volume of 1 mL. The rates of the reactions were followed by monitoring the absorbance change associated with the appearance of the reduced dinucleotide at 340 nm for NAD and NADP (ϵ_{340}, 6.22 mM^{-1} cm^{-1}), 363 nm for 3-APAD (ϵ_{363}, 9.1 mM^{-1} cm^{-1}), or 358 nm for PAAD (ϵ_{358}, 9.3 mM^{-1} cm^{-1}). Deuterium isotope effects were determined at pH 7.0 in 100 mM Hepes buffer by direct comparison of initial velocities varying L-malate-*h*(*d*) at a fixed saturating concentration of the free dinucleotide substrate ($20K_m$) and 20 mM free magnesium. *A. suum* NAD–malic enzyme utilizes the unliganded metal and reactants and not the chelate complexed form, and consequently, correction for the metal–chelate complex was made according to Park et al. (1984). The following dissociation constants were used, Mg–malate, 25.1 mM, and Mg-NAD, 19.1 mM (Martell & Smith, 1979). The dissociation constants for the alternative dinucleotides were assumed to be the same as those for Mg–NAD. Stock solutions of L-malate were calibrated by enzymatic endpoint assays using 2 units of chicken liver malic enzyme, 1 mM NADP, 2 mM $MgSO_4$, and 0.2 mM DTT at pH 9.0 according to the method of Cook et al. (1980).

Tritium Isotope Effects. Tritium isotope effects were determined after the method of Schimerlik et al. (1977). Assays were performed in a 1-mL total volume containing 100 mM Hepes, pH 7.0, 10 mM dinucleotide substrate, 2 mM tritiated malate (1.35×10^6 cpm/μmol), and 50 mM $MgSO_4$. 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 (1 × 18 cm) equilibrated with 25 mM Tris, pH 8.7. The reduced dinucleotide was eluted with a linear gradient of 0–250 mM NaCl in the same buffer, with the exception of NADPH(T) which was eluted with a linear gradient from 0–350 mM NaCl. Radioactivity was determined by liquid scintillation counting in a Beckman LS 7500 by adding 50- or 100-μL aliquots to 10 mL of scintillation cocktail. Those fractions containing constant specific radioactivities were used to determine the tritium isotope effects. Samples run to a percent completion other than 10% (8–19%) yielded isotope effects identical to the 10% conversion samples. Several complete conversion reactions were run, and the isolated reduced dinucleotide was found to have a specific radioactivity identical to malate-2-*t*. Therefore, the malate-2-*t* specific activity was used in subsequent calculations of the tritium isotope effects. As a test of our method, the tritium isotope effect was redetermined for the chicken liver malic enzyme which had been reported previously to be about 2.0 (Schimerlik et al., 1977; Grissom & Cleland, 1988). On the basis of three separate determinations, we calculated an average value of 2.02.

Oxalacetate Partitioning. Oxalacetate-partitioning experiments were performed according to the method of Grissom and Cleland (1985) with several modifications. In their procedure, Grissom and Cleland used glutamate–pyruvate transaminase to remove the pyruvate produced to prevent spurious absorbance at 282 nm as a result of Mg–pyruvate. In the present procedure, the glutamate–pyruvate transaminase was excluded since inclusion resulted in unreasonably high partitioning ratios. No significant A_{282} resulted from the pyruvate produced. Assays were carried out in 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}, 0.562 mM^{-1} cm^{-1} with 4 mM oxalacetate and 5 mM Mg^{2+}) under the exact partitioning conditions for each experiment. The concentrations of oxalacetate solutions were determined by enzyme end-point assays using malate dehydrogenase and NADH. In the partitioning experiments, changes in oxalacetate concentration were followed at 282 nm, the isosbestic point for conversion of NAD to NADH or 3-APAD to 3-APADH as determined by Grissom and Cleland (1985).[2] Partitioning reaction mixtures contained, in a 1-mL total volume, 100 mM Hepes, pH 7.0, 0.4 mM reduced dinucleotide (NADH or 3-APADH), 4 mM oxalacetate, 2 or 5 mM $MgSO_4$, and 1–5 units of NAD–malic enzyme. A control reaction mixture containing the same components with the exception of enzyme was used to monitor the background nonenzymatic rate of metal-ion-catalyzed degradation of oxalacetate. At higher concentrations of enzyme and with NADH as substrate, it was necessary to correct for a background rate due to a slight amount of contaminating malate dehydrogenase activity in the malic enzyme preparations. These control reactions were identical to the partitioning reactions with the omission of $MgSO_4$ and inclusion of 2.5 mM EDTA. Typical partitioning reactions were followed for 20 min, over which time the reaction rates were linear. The disappearance of oxalacetate was monitored at 282 nm while the formation of malate was followed by the decrease in absorbance at 340 nm (363 nm for 3-APADH) associated with the disappearance of reduced dinucleotide. After subtraction of appropriate background control rates, the partitioning ratio (r_H) expressed as pyruvate/malate was calculated from:

$$(d[\text{oxalacetate}]/dt)-(d[\text{NADH}]/dt) = d[\text{pyruvate}]/dt \quad (1)$$

$$(d[\text{pyruvate}]/dt)/(d[\text{NADH}]/dt) = r_H \quad (2)$$

The partitioning experiments using reduced dinucleotides deuterated in the *pro-R* position were performed in a manner identical to those with unlabeled dinucleotides.

Data Processing. Data were fit using BASIC versions of the Fortran computer programs of Cleland (1979). The data for deuterium isotope effects were fit using

$$v = VA/[K(1 + F_iE_{V/K}) + A(1 + F_iE_V)] \quad (3)$$

where V is the maximum velocity, K is the Michaelis constant for A, A is the variable substrate concentration, F_i is the fraction of deuterium in the labeled compound, and $E_{V/K}$ and E_V are the isotope effects minus 1 on V/K and V.

Tritium isotope effects were determined according to

$$^T(V/K) = \log(1 - f)/\log(1 - fR_p/R_o) \quad (4)$$

where f is the fraction of reaction, R_o is the specific radioactivity of malate-2-*t*, and R_p is the specific radioactivity of the reduced dinucleotide at f fractional conversion of substrate to product.

The isotope effect nomenclature of Northrop (1977) 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 ($^D(V/K)$, $^T(V/K)$, and $^{13}(V/K)$, etc.) and a following subscript refers to the constant isotopic species in a multiple isotope effect experiment, for example, $^{13}(V/K)_H$ reflects a ^{13}C isotope effect on V/K with malate-2-*h*.

Calculation of Intrinsic Isotope Effects and Commitment Factors. The kinetic mechanism of the *A. suum* malic enzyme is steady-state random but with the requirement that the divalent metal ion must bind to the enzyme prior to malate or pyruvate in order to form a catalytically competent complex. Under the conditions employed in the present work, the kinetic mechanism may be described as in Scheme 1 where M is Mg^{2+}, A is oxidized dinucleotide, B is malate, X is the enzyme-bound oxalacetate intermediate, and R is reduced dinucleotide.

Scheme 1

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

The rate constants k_5 and k_6 are for the malate-binding and -release steps, and the rate constants k_7 and k_8 represent any precatalytic conformational change leading to a complex poised for catalysis. The rate constants k_9 and k_{10} represent hydride-transfer and reverse-hydride-transfer steps, respectively. The rate constant k_{11} represents the decarboxylation step. The affinity of the enzyme for CO_2 is low (K_m = 50 mM), and the initial velocity studies of Mallick et al. (1991) suggest that CO_2 reacts upon collision with enol pyruvate, indicating there is no binding site for CO_2. These results indicate that the release of CO_2 is likely very fast making the decarboxylation step irreversible. For this mechanism, the equations for the isotope effects are as follows:

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

[2] The actual isosbestic point for conversion of NAD to NADH determined by Grissom and Cleland (1985) is 281.5 nm. A value of 282 nm was used in this work due to the technical limitations of the diode array spectrophotometer used in these studies.

$$^{T}(V/K) = [^{D}k_9^{1.44} + c_f + {}^{D}K_{eq}^{1.44}(c_r)]/[1 + c_f + c_r] \quad (6)$$

$$^{13}(V/K)_H = [^{13}k_{11} + (k_{11}/k_{10})(1 + c_f)]/[1 + (k_{11}/k_{10})(1 + c_f)] \quad (7)$$

$$^{13}(V/K)_D = [^{13}k_{11} + (k_{11}{}^{D}k_9/{}^{D}K_{eq}k_{10})(1 + c_f/{}^{D}k_9)]/[1 + (k_{11}{}^{D}k_9/{}^{D}K_{eq}k_{10})(1 + c_f/{}^{D}k_9)] \quad (8)$$

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}, $^{D}k_9$ is the intrinsic primary deuterium isotope effect, $^{13}k_{11}$ is the intrinsic primary ^{13}C isotope effect, and $^{D}K_{eq}$ is the deuterium isotope effect on the equilibrium constant which has been determined by Cook et al. (1980) to be 1.18. There are four equations and four unknowns (c_f, c_r, ^{13}k, and ^{D}k), and it is therefore possible to calculate an exact solution. However, the value of $^{13}k_{11}$ can be accurately calculated using eqs 13 and 14 (see Results), and thus, the value of $^{13}k_{11}$ was fixed, decreasing the number of unknowns in eqs 5–8 to three. A computer program was written to make these calculations, and the results are presented in Table 4.

When the dinucleotide used in the malic enzyme reaction has a redox potential more positive than NAD or NADP, the ^{13}C isotope effect on V/K increases when malate-2-*d* is used as a substrate (Table 2). Although this result is consistent with a concerted chemical mechanism, it was proposed by Weiss et al. (1991) that this result could be explained if there was an expression of a β-secondary ^{13}C isotope effect on the hydride-transfer step rather than a change to a concerted chemical mechanism with these alternative dinucleotides. These authors chose this mechanism to be more consistent with the trend in deuterium and ^{13}C isotope effects (Table 2) and the oxalacetate-partitioning data (Grissom & Cleland, 1988). If a β-secondary ^{13}C isotope effect is included on the rate constants k_9 and k_{10} of Scheme 1, eqs 5 and 6 will remain unchanged but eqs 7 and 8 will be modified in the following manner:

$$^{13}(V/K)_H = (^{13}k_9' + c_f + {}^{13}K_{eq}'\,{}^{13}k_{11}c_r)/(1 + c_f + c_r) \quad (9)$$

$$^{13}(V/K)_D = (^{13}k_9' + c_f/{}^{D}k_9 + {}^{13}K_{eq}'\,{}^{13}k_{11}\,{}^{D}K_{eq}c_r/{}^{D}k_9)/(1 + c_f/{}^{D}k_9 + {}^{D}K_{eq}c_r/{}^{D}k_9) \quad (10)$$

where $^{13}k_9'$ is the β-secondary ^{13}C isotope effect and $^{13}K_{eq}'$ is the β-secondary ^{13}C equilibrium isotope effect. The other parameters are as described previously. With eqs 9 and 10, four equations are again available, but two new unknowns for the secondary isotope effects have been added ($^{13}k_9'$ and $^{13}K_{eq}'$). As a result, an exact solution cannot be estimated.

Two additional experimentally determined parameters may be determined from oxalacetate-partitioning experiments. Grissom and Cleland (1985) have shown the utility of directly observing the partitioning of oxalacetate to malate and pyruvate and using these data to calculate intrinsic deuterium and ^{13}C isotope effects. The equations for oxalacetate partitioning, based on Scheme 1, are the following:

$$r_H = (k_{11}/k_{10})(1 + c_f) \quad (11)$$

$$r_D = (k_{11}/k_{10})(^{D}k_9 + c_f)/{}^{D}K_{eq} \quad (12)$$

where r_H is the partitioning ratio [pyruvate]/[malate] with unlabeled reduced dinucleotide and r_D is the partitioning ratio if determined using A-side-deuterated reduced dinucleotide.

Although an exact solution is not possible when a β-secondary ^{13}C isotope effect is included, with the use of eqs 5, 6, and 9–12, it is possible to calculate estimates for the commitment factors and the intrinsic isotope effects. The method employed to calculate the unknown parameters is described. The commitment factors and the intrinsic deuterium isotope effects were calculated using eqs 5, 6, 11, and 12 by the method described in Grissom and Cleland (1985). These calculated parameters were then used to determine values for the intrinsic ^{13}C isotope effects using eqs 9 and 10 by stepping through different entered values of the β-secondary ^{13}C equilibrium isotope effect and calculating $^{13}k_{11}$ and $^{13}k_9'$. The criterion employed for choosing the correctly calculated values for $^{13}K_{eq}'$ and $^{13}k_9'$ with NAD was convergence of the primary ^{13}C intrinsic isotope effect at a value of 1.052, the average value calculated for this parameter from eqs 13 and 14. Since for 3-APAD eqs 13 and 14 do not provide agreement for estimates of $^{13}k_{11}$, a different criterion was employed for choosing correctly calculated values for $^{13}k_9'$ and $^{13}k_{11}$. For 3-APAD, the criterion for choosing the correctly calculated values for $^{13}k_9'$ and $^{13}k_{11}$ was convergence of $^{13}K_{eq}'$ to a value of 1.0. $^{13}K_{eq}'$ is likely to be small and nearly equal to 1.0, and if the same criterion is employed with NAD, as $^{13}K_{eq}'$ converges to a value of 1.0, $^{13}k_{11}$ converges to a value of 1.052. A computer program has been written to make these calculations, and the calculated values are reported in Table 5.

If Scheme 1 is modified to reflect a concerted mechanism, then k_9 will represent both the deuterium- and ^{13}C-isotope-sensitive steps, while k_{10} will be zero since decarboxylation is irreversible and k_{11} will no longer be present; thus, there will be no reverse commitment, and the forward commitment will remain unchanged. There are now three unknown parameters, $^{D}k_9$, $^{13}k_9$, and c_f. Using eqs 5–8, modified to reflect a concerted mechanism, an absolute solution is possible. A computer program has been written to make these calculations, and the calculated values are reported in Table 6.

RESULTS

Purification. When *A. suum* malic enzyme is isolated by the purification procedure of Allen and Harris (1982), the resulting enzyme typically contains 0.5–1% contaminating malate dehydrogenase activity. For the great majority of kinetic studies, this level of contaminant poses no problem. The oxalacetate-partitioning experiments reported here, however, require large amounts of malic enzyme in the assay mix (1–5 units), and consequently, even a 0.5% contamination with malate dehydrogenase activity interferes with these experiments. Therefore, a new purification scheme was needed that eliminated or greatly reduced the MDH activity. The dye ligand affinity gels from Amicon (Green-A, Red-A, Blue-B, Blue-A, and Orange-A agarose) were screened for their ability to bind *A. suum* MDH and malic enzyme. Both enzymes bound to each dye ligand gel tested with the exception of Blue-B which displayed no apparent affinity for malic enzyme yet bound a significant fraction of the MDH activity in the samples tested. A Blue-B–Orange-A-tandem-column-chromatography step was subsequently incorporated into a purification procedure for malic enzyme simpler and faster than the previously used method of Allen and Harris (1982). The resulting purified enzyme has a specific activity equivalent to the enzyme purified by the older procedure and appears to be >90% pure based on visual inspection of Coomassie blue stained polyacrylamide gels. The purified enzyme does still typically contain about 0.2% MDH activity, but this can be reduced by one or two additional passes through the Blue-B

Table 1: Purification of Mitochondrial NAD–Malic Enzyme from *A. suum*[a]

	total units	total protein[b] (mg)	specific activity[c]	yield (%)
crude extract	386	2817	0.14	100
ammonium sulfate fractionation (30–50%)	368	694	0.53	95
DEAE–cellulose	307	151	2.03	80
Blue-B–Orange-A agarose tandem chromatography	193	6	32	50

[a] Starting with 100 g of *A. suum*. [b] Protein concentrations are determined by the method of Bradford (1976). [c] μmol/min/mg of protein. Assay conditions: 100 mM Hepes, pH 7, 50 mM L-malate, 0.5 mM NAD, and 5 mM $MnSO_4$.

agarose column to a level of less than 0.01% of the malic enzyme activity, and that level is suitable for the partitioning experiments. The purification procedure is summarized in Table 1.

Isotope Effects. In Table 2 are presented the deuterium and tritium isotope effect data. The individual values for ^{D}V and $^{D}(V/K)$ reported in Table 2 are the mean averages of at least six separate determinations. The deuterium isotope effects on V/K_{malate}, $V/K_{dinucleotide}$, and V_{max} for the NAD–malic enzyme have been reported to be finite and equal within experimental error with Mg^{2+} as the divalent metal ion activator (Weiss et al., 1991). The values reported in this study show that ^{D}V is apparently slightly greater than or equal to $^{D}(V/K_{malate})$, dependent on which dinucleotide substrate is used. The differences in the V and V/K_{malate} isotope effects are average values based on multiple determinations. The larger value of ^{D}V compared to $^{D}(V/K_{malate})$, in those cases where it is observed, may be real and not the result of a contaminant in the L-malate-2-*d* preparations. If the latter were true, there would be a larger ^{D}V than $^{D}(V/K_{malate})$ with all dinucleotide reactants used, and this is not the case as seen in Table 2. In all cases, however, the values are within error equal to those measured previously (Weiss et al., 1991). Consistent with the findings in this study is the deuterium isotope effect of about 2 on V determined with NAD by Rajapaksa et al. (1993) during the course of pre-steady-state kinetic studies on the *A. suum* malic enzyme. The greater isotope effect on V compared to that on V/K_{malate} is still consistent with a steady-state random kinetic mechanism for the NAD–malic enzyme but one in which malate release from the central E:NAD:Mg:malate complex is slower than isomerization of the E:NAD complex (Rajapaksa et al., 1993). A tritium isotope effect of about 2.5 is observed with NAD or NADP and increases to about twice this value with 3-PAAD or 3-APAD.

Oxalacetate Partitioning. The partitioning data obtained with NADH or 3-APADH are summarized in Table 3. The partitioning ratio with NADH as the dinucleotide substrate is 0.4 and increases to 2.6 with 3-APADH. The ratio of pyruvate to malate increases when a deuterated dinucleotide is used in these experiments, the result of slowing down the reverse-hydride-transfer step. The deuterium isotope effect on the partitioning ratio r_H/r_D is 0.4 for NADH(D) and 0.25 for 3-APADH(D). The primary intrinsic ^{13}C isotope effect may be calculated using the partitioning ratios (Grissom & Cleland, 1985) and eqs 13 and 14 to obtain two separate values.

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

$$^{13}k_{11} = {}^{13}(V/K)_D + r_D({}^{13}(V/K)_D - 1) \quad (14)$$

With the values reported in Tables 2 and 3, eqs 13 and 14 give respectively $^{13}k_{11} = 1.049$ and $^{13}k_{11} = 1.055$ for NAD. There is reasonable agreement between these calculated values for $^{13}k_{11}$ with an average value equal to 1.052. In contrast, if eqs 13 and 14 are used to calculate $^{13}k_{11}$ with 3-APAD, significantly different values of $^{13}k_{11} = 1.0252$ and $^{13}k_{11} = 1.1368$ are obtained, with the latter being unreasonably high.

DISCUSSION

Mechanism with NAD(P) as the Dinucleotide Substrate. A stepwise chemical mechanism as depicted in Scheme 1 in which hydride transfer precedes decarboxylation has long been assumed for malic enzyme (Viega Salles & Ochoa, 1950; Hsu, 1970; Tang & Hsu, 1973). With NAD(P) as dinucleotide substrate in the NAD–malic enzyme reaction, several criteria may be used to support this assumption. (1) The multiple isotope effects reported by Weiss et al. (1991) and reproduced in Table 1 show that $^{13}(V/K_{malate})$ decreases when malate-2-*d* is used as the substrate compared to unlabeled malate. This result suggests that as the hydride-transfer step is slowed down by deuterium substitution, there is a concomitant increase in the forward commitment factor and a resulting decrease in the observed value of $^{13}(V/K_{malate})$. (2) Equations 13 and 14, which may be used to calculate primary intrinsic ^{13}C isotope effects from partitioning ratios, were derived on the basis of a stepwise mechanism with hydride transfer preceding decarboxylation. These equations should give agreement between the calculated values of $^{13}k_{11}$ with NADH or NADD, and this is what is observed with the NAD–malic enzyme. (3) If hydride transfer precedes decarboxylation, Hermes et al. (1982) showed that the following equality should be satisfied.

$$({}^{13}(V/K_{malate})_H - 1)/({}^{13}(V/K_{malate})_D - 1) = {}^{D}(V/K_{malate})/{}^{D}K_{eq} \quad (15)$$

With the values reported in Table 2 for the ^{13}C and deuterium isotope effects and a value of 1.18 for $^{D}K_{eq}$ (Cook et al., 1980),

Table 2: Primary Deuterium, Tritium, and ^{13}C Isotope Effects for the *A. suum* NAD–Malic Enzyme with Mg^{2+} as the Divalent Metal Ion Activator[a]

parameter	NAD	NADP	PAAD	3-APAD
E_o' (volts)	–0.32	–0.32	–0.262	–0.258
^{D}V	2.02 ± 0.07	2.11 ± 0.33	3.37 ± 0.70	2.82 ● 0.48
$^{D}(V/K)$	1.57 ± 0.07	1.70 ± 0.22	2.69 ± 0.52	2.56 ± 0.44
$^{T}(V/K)$	2.55 ± 0.23	2.54 ● 0.06	5.22 ± 0.55	4.54 ± 0.53
$^{13}(V/K)_H$[b]	1.0342 (0.0002)[c]	1.0399 (0.0007)	1.0028 (0.0002)	1.007 (0.0001)
$^{13}(V/K)_D$	1.0252 (0.0001)	1.0279 (0.0004)	1.0053 (0.0004)	1.0120 (0.0002)

[a] All assays were carried out as described in Materials and Methods. The deuterium isotope effects are an average of at least six separate determinations, and the tritium isotope effects are an average of at least five separate determinations. [b] The ^{13}C isotope effects are from Weiss et al. (1991). [c] Values in parentheses are ± standard error for the ^{13}C effects.

Table 3: Pyruvate/Malate Partition Ratios for Oxalacetate Partitioning for the NAD–Malic Enzyme from *A. suum*[a]

dinucleotide	r_H	r_D	r_H/r_D
NADH(D)	0.4 ± 0.2	1.2 ± 0.2	0.4 ± 0.2
3-APADH(D)	2.6 ± 0.4	10.4 ± 0.4	0.25 ± 0.04

[a] Partition ratios are the mean average of at least three separate determinations for 3-APADH(D) and at least eight separate determinations for NADH(D).

Table 4: Commitment Factors and Intrinsic Isotope Effects for NAD–Malic Enzyme with NAD(P) and Mg^{2+} as the Divalent Metal Ion Activator[a]

dinucleotide	c_f	c_r	$^Dk_9$	$^{13}k_{11}$
NAD	6.6	14.3	10.8	1.052
NADP	0.9	6.2	5.6	1.052

[a] The value of $^{13}k_{11}$ is an average value calculated from eqs 13 and 14. The other parameters were calculated on the basis of setting $^{13}k_{11}$ = 1.052.

Table 5: Commitment Factors and Intrinsic Isotope Effects for the NAD–Malic Enzyme from *A. suum*

dinucleotide	c_f	c_r	$^Dk_9$	$^{13}K_{eq}'$	$^{13}k_9'$	$^{13}k_{11}$
NAD	5.8	13	10.9	1.0	1.0009	1.052
3-APAD	0.9	0.5	5.0	1.0	1.0152	1.0034

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

dinucleotide	c_f	$^Dk_9$	$^{13}k_9$
3-APAD	1.49	4.9	1.0175
PAAD	2.20	6.4	1.0089

the equality gives 1.36 = 1.33 when NAD is the dinucleotide substrate and 1.43 = 1.44 when NADP is used. These results are consistent with a two-step chemical mechanism with hydride transfer preceding decarboxylation. The errors on the experimentally determined $^{13}(V/K_{malate})$ values are small and indicate that these values are well determined. Since the equality expressed in eq 15 is well satisfied using the average $^D(V/K_{malate})$ value, this serves to strongly suggest that the average values for the deuterium isotope effects are also very well determined. These results all support a stepwise chemical mechanism for the NAD–malic enzyme when NAD(P) is the dinucleotide substrate.

Estimates of intrinsic isotope effects and commitment factors calculated according to the stepwise mechanism of Scheme 1 for NAD(P) are shown in Tables 4 and 5, while those calculated according to the concerted mechanism (see above) are shown in Table 6. Commitment factors are reduced significantly as the redox potential of the dinucleotide substrate becomes more positive. The reverse commitment decreases from about 14 with NAD to a value below 1, while the forward commitment also decreases from a value of around 6 to about 1. The intrinsic deuterium isotope effect is determined within relatively narrow limits for each dinucleotide. The ^Dk value is around 5.6 with NADP, increases to about 6.5 with 3-PAAD, and then decreases to 4.9 with 3-APAD. The value of the intrinsic deuterium isotope effect of about 5.6 with NADP is essentially identical to the value of 5.6 reported by Grissom and Cleland (1988) for the chicken liver NADP–malic enzyme using NADP and Mg^{2+}. The larger value of about 11 determined for ^Dk with NAD will be discussed below.

The calculated intrinsic deuterium isotope effects may be compared to the values determined for the chicken liver NADP–malic enzyme. With Mg^{2+} as the divalent metal ion activator, Grissom and Cleland (1988) determined an intrinsic deuterium isotope effect of 5.6 with NADP as the dinucleotide substrate. This value is the same as the intrinsic isotope effect of 5.6 with NADP calculated for the NAD–malic enzyme from *A. suum* and suggests similar transition-state structures for both of these enzymes as suggested previously (Weiss et al., 1991). In contrast, an intrinsic isotope effect of about 11 is calculated for the NAD–malic enzyme with the dinucleotide substrate NAD. Given the errors on the experimentally determined parameters and the extreme sensitivity of the calculated value of $^Dk_9$ on the precision of the experimental parameters resulting from the exponential relationship between the tritium and deuterium isotope effects, it is not possible to state with confidence that this value is really different from the value determined with NADP. However, if there is a real difference in $^Dk_9$ with NAD compared to with NADP, it suggests that there is a significant tunneling contribution to the reaction with NAD since a value for $^Dk_9$ of 11 is greater than the semiclassical limit for a deuterium isotope effect. The difference in the intrinsic isotope effects could not be explained on the basis of redox potential differences, and therefore, the most likely explanation would be a difference in the mode of binding of NAD to the enzyme compared to that of NADP. The difference in binding of the dinucleotides may be reflecting in the nearly 10-fold increase in K_{NADP} compared to K_{NAD} (Weiss et al., 1991).[3]

The average value of the primary ^{13}C intrinsic isotope effect is 1.052 with NAD and is essentially equal to the value of 1.049 reported previously (Grissom & Cleland, 1988) for the NADP–malic enzyme from chicken liver. The agreement between these two values for the intrinsic ^{13}C isotope effect suggests that both enzymes catalyze the decarboxylation reaction in a very similar manner. The ^{13}C isotope effect value reported for the nonenzymatic Mg^{2+}-ion-catalyzed decarboxylation of oxalacetate is 1.049 (Grissom & Cleland, 1986). These results suggest similar transition states for decarboxylation of oxalacetate catalyzed by NAD- and NADP–malic enzymes and the nonenzymatic reaction. It has been postulated, on the basis of these similar isotope effects, that the role of the enzyme in decarboxylation is to position the metal ion near the carbonyl of the enzyme-bound oxalacetate and to stabilize the oxalacetate intermediate (Grissom & Cleland, 1988).

Stepwise Oxidative Decarboxylation Mechanism with Alternative Dinucleotide Substrates. With alternative dinucleotide substrates, Weiss et al. (1991) observed that the multiple isotope effect results did not conform to a stepwise oxidative decarboxylation mechanism but were more consistent with a concerted mechanism. Rather than proposing a change in chemical mechanism from stepwise to concerted, they proposed, based on considerations discussed above, the existence of a β-secondary ^{13}C effect on the hydride-transfer step. The data obtained with 3-APAD presented here have been fit using a model (eqs 5, 6, 9, and 10) that includes a β-secondary ^{13}C isotope effect on the hydride-transfer step (Table 5). Data obtained with NAD fit using the same model are included for comparison.

Calculated values of the intrinsic β-secondary ^{13}C isotope effect are about 1–2% with 3-APAD as the dinucleotide

[3] A tunneling correction may also be present with NADP as the dinucleotide substrate; there may simply be a difference in the degree of tunneling that is involved in the hydride-transfer reactions catalyzed with NAD or NADP.

substrate.[4] From ab initio and semiempirical calculations, model studies suggest that no more than a 20% reduction in bond order between C3 and C4 is required to produce a secondary isotope effect of about 1% (Weiss et al., 1991). The calculated value of 1.0152 with 3-APAD would require a larger reduction in the bond order. The most likely reason for the larger value of $^{D}(V/K_{malate})$ obtained with 3-APAD compared to that obtained with NAD is a result of a decrease in the c_r term as reverse hydride transfer becomes less favored as the thermodynamics favor oxidation. In agreement, a larger oxalacetate partitioning ratio (2.6) is observed with 3-APAD, that is, pyruvate formation is favored, and this would explain the low observed value of $^{13}k_{11}$. However, the small calculated intrinsic ^{13}C isotope effect (1.0034) with 3-APAD, which perhaps actually could be 1.0, suggests there is little bond breaking in the transition state for decarboxylation. If the stepwise model holds, the ^{13}C isotope effect measured on V/K_{malate} would most likely predominantly be an expression of the β-secondary ^{13}C isotope effect on the hydride-transfer step as argued by Weiss et al. (1991) rather than an expression of the primary ^{13}C isotope effect on the decarboxylation step. The latter does not, however, explain the dramatic reduction in the intrinsic ^{13}C isotope effect. In a stepwise mechanism, decarboxylation of the oxalacetate intermediate will occur in a step discrete from the hydride-transfer step. The very small calculated value for the primary intrinsic ^{13}C isotope effect with 3-APAD assuming a stepwise mechanism is very small compared to the value of 5.2% with NAD, suggesting that the identity of the dinucleotide substrate can have a significant influence on the transition state for decarboxylation. This does not seem very reasonable. Taken together, these results suggest that, at the very least, there is a significant change in transition-state structure as the dinucleotide is changed from NAD to 3-APAD. A mechanism more consistent with these data obtained with 3-APAD and PAAD is a concerted oxidative decarboxylation.

Concerted Oxidative Decarboxylation Mechanism with Alternative Dinucleotide Substrates. Values for $^{D}k_9$, $^{13}k_{11}$, and c_f were calculated using eqs 5–8 modified to reflect a concerted oxidative decarboxylation reaction (see Materials and Methods), Table 6, for 3-APAD and PAAD. A change in the dinucleotide substrate to one with a greater 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 the calculated intrinsic isotope effects for the various dinucleotide substrates suggests that this may be the case with the value of 6.4 (Table 6) estimated for PAAD ($\epsilon^{o\prime}$, −262 mV) greater than the value of 5.5 estimated for NAD(P) (Table 4) ($\epsilon^{o\prime}$, −320 mV) and the value of 4.9 estimated for 3-APAD (Table 6) ($\epsilon^{o\prime}$, −258 mV). The value of the intrinsic deuterium isotope effect is expected to be maximal for a symmetric transition state and decrease from the maximal value as the transition state becomes early or late (Westheimer, 1961). However, an earlier transition state for hydride transfer would not seem to provide a better driving force for C3–C4 bond cleavage. A possible explanation for the switch from a stepwise to a concerted mechanism is a change in the binding of malate allowed as a result of the conformational change induced by the different dinucleotide substrates, that is, NAD(P) may provide a conformation such that malate binds with C4 in the C2–C3 plane, a configuration not amenable to decarboxylation concomitant with oxidation. Once oxidation has occurred, the oxalacetate intermediate is bound with C4 out of the C2–C3 plane, facilitating decarboxylation. With 3-APAD, however, malate may be bound with C4 out of the C2–C3 plane and trans to the hydride to be transferred to C4 of the nicotinamide ring of the dinucleotide substrate, allowing a concerted oxidative decarboxylation. The above suggestion requires distinctly different active site conformations induced by the different dinucleotide substrates. This aspect is currently being tested using CD and fluorescence spectrophotometry.

There is a significant decrease in the estimated value of the intrinsic ^{13}C isotope effect as the dinucleotide substrate is changed, going from a value of about 1.05 with NAD(P) to values of about 1.018 with 3-APAD and 1.009 with PAAD. The decrease in the value of the intrinsic ^{13}C isotope effect would not appear to be consistent with the change in the redox potential of the dinucleotide substrate which should give less of a decrease in the electron density at C3–C4 in the transition state. The latter should give a late, not early, transition state. Thus, if the mechanism is truly concerted with respect to the oxidative decarboxylation of malate as it appears, cleavage of the C3–C4 bond may lag behind cleavage of the C2–H bond, that is, the transition state may be concerted but asynchronous.

Two lines of reasoning had been used to suggest that there is an expression of a β-secondary ^{13}C isotope effect in a two-step chemical mechanism with 3-APAD rather than a switch from a stepwise to a concerted mechanism. First, as the observed deuterium isotope effect increases, there is a concomitant decrease in the ^{13}C isotope effect. However, this observation could simply be a reflection of the difference in the relative magnitudes of the two isotope effects in a two-step chemical mechanism compared to in a concerted mechanism as discussed above. The second line of reasoning in support of a two-step mechanism with 3-APAD is the observation that oxalacetate still partitions to malate and pyruvate with this dinucleotide. However, in this case, if malic enzyme is incubated with Mg^{2+}, oxalacetate, and 3-APADH, the enzyme is essentially being forced to do something with oxalacetate. As long as oxalacetate can bind to the enzyme in a productive mode, partitioning to malate and pyruvate could occur even if it is not along the reaction pathway for the overall reaction. Consequently, a concerted chemical mechanism is a possibility with 3-APAD and, also, with the other alternative dinucleotides with redox potentials more positive than NAD(P).

Are there other indications that a change from a stepwise to a concerted oxidative decarboxylation of malate occurs with malic enzyme? A thiol group in or near the malate-binding site of the *Ascaris* malic enzyme may be modified to form an active thiocyanate enzyme (Gavva et al., 1991). The major effect of this modification on the kinetic parameters is an approximately 10-fold increase in K_{malate}, that is, malate binds differently to the modified than to the native enzyme even when NAD(P) is the dinucleotide substrate. For the thiocyanate enzyme, with Mg^{2+} and NAD as the dinucleotide substrate, the equality of eq 8 is not satisfied and suggests that the reaction does not proceed in a stepwise fashion. In addition, the multiple isotope effect results lead to only a small decrease in $^{13}(V/K_{malate})$ of less than 0.2% when deuterated malate is the substrate ($^{13}(V/K_{malate})_H = 1.0191$; $^{13}(V/K_{malate})_D = 1.0172$). This can be compared to the average change of about 1% observed in these values for the unmodified malic enzyme from *A. suum* or the chicken liver enzyme with NAD(P) (Weiss et al., 1991). The nearly equal multiple ^{13}C

[4] The calculated value of the β-secondary intrinsic ^{13}C isotope effect with NAD is small (1.0009) and, therefore, would not contribute significantly to the measured value of $^{13}(V/K_{malate})$.

isotope effects and the inability to satisfy eq 8 are consistent with a change from a stepwise mechanism with the unmodified malic enzyme to a concerted mechanism with the thiocyanate enzyme. Since the major effect of the thiocyanate modification is on the binding interactions of malate with enzyme, data suggest that the binding interactions that the enzyme uses to enforce a stepwise chemical mechanism are lost in the thiocyanate enzyme as a result of the difference in binding and that this change leads to a switch from a stepwise to a concerted mechanism.

It seems likely that with the unmodified malic enzyme a change occurs from a stepwise oxidative decarboxylation with NAD(P) to a concerted mechanism when 3-APAD or PAAD is the dinucleotide substrate. A change in configuration of bound malate with these alternative dinucleotides compared to that with NAD(P) may be the reason for the change in chemical mechanism from stepwise to concerted. The same change is apparently observed when the thiocyanate-modified malic enzyme is used with NAD(P).

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