Determination of the Chemical Mechanism of Malic Enzyme by Isotope Effects†

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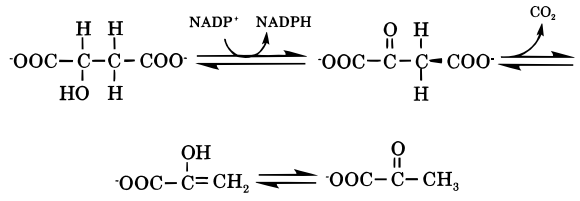
ABSTRACT: Carbon-13 isotope effects have been determined for all four carbons of L-malate as a substrate for chicken liver malic enzyme, using either NADP or acetylpyridine−NADP as the other substrate. The effect of deuteration at C2 of malate was then used to tell whether the chemical mechanism of this oxidative decarboxylation was stepwise, with oxaloacetate as an intermediate, or concerted. With NADP, the ^{13}C isotope effects at C3 and C4 both decrease with deuteration of malate, showing a stepwise mechanism, as previously determined [Hermes, J. D., Roeske, C. A., O'Leary, M. H., & Cleland, W. W. (1982) *Biochemistry 21*, 5106−5114]. With acetylpyridine−NADP, however, the ^{13}C isotope effects at both C3 and C4 increase with deuteration of malate. While the increase at C4 could be explained by a secondary ^{13}C isotope effect on hydride transfer, the increase at C3 proves that the chemical mechanism has changed to a concerted one, presumably because hydride transfer is more rate-limiting and the overall equilibrium constant is more favorable by 2 orders of magnitude. The transition state for this concerted reaction is asynchronous, however, with an intrinsic deuterium isotope effect of ∼5 and a ^{13}C isotope effect of only 1.010−1.015. Equilibrium ^{13}C isotope effects for conversion of carbons 2, 3, and 4 of malate to pyruvate or CO_2 are 1.010, 1.011, and 0.988, respectively. Measured ^{13}C isotope effects at C2 of malate are slightly inverse, but no explanation for this is obvious. With NADP, deuterium isotope effects at C3 of 1.17 and 1.08 for di- and monodeuteration and an increase in the ^{13}C isotope effect at C4 upon dideuteration at C3 are consistent with a stepwise mechanism with the deuterium isotope effect at C3 being only on the decarboxylation step. Smaller deuterium isotope effects of 1.03−1.04 from dideuteration at C3 with acetylpyridine−NADP are consistent with a concerted but asynchronous mechanism where C−C cleavage is not far advanced in the transition state.

Malic enzyme, which is found in all plants, animals, and bacteria, catalyzes the biologically important reaction

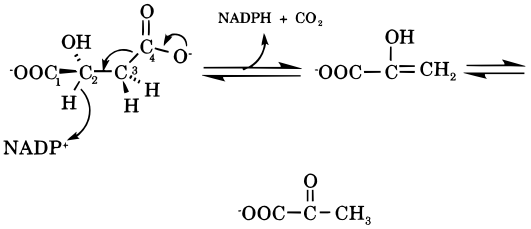
$$\text{L-malate} + \text{NADP}^+ \overset{Mg^{2+}}{\rightleftharpoons} CO_2 + \text{pyruvate} + \text{NADPH} \quad (1)$$

There are two plausible chemical mechanisms for the conversion of malate to pyruvate catalyzed by malic enzyme. The first is a stepwise mechanism in which hydride transfer precedes decarboxylation via the intermediate oxaloacetate, as shown in reaction 2.



(2)

This mechanism is supported by the fact that oxaloacetate has been shown to partition between malate and pyruvate with all the dinucleotide substrates tested thus far (Grissom & Cleland, 1988). The second plausible mechanism is a concerted one in which hydride transfer occurs simultaneously with decarboxylation, as shown in reaction 3. A stepwise mechanism in which decarboxylation precedes hydride transfer has no chemical precedent.



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The multiple isotope effect method was employed by Hermes et al. (1982) to distinguish between the two plausible chemical mechanisms. In this technique the deuterium kinetic isotope effect is measured for the hydrogen that participates in hydride transfer, while the ^{13}C isotope effect is measured for the carbon of the carboxyl leaving group. If neither hydride transfer nor decarboxylation is completely rate-limiting, a stepwise mechanism can be differentiated from a concerted mechanism by measuring the ^{13}C isotope effect with both protiated and deuterated malate. Deuteration makes the hydride transfer more rate-limiting so that in a stepwise mechanism the ^{13}C kinetic isotope effect with deuterated malate will decrease relative to that with unlabeled malate. In a concerted mechanism, however, substitution of deuterium for protium will slow the chemical step of the reaction with respect to the binding and conformational steps, resulting in an enhanced ^{13}C kinetic isotope effect if decarboxylation is not totally rate-limiting and no change if the chemistry is totally rate-limiting.

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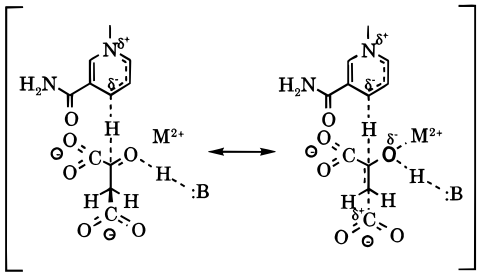
Multiple isotope effect studies with NADP as the substrate have indicated that both the hydride transfer and decarboxylation are partially rate-limiting and that malic enzyme catalyzes the malate to pyruvate reaction by a stepwise mechanism. A decrease from 1.0302 ± 0.0005 to 1.0250 ± 0.0007 in the ^{13}C isotope effect at C4 was found when malate-2-*d* was used as the substrate in place of unlabeled malate (Hermes et al., 1982). However, later studies using alternative dinucleotide substrates suggested a concerted mechanism. An increase in the ^{13}C isotope effect at C4 was found when malate-2-*d* was used as the substrate in place of malate for reactions using thionicotinamide adenine dinucleotide (thio-NAD; from 1.0102 ± 0.0002 to 1.0180 ± 0.0003), 3-acetylpyridine adenine dinucleotide (APAD; from 1.0070 ± 0.0001 to 1.0120 ± 0.0002), and 3-pyridinealdehyde adenine dinucleotide (PAAD; from 1.0028 ± 0.0002 to 1.0053 ± 0.0004) (Weiss et al., 1991). Later work by Karsten and Cook (1994) and Karsten et al. (1995) also supported a change from a stepwise mechanism with NAD(P) to a concerted one with alternate nucleotide substrates.

There is another possible explanation besides a concerted mechanism for the data observed with the alternative dinucleotide substrates. If the mechanism is stepwise, there may be a β-secondary ^{13}C isotope effect on the hydride transfer step (Weiss et al., 1991). This β-secondary effect would be the result of a hyperconjugation-like effect on the β-carboxyl group of malate as the transition state for hydride transfer is approached. The electrons that would normally form the carbonyl group would be to a large extent held on the oxygen by the coordinated metal ion. The developing positive charge at C2 as the hydride is removed could be satisfied by borrowing electron density from the C3−C4 bond, as shown in resonance forms 4. This decrease in the



(4)

C3−C4 bond order could not be matched by increased C−O bond order in the carboxyl group because the geometry of that group would still be trigonal (C−O bond order can increase only when the three atoms are collinear). As a result there would be a decrease in the fractionation factor of C4 accompanying hydride transfer.

This secondary ^{13}C isotope effect would not be seen with NADP as the substrate, since decarboxylation is much more rate-limiting than hydride transfer, and thus the ^{13}C isotope effect on decarboxylation dominates the observed isotope effect. With the alternate nucleotide substrates, however, hydride transfer is the major rate-limiting step, and thus the secondary ^{13}C isotope effect on this step would make up most of the observed isotope effect.

While the possibility of hyperconjugation causes some ambiguity in interpreting the ^{13}C isotope effects at C4, there would be no such problem with the ^{13}C isotope effects at C3, since any hyperconjugation effect would increase the C2 to C3 bond order to the same extent that it decreases the C3 to C4 bond order so that no secondary ^{13}C isotope effect on hydride transfer is expected.

It was the goal of the present work to determine if the chemical mechanism of malic enzyme changes from stepwise to concerted when NADP is replaced by alternative dinucleotides by measuring ^{13}C isotope effects at C3 with deuterated and unlabeled malate. We also report the deuterium isotope effects at C3 and the effect of deuteration at C3 on the ^{13}C isotope effect at C4.

EXPERIMENTAL PROCEDURES

Materials. Malate, oxidized glutathione, reduced glutathione, dithiothreitol, oxaloacetate, chicken liver malic enzyme, glutathione reductase, alcohol dehydrogenase, NAD, NADP, APADP, and NADPH were from Sigma. Octane and ethanol-d_6 were from Aldrich. D_2O was from Cambridge Isotope Labs. ^{13}C sodium bicarbonate, ^{12}C sodium bicarbonate, and 2-[^{13}C]pyruvate were from Isotech. Malate-2-*d* was synthesized with the method of Viola et al. (1979) using 50 units/mL of malic and alcohol dehydrogenases. Malate-3-d_2 was prepared by reduction of oxalacetate deuterated by exchange in D_2O in the presence of malate dehydrogenase (Cook et al., 1980). Malate-(3*R*)-*d* was synthesized from fumarate and D_2O in the presence of fumarase.

2,4-[$^{13}C_2$]Malate. 100 mL of 15 mM 2-[^{13}C]pyruvate, 1 mM NADPH, 1 mM $MgCl_2$, 15 mM reduced glutathione, 50 mM dithiothreitol, and 100 mM potassium phosphate were sparged for 2 h at pH 3. The pH was then quickly raised to 7 with saturated sodium hydroxide. A dry addition apparatus was then used to add sodium [^{13}C]bicarbonate so that the final concentration was 20 mM. 20 units of malic enzyme and 200 units of glutathione reductase were then used to start the reaction, which was allowed to proceed over night.

The reaction was quenched with the addition of 6M HCl to pH 3 and the enzyme was removed by filtering with an Amicon filter. The reaction mixture was then titrated back to pH 7 and loaded on a Dowex 1-X8 ion exchange column. The malate was eluted with a 500 mL gradient from 0 to 6 M formic acid. The column was then rinsed with another 250 mL of 6 M formic acid. The fractions containing malate were located using a malic enzyme assay that consisted of 2.38 mL of 50 mM phosphate buffer, pH 7, 300 μL of 10 mM $MgCl_2$, 300 μL of 5 mM NADP, 1 unit of malic enzyme, and 20 μL of sample. These fractions were then evaporated to dryness, dissolved in 100 mL of water and evaporated to dryness again. The residue was dissolved in 50 mL of water and evaporated to a thick solution which was stored in the freezer.

4-[^{12}C]Malate. The reaction conditions and purification were similar to the synthesis of the 2,4-[$^{13}C_2$]malate except that pyruvate and [^{12}C]bicarbonate replaced 2-[^{13}C]pyruvate and [^{13}C]bicarbonate. Because more of this compound was needed, the reaction was also scaled up to 5 times the size of the 2,4-[$^{13}C_2$]malate preparation.

Data Analysis. Isotopic ratios were determined with a Finnigan MAT Delta isotope ratio mass spectrometer. Kinetic isotope effects were calculated using the isotopic ratios from the product at partial reaction (R_p) and the isotopic ratio in the starting material (R_0). The latter was determined by complete conversion of substrate to product. Equation 5 was used to calculate the observed isotope effect from the isotopic ratios of the product and starting material at known

fractions of reaction, *f*.

$$\text{isotope effect} = \log(1 - f)/\log(1 - f(R_p/R_0)) \quad (5)$$

Kinetic Deuterium Isotope Effects. All kinetic deuterium isotope effects were determined by direct comparison of initial velocities of malic enzyme enzyme catalysis with malates varying in deuterium composition at a defined position in the molecule. The reactions were performed at 25 °C, with absorbance changes followed at 340 nm (NADP) or 363 nm (APADP). Final concentrations were 1.0 mM NADP or APADP, 5.0 mM Mg^{2+}, 200 mM Hepes, and 100 mM dithiothreitol, pH 7.25. The concentration of malate was varied from 0.1 to 1.0 mM.

The data for the deuterium isotope effects were fitted to equations describing an isotope effect on *V* only, on *V*/*K* only, equal isotope effects on *V* and *V*/*K*, or different isotope effects on *V* and *V*/*K* (Cleland, 1979). The reported values are those from the best fit as judged by the value of σ as well as the standard errors of the fitted parameters.

Degradation of Malate. For determination of the isotopic content of each carbon of malate two separate sets of reactions were used. The following reaction conditions were used for the first set of reactions: 200 μmol of malate, 0.5 mM NADP, 250 μmol of oxidized glutathione, 1 mM $MnCl_2$, 1 mM dithiothreitol, 1 unit of malic enzyme, and 10 units of glutathione reductase in 20 mL of 25 mM potassium phosphate buffer titrated to pH 7.0 with KOH.

The first set of these reactions was carried out in sealed flasks that were sparged with nitrogen overnight prior to addition of enzymes. Once the enzymes were added the reactions were allowed to run for 14 h for total conversion of reactants to products. The reactions were quenched by injecting 1 mL of 5 N H_2SO_4, and the CO_2 from C4 was isolated by a freeze−thaw distillation. The flasks and their contents were frozen in liquid nitrogen and then evacuated on the vacuum lines. The flasks were then warmed with room temperature water until their contents melted. The flasks were then refrozen in dry ice−isopropyl alcohol baths. The gaseous contents of the flask were distilled through two dry ice−isopropyl alcohol traps and into a liquid nitrogen trap. The flasks were then thawed, frozen, and distilled again. This process was repeated three times.

The freeze−thaw method was used to prevent any of the pyruvate from distilling into the dry ice−isopropyl alcohol traps. The collected CO_2 was analyzed with the isotope ratio mass spectrometer to determine the isotopic composition at the C4 position.

The reactions were then put on ice and stirred as 1 mL of a saturated NaOH solution was added. Upon addition of the saturated NaOH solution the reaction mixtures slowly turned light brown. Balloons flushed with nitrogen were then used as vents for the flasks as 3 mL of 30% hydrogen peroxide was added to oxidize pyruvate to acetate and CO_2. The vent was then removed from the reactions, and the reactions were stirred overnight. The pH of the reactions was then lowered by adding 5 mL of 18 N H_2SO_4 and the reactions were subjected to the freeze−thaw procedure again. This time the freeze−thaw method was used to prevent any acetate from distilling into the dry ice−isopropyl alcohol traps. The collected CO_2 was analyzed by isotope ratio mass spectrometry to determine the isotopic composition of C1.

The reactions were then removed from the sealed flasks, and the following procedure was used to isolate and purify the acetate. The pH of the reaction mixtures was checked to make sure that it was below 2.5. The reaction mixtures were added to 1 L flasks with 600 mL of distilled octane and boiling chips and then were distilled until the vapor temperature reached 124 °C for 5 min. Ten drops of saturated NaOH were then added to the receiving flasks, followed by vigorous stirring for 1 min. The aqueous layers in the receiving flasks were separated from the octane layers, 3 g of activated charcoal was added to each aqueous layer, and the resulting mixtures were stirred for 10 min. The charcoal was filtered with 0.2 micron syringe top filters, and the solutions were adjusted to pH 7.2 with H_2SO_4. They were placed in sealed flasks and sparged with N_2 gas overnight and then evaporated to dryness. The resultant powders were stored in sealed flasks in a desiccator flushed with N_2 gas.

The purified acetate from each reaction was then divided into two portions; one portion for combustion to give the isotopic composition of both C2 and C3 and the other portion for the acetate degradation system to give methane from C3.

For combustion, 40 μmol samples of the purified acetate were placed inside 2.5 cm long quartz boats with 6 and 4 mm outer and inner diameters, respectively. These boats were then placed inside 25 cm long, 2 mm thick quartz tubes with an inner diameter of 7 mm. Layers of diatomaceous earth (0.5 g), CuO (10 g), and silver (0.2 g), in that order, were then placed inside the quartz tube on top of the boat. The tubes were evacuated, sealed, and heated to 750 °C for 2 h. The quartz boats and diatomaceous earth were needed so the salts that precipitated along with the acetate would not react with the walls of the quartz tube and cause it to explode during heating. After the combustion the tubes were cracked and the resulting CO_2 was distilled through two dry ice−isopropyl alcohol traps and collected in a liquid nitrogen trap. The collected gas was then analyzed with the isotope ratio mass spectrometer.

For the acetate degradation system (Canellas & Cleland, 1991), 60 μmol of purified acetate and 0.3 g of NaOH were heated in an evacuated vessel to 200 °C for 30 min. This heating was performed in the presence of two dry ice−isopropyl alcohol traps to collect water from the NaOH. An additional liquid N_2 trap was then added to the vacuum line, and the acetate and NaOH were heated to 400 °C for 15 min. At this temperature pyrolysis occurs, producing CO_2 and methane from the C2 and C3 positions, respectively. Any CO_2 liberated was collected in the liquid N_2 trap, and the methane, which is not condensable, was collected using molecular sieves cooled with liquid nitrogen. The methane was then allowed to equilibrate for 15 min into a specially designed combustion tube containing 20 g of CuO by heating the sieves to 200 °C. The lower portion of this 1 m long combustion tube was composed of 30 cm of quartz, the middle 30 cm was a quartz to glass gradient, and the top 40 cm was glass with a ground glass stopcock and a ground glass joint for connection to the vacuum line. The outer and inner diameters of this tube were 9 and 7 mm, respectively. Once the methane was equilibrated into the combustion tube the stopcock was closed, the tube was disconnected from the vacuum line and the quartz portion of the tube (containing the CuO) was placed in an oven and heated to 750 °C for 12 h. Approximately 80% of the methane gas produced

could be collected in this manner. The resulting CO_2 gas from combustion was distilled and analyzed in the same manner as the gas produced from the acetate combustion samples. The isotopic composition of C2 was then calculated from the ratios determined for C3 and C2 plus C3 by using eq 6,

$$R_{C2} = (R_{C2C3} \times 2) - R_{C3} \quad (6)$$

where R_{C2} is the calculated isotopic ratio for the C2 position, R_{C3} is the measured isotopic ratio for the C3 position, and R_{C2C3} is the measured isotopic ratio from the acetate combustion.

To ensure that no fractionation was occurring in the acetate due to the distillation of CO_2 in determining the C1 and C4 mass ratios, separate control reactions were run. These reactions were run under the same conditions as listed above except that the CO_2 was not isolated so that none of the pyruvate or acetate could possibly be lost during the freeze−thaw procedure. After running overnight the malic enzyme reactions were quenched by the addition of the saturated NaOH solution and the 30% hydrogen peroxide for the conversion of pyruvate to acetate. Results from the acetate collected in this procedure did not differ from those where the acetate was purified after the freeze−thaw procedure.

The reactions for the determination of the isotopic composition of malate after partial conversion with malic enzyme were similar to those for the total conversion, except that 400 μmol of malate and 300 μmol of oxidized glutathione were used. Reaction progress was monitored by removing aliquots and assaying for the amount of reduced glutathione produced with Ellman's reagent (extinction coefficient at 412 nm = 1.14×10^4 L/mol cm). The reactions were stopped between 30% and 60% conversion by adding 1 mL of 5 N H_2SO_4.

The CO_2 from all four carbon positions was collected and analyzed in the same manner as in the total conversion reactions. The control to check for fractionation in the acetate was also run with the partial conversions. As a final control for this procedure, a sample of malate was combusted and its isotopic composition was analyzed with the isotope ratio mass spectrometer. The combined ratio of the total conversion samples was calculated using equation 7,

$$R_{comb} = 0.25(R_{C1} + R_{C2} + R_{C3} + R_{C4}) \quad (7)$$

where R_{C1}, R_{C2}, R_{C3}, and R_{C4} are the $^{13}C/^{12}C$ ratios for total conversions at carbons 1, 2, 3, and 4 (−34.355, −24.651, −29.987, and −30.507, respectively, in δ notation) and R_{comb} is therefore the calculated value of the $^{13}C/^{12}C$ ratio of the malate used in these experiments. The isotopic ratio of the malate combustion sample (−29.875) was then compared to the R_{comb} value of −30.132.

The isotope effects with malate-2-*d* and APADP as substrates were determined in a similar manner to that described above by simply using the desired alternative substrate.

Remote Label Isotope Effects on Malic Enzyme. The 2,4-[$^{13}C_2$]malate and 4-[^{12}C]malate were mixed to have approximately 1% ^{13}C and 99% ^{12}C at the C4 position. Effects at the C2 position could then be measured by isolating the C4 carbon from both total and partial conversions, using the techniques described above. The remote label experiments yield an observed isotope effect which is the product of the kinetic isotope effect at C4 and of the kinetic isotope effect at C2. The observed effects from these experiments were corrected for the kinetic isotope effect at C4 to determine the kinetic isotope effect at C2.

Equilibrium Isotope Effects on Malic Enzyme. The equilibrium isotope effects at the C2, C3, and C4 positions of malate were determined in two different ways. The first way was to begin the reaction with only malate present and allow the reaction to come to equilibrium. The second way was to start the reaction with pyruvate and bicarbonate. The conditions for starting with malate were as follows: 200 μmol of malate, 1 mM NADP, 1 mM $MnCl_2$, 1 μM dithiothreitol, and 3.5 units of malic enzyme at room temperature in 50 mL of 50 mM potassium phosphate buffer titrated to pH 7.0 with KOH. The conditions for starting with pyruvate were similar, with 200 μmol of pyruvate and 100 μmol of sodium bicarbonate replacing malate and 1 mM NADPH replacing NADP.

Both sets of reactions were allowed to sit for 2 days to ensure sufficient time to come to isotopic equilibrium. The reactions were then quenched with acid and the CO_2 from the reactions was distilled on vacuum lines by freezing and thawing the reaction mixtures so that no residual malate would be lost. The gas was then analyzed with the isotope ratio mass spectrometer. The solution containing the residual malate and pyruvate was titrated to pH 7 and loaded on a AG 1-X8 200−400 mesh ion exchange column. The malate and pyruvate were then eluted from the column with a 500 mL gradient from 0 to 6 M formic acid. The fractions containing malate were located using a malic enzyme assay as detailed above. The fractions containing pyruvate were located using a pyruvate decarboxylase assay that consisted of 2.68 mL of 50 mM phosphate buffer, pH 6, 300 μL of 10 mM $MgCl_2$, 1 unit of pyruvate decarboxylase, and 20 μL of sample. The isotopic composition of the C2 and C3 positions of pyruvate and the C2, C3, and C4 of malate were determined using the same techniques described in the previous section.

The observed value for the C4 equilibrium isotope effect must be corrected for the equilibrium between bicarbonate and free CO_2, since the reactions were run at pH 7 and bicarbonate has a pK_a of 6.37. The ^{13}C isotope effect for the conversion of bicarbonate to free CO_2 is 1.009 (Mook et al., 1974) or, when adjusted for the pH, 1.007. This value was subtracted from the observed value at C4 to give the adjusted value for CO_2 as the reactant.

RESULTS AND DISCUSSION

Kinetic Isotope Effects. The ^{13}C isotope effects on malic enzyme were determined at all four carbon positions of malate (Table 1). The kinetic isotope effect at the C1 position of malate with $NADP^+$ was 1.0011, which indicates very little change in bond order over the course of the reaction at that position. The kinetic isotope effect of 1.0009 with malate and APADP at the C1 position shows that little change in bond order was occurring even when the chemistry of the hydride transfer was made more rate-limiting.

The kinetic isotope effect at the C4 position with malate and $NADP^+$ was 1.0324. When this isotope effect was determined with malate-2-*d* and NADP, the value dropped to 1.0243, which suggests that the mechanism is stepwise

Table 1: ^{13}C Kinetic Isotope Effects

carbon position	malate + NADP $^{13}(V/K)_H$	malate-2-*d* + NADP $^{13}(V/K)_D$
C1	1.0011 ± 0.0004	
C2	0.9871 ± 0.0021	0.9892 ± 0.0013
C3	1.0210 ± 0.0005	1.0130 ± 0.0006
C4	1.0324 ± 0.0003	1.0243 ± 0.0004
carbon position	malate + APADP $^{13}(V/K)_H$	malate-2-*d* + APADP $^{13}(V/K)_D$
C1	1.0009 ± 0.0007	
C2	0.9941 ± 0.0008	0.9961 ± 0.0004
C3	1.0067 ± 0.0003	1.0125 ± 0.0002
C4	1.0056 ± 0.0005	1.0087 ± 0.0007

with hydride transfer preceding decarboxylation. These data agree with those previously obtained by Hermes et al. (1982). When the alternative dinucleotide APADP was substituted into this reaction, however, the C4 kinetic isotope effects for malate and malate-2-*d* became 1.0056 and 1.0087, respectively. This increase in the C4 isotope effect with malate-2-*d* suggests a concerted mechanism with the alternative dinucleotide, APADP. The trend of these isotope effects is consistent with those previously observed by Weiss et al. (1991). As stated earlier there are two reasonable explanations for this outcome. First, malic enzyme may change its chemical mechanism with the alternative substrate. Second, there may be a β-secondary kinetic ^{13}C isotope effect on the hydride transfer step as the result of a hyperconjugation-like effect on the C3−C4 bond order as the transition state for hydride transfer was approached.

If this hypothesis is correct the amount of electron density borrowed from the C3−C4 bond would be transferred to the C2−C3 bond and therefore the net change in bond order at the C3 position would be close to zero. Thus the ^{13}C kinetic isotope effect at the C3 position should be free of the secondary isotope effect. If the mechanism were stepwise with the alternative substrate, the change from malate to malate-2-*d* should show a decrease in the observed ^{13}C kinetic isotope effect. If the mechanism is concerted, then malate-2-*d* should cause an increase in the isotope effect.

The kinetic isotope effect at the C3 position with malate and NADP was 1.0210. This is smaller than the effect observed at the C4 position, as expected because C3 undergoes less movement than C4 upon bond cleavage. When C4 is released from malate its geometry changes from trigonal to linear and the carbon undergoes most of the motion, as it is connected to two oxygens which are more massive than it is. However, at the C3 position there is only a slight bond shortening as a double bond is formed with C2. When malate-2-*d* was used with NADP, the C3 kinetic isotope effect dropped to 1.0130, which parallels the trend at the C4 position. This confirms the stepwise mechanism with NADP. With APADP as the dinucleotide and malate as substrate the kinetic isotope effect at the C3 position was 1.0067. When malate-2-*d* was used with APADP the C3 kinetic isotope effect became 1.0125. This was nearly double the effect seen with normal malate and confirms the change to a concerted chemical mechanism with APADP, in agreement with Karsten and Cook (1994).

This change in mechanism with APADP is most likely due to combination of a higher energy barrier for hydride transfer and lower overall free energy levels of products.

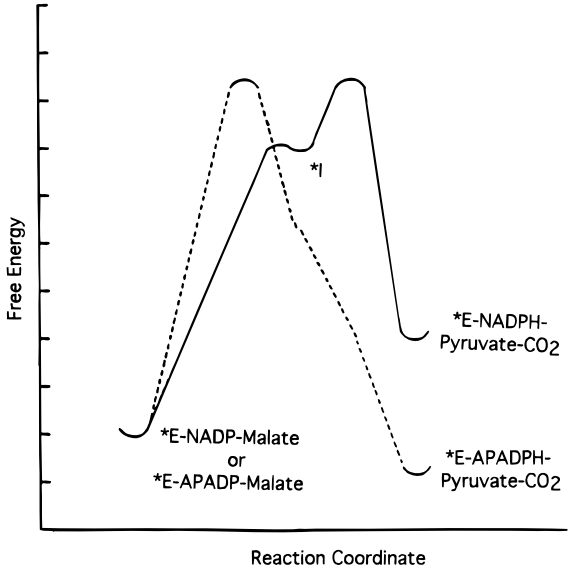


FIGURE 1: Free energy profiles for the enzymatic reaction of malate to pyruvate and CO_2 with NADP or APADP as the dinucleotide. *E−NADP−malate and *E−APADP−malate represent the activated enzyme−substrate complex, *I is the E−NADPH−oxaloacetate intermediate for the stepwise reaction with NADP, *E−NADPH−Pyruvate−CO_2 is the activated enzyme−product complex for the reaction with NADP, and *E−APADPH−Pyruvate−CO_2 is the activated enzyme−product complex for the reaction with APADP.

Figure 1 shows two plausible energy level diagrams, one for the malate−NADP reaction and one for the malate-APADP reaction. Grissom and Cleland (1985) showed that with NADPH present malic enzyme will convert oxaloacetate to both enzyme bound malate and pyruvate, in a ratio of approximately 10 to 1. This means that the energy barrier to decarboxylation is larger than that for hydride transfer, as shown by the solid line in Figure 1. While the relative heights of these barriers are known, the level of the well corresponding to the intermediate is not. It is conceivable that if one were to substantially raise the barrier to hydride transfer while lowering the energy level of the final products, the energy well normally found for oxaloacetate could collapse, as shown by the dashed line in Figure 1. Since the equilibrium constant for the reaction is 2 orders of magnitude more favorable when APADP is the dinucleotide ($E^{o\prime}_{pH7}$ = −0.258 V vs −0.32 V for NADP), this could explain the switch from a stepwise to a concerted mechanism.

The ^{13}C kinetic isotope effects at the C2 position with NADP as the dinucleotide and malate or malate-2-*d* as the substrates are 0.9871 and 0.9892, respectively. This was unexpected, as the C2 position is where the hydride transfer occurs. Since hydride transfer is a bond breaking process, a normal isotope effect would be expected. It is interesting that when the hydride transfer is slowed down with malate-2-*d* the effect becomes less inverse. This suggests that an aspect of the chemistry other than the hydride transfer is dominating these C2 effects, and when hydride transfer is made more rate-limiting the normal effect due to the bond breaking begins to become expressed. When APADP is used as the dinucleotide, ^{13}C kinetic isotope effects of 0.9941 and 0.9961 are observed for malate and malate-2-*d*, respectively. Again the isotope effect is inverse and again as the hydride transfer is slowed down the effect moves closer to being a normal effect. It was conceivable that these effects were incorrect due to a flaw in the technique for determining the

Table 2: Deuterium Kinetic Isotope Effects[a]

	NADP	APADP
$^{C2D}(V/K)$	1.47 ± 0.02	1.98 ± 0.05
$^{C3D2}(V/K)$	1.17 ± 0.01	1.03 ± 0.02
$^{C(3R)D}(V/K)$	1.076 ± 0.009	
$^{D3}(V/K)$	1.65 ± 0.02	2.07 ± 0.07
$^{C2D}(V/K)_{C3D2}$	1.48 ± 0.08	2.02 ± 0.06
$^{C3D2}(V/K)_{C2D}$	1.15 ± 0.02	1.04 ± 0.02

[a] Since malate was the varied substrate, all isotope effects are on V/K_{malate}. In every case equal isotope effects were seen on V and V/K. Most values represent three or four replicate experiments. The leading superscripts indicate the number and location of deuteriums producing the isotope effect, while the trailing subscripts indicate deuteration present in both molecules being compared.

isotopic ratio. When the acetate is degraded, the methane generated is solely from the C3 position. However, when the acetate is combusted any source of carbon could cause contamination. This is unlikely in view of the controls described in the methods section, but as a check against that possibility the remote label method was employed. Using C4 as the indicator position the C2 kinetic isotope effect was redetermined. An isotope effect of 0.9898 ± 0.0006 was observed for the C2 position with the remote label method when NADP was the substrate. This confirms the inverse kinetic isotope effect observed earlier at the C2 position.

Deuterium Isotope Effects at C3 and Their Effect on the ^{13}C Isotope Effect at C4. Table 2 shows the effects of deuteration on the reaction with NADP or APADP. With NADP the secondary isotope effect resulting from deuteration at C3 is 17% normal for two deuteriums and 8% for one, so the isotope effects are not different in the *R* and *S* positions. One expects an isotope effect at this position only on the decarboxylation step, since there should be little hyperconjugation with the carbonyl group of an oxalactate intermediate after hydride transfer because C4 must be out of plane for decarboxylation, and thus the hydrogens at C3 are nearly in plane in the intermediate. The equilibrium isotope effect for decarboxylation at C3 to give enolpyruvate can be estimated as 1.26 for mono- and 1.58 for dideuteration (Cleland, 1980). If one uses the commitments estimated by Grissom and Cleland (1985) to correct the observed isotope effect to an intrinsic one on decarboxylation, one gets 1.22 ± 0.03 for dideuteration. We are thus observing 38% of the equilibrium isotope effect, which may provide an estimate of the degree of decarboxylation in the transition state.

With APADP as the nucleotide, the deuterium isotope effect at C3 of malate is only 3−4%, and hardly significantly different from unity. After correction for an average forward commitment of 1.0 (Table 5), the intrinsic isotope effect of 6−8% is consistent with a concerted reaction showing 10−14% of the equilibrium isotope effect in the transition state. These data thus support our conclusion and that of Karsten and Cook (1994) that the reaction with APADP is asynchronous with C−C cleavage less advanced than C−H cleavage in the transition state.

Table 3 shows ^{13}C isotope effects at C4 for three nucleotide substrates with malates deuterated at C2 or C3. The decrease due to deuteration at C2 with NADP, but increases with thio-NADP or APADP, confirm the observations of previous workers and the results in Table 1. The increases in the observed ^{13}C isotope effect with NADP when C3 is deuterated is consistent with the expected decrease in rate of decarboxylation relative to hydride transfer caused by deuteration at C3. On the other hand, the insignificant effect of deuteration at C3 on the ^{13}C isotope effect in the reactions with thio-NADP or APADP is consistent with asynchronous concerted reactions with little C−C cleavage in the transition state.

Table 3: ^{13}C Isotope Effects As a Function of Deuteration

	NADP[a]	thio-NADP[b]	APADP[b]
$^{13}(V/K)_H$	1.0339 ± 0.0003	1.0089 ± 0.0001	1.0051 ± 0.0002
$^{13}(V/K)_{C2D}$	1.0292 ± 0.0001	1.0147 ± 0.0001[c]	1.0084 ± 0.0001[c]
$^{13}(V/K)_{C3D2}$	1.0354 ± 0.0001	1.0087 ± 0.0005	1.0053 ± 0.0001

[a] The isotope effects with NADP were determined in a single experiment. [b] Values for $^{13}(V/K)_H$ and $^{13}(V/K)_{C3D2}$ with thio-NADP and APADP were determined in a single experiment. [c] These values are from Weiss et al. (1991).

Table 4: Equilibrium Isotope Effects for the Conversion of Malate to Pyruvate and CO_2[a]

initial reactants	C2	C3	C4[b]
malate	1.0114 ± 0.0001	1.0111 ± 0.0001	0.9941 ± 0.0001
pyruvate + bicarbonate	1.0099 ± 0.0001	1.0115 ± 0.0001	0.9949 ± 0.0009
weighted average	1.0102 ± 0.0001	1.0113 ± 0.0001	0.9943 ± 0.0003 0.9880 ± 0.0003[c]

[a] The value is the $^{13}C/^{12}C$ ratio in malate divided by that in pyruvate or CO_2. [b] The observed value at pH 7 for the equilibrium mixture of CO_2 and bicarbonate, except as noted. [c] The value corrected to CO_2 alone.

Equilibrium Isotope Effects. Because we thought that an internal equilibrium isotope effect might be inverse and dominate the observed kinetic isotope effect at C2, equilibrium ^{13}C isotope effects were determined for conversion of C2, C3, and C4 of malate to pyruvate or CO_2 (Table 4). Mechanism 8 was used to explore this possibility.

$$\mathrm{EAB} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{EAB^*} \underset{k_4}{\overset{\text{hydride transfer } k_3}{\rightleftharpoons}} \mathrm{EC^*} \xrightarrow{\text{decarboxylation } k_5} \qquad (8)$$

In mechanism 8 EAB is the initial enzyme−NADP−malate complex, EAB* is the activated enzyme−NADP−malate complex, and EC* is the activated enzyme−oxaloacetate complex. From mechanism 8, eq 9 can be derived.

$$^{13}(V/K)_{C2} = [^{13}K_{eq3}\,{}^{13}k_5 + (^{13}k_3k_5)/k_4 + (k_3k_5)/(k_2k_4)]/ [1 + k_5/k_4 + (k_3k_5)/(k_2k_4)] \qquad (9)$$

Grissom and Cleland (1985) determined that $k_5/k_4 = 0.1$ and $k_3/k_2 = 3.3$. Assuming $^{13}k_5 = 1.00$, $^{13}k_3 = 1.01$, and that $^{13}K_{eq3}$ is between 0.99 and 0.98, the calculated value for $^{13}(V/K)_{C2}$ ranges from 0.9937 to 0.9867. Thus an inverse equilibrium effect could explain the inverse kinetic effect found experimentally with NADP as substrate. When the equilibrium isotope effect at C2 was measured, however, it was found to be 1.0102. This value is a measure of the equilibrium from malate to pyruvate and must be corrected to yield a value for oxaloacetate. Replacing H with C on a carbon next to the carbon of interest raises the ^{13}C fractionation factor by 1.004 (Cleland, 1980), and applying this rule gives 1.006 for the equilibrium isotope effect at C2 for conversion of malate to oxaloacetate. There must be

considerable stiffening of vibrational modes involving C2 upon binding of malate to the enzyme if the fractionation factor of this carbon is to be raised by the additional factor of 1.025 needed to match the experimental results. Freezing the rotation of the β-carboxyl group will elevate the torsional force constants involving C2, but it is difficult to believe that this effect can be this large. The inverse ^{13}C isotope effects at C2 remain a puzzle.

The equilibrium isotope effects at the C3 and C4 positions were determined to be 1.0113 and 0.9880, respectively. The equilibrium isotope effect at the C4 position is slightly more inverse than predicted by calculations based on the equilibrium isotope effect observed with isocitrate dehydrogenase by O'Leary and Yapp (1978). The calculated value for malic enzyme was reported to be 0.999 by Hermes et al. (1982). The fact that the isotope effect is inverse indicates that C4 is more tightly bound in CO_2 than when it is in malate. The C3 effect is the first equilibrium isotope effect measured on a decarboxylation reaction for the carbon that is involved in the bond breaking and is not the leaving group. The observed normal equilibrium effect at the C3 position indicates that this carbon is bound more tightly in malate than when it is in pyruvate. This is the expected result of replacing a proton in pyruvate with a carboxyl group (Cleland, 1980).

Intrinsic Isotope Effects. The intrinsic isotope effects at C2, C3, and C4 for a concerted mechanism with APADP as the dinucleotide can be calculated. Mechanism 10 shows the rate constants involved.

$$\mathrm{EAB} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} {}^{*}\mathrm{EAB} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} {}^{*}\mathrm{EPQR} \xrightarrow{k_5} \mathrm{EPQ} \qquad (10)$$

EAB is the initial malate and APADP complex, *EAB is the active enzyme conformation with both substrates bound, *EPQR is the active enzyme conformation with pyruvate, APADPH, and CO_2 bound, and EPQ is enzyme-bound pyruvate and APADPH. Based on mechanism 10, eqs 11−13 can be derived.

$$^{D}(V/K) = [^{D}k_3 + k_3/k_2 + {}^{D}K_{eq}(k_4/k_5)]/(1 + k_3/k_2 + k_4/k_5) \quad (11)$$

$$^{13}(V/K)_H = [^{13}k_3 + k_3/k_2 + {}^{13}K_{eq}(k_4/k_5)]/(1 + k_3/k_2 + k_4/k_5) \quad (12)$$

$$^{13}(V/K)_D = [^{13}k_3 + k_3/{}^{D}k_3k_2 + {}^{13}K_{eq}({}^{D}K_{eq}k_4/{}^{D}k_3k_5)]/(1 + k_3/{}^{D}k_3k_2 + {}^{D}K_{eq}k_4/{}^{D}k_3k_5) \quad (13)$$

Since CO_2 is rapidly released (Weiss et al., 1991) the k_4/k_5 term is insignificant and eqs 11−13 reduce to three equations in three unknowns (k_3/k_2, $^{D}k_3$, and $^{13}k_3$). Solving these equations simultaneously allows calculation of the values shown in Table 5. The values determined here from the isotope effects at C4 differ from those previously calculated by Weiss et al. (1991) only for $^{D}k_3$ as the result of those authors using a higher value of $^{D}(V/K)$ than the one we have determined and the value determined earlier by Hermes et al. (1982). The differences in values calculated in Table 5 from C4 and C3 ^{13}C isotope effects can be accounted for by the error in the isotope effects used for the calculations. The ^{13}C isotope effects at C2 are not sufficiently different from unity or each other to permit a useful calculation.

Table 5: Intrinsic Isotope Effects for the Concerted Mechanism with APADP

	calculated from ^{13}C isotope effects at	
	C4	C3
k_3/k_2[a]	1.12	1.74
$^{D}k_3$[b]	3.1	3.7
$^{13}k_3$[c]	1.012	1.018

[a] k_3/k_2 is the forward commitment and should be the same when determined from values at C4 and C3. [b] Intrinsic isotope effect resulting from deuteration at C2; should be the same when determined from values at C4 and C3. [c] Intrinsic isotope effects from ^{13}C substitution at C4 or C3; the values need not be the same.

The values for $^{D}k_3$ above 3, with comparatively small values for the two $^{13}k_3$ isotope effects at C4 and C3, indicate that the concerted mechanism is asynchronous with decarboxylation less advanced than hydride transfer in the transition state. The results of deuteration at C3 agree with this conclusion. There is precedent for this type of asynchronous mechanism in prephenate dehydrogenase (Hermes et al., 1984).

In conclusion, the work reported here shows that malic enzyme does change its chemical mechanism from stepwise to concerted when the alternative dinucleotide APADP is used as a substrate. However, the unexpected inverse ^{13}C isotope effect at the C2 position continues to be a puzzle at this time.

REFERENCES

Canellas, P. F., & Cleland, W. W. (1991) *Biochemistry 30*, 8871−8876.

Cleland, W. W. (1979) *Methods Enzymol. 63* 103−138.

Cleland, W. W. (1980) *Methods Enzymol. 64*, 104−125.

Cook, P. F., Blanchard, J. S., & Cleland, W. W. (1980) *Biochemistry 21*, 4853−4858.

Grissom, C. B., & Cleland, W. W. (1985) *Biochemistry 24*, 944−948.

Grissom, C. B., & Cleland, W. W. (1988) *Biochemistry 27*, 2927−2934.

Hermes, J. D., Roeske, C. A., O'Leary, M. H., & Cleland, W. W. (1982) *Biochemistry 21*, 5106−5114.

Hermes, J. D., Tipton, P. A., Fisher, M. A., O'Leary, M. H., Morrison, J. F., & Cleland, W. W. (1984) *Biochemistry 23*, 6263−6275.

Karsten, W. E., & Cook, P. F. (1994) *Biochemistry 33*, 2096−2103.

Karsten, W. E., Gavva, S. R., Park, S.-H., & Cook, P. F. (1995) *Biochemistry 34*, 3253−3260.

Mook, W. G., Bommerson, J. C., & Staverman, W. H. (1974) *Earth Planet. Sci. Lett. 22*, 169−176.

O'Leary, M. H., & Yapp, C. J. (1978) *Biochem. Biophys. Res. Commun. 80*, 155−160.

Viola, R. E., Cook, P. F., & Cleland, W. W. (1979) *Anal. Biochem. 96*, 334−340.

Weiss, P. M., Gavva, S. R., Harris, B. G., Urbauer, J. L., Cleland, W. W., & Cook, P. F. (1991) *Biochemistry 30*, 5755−5763.

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