Determination of the Kinetic and Chemical Mechanism of Malic Enzyme Using (2*R*,3*R*)-*erythro*-Fluoromalate as a Slow Alternate Substrate†

Jeffrey L. Urbauer,‡ Debra E. Bradshaw, and W. W. Cleland*

Institute for Enzyme Research and Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53705

Received July 29, 1998; Revised Manuscript Received September 18, 1998

ABSTRACT: (2*R*,3*R*)-*erythro*-Fluoromalate, but not the threo isomer, is a slow substrate for chicken liver malic enzyme with either NADP or 3-acetylpyridine-NADP (APADP) as the other substrate. The K_m for *erythro*-fluoromalate is similar to that of malate, but the turnover number with NADP is 3300-fold slower, although 5.5-fold faster with APADP than with NADP. Deuteration of fluoromalate at C-2 gave an isotope effect on *V*/*K* of 1.39 with NADP and 3.32 with APADP. With NADP, the ^{13}C isotope effects at C-4 were 1.0490 with unlabeled and 1.0364 with deuterated fluoromalate. With APADP, the corresponding values were 1.0138 and 1.0087. These data show that the mechanism is stepwise with both nucleotide substrates, in contrast to the reaction of malate and APADP, which was postulated to be concerted by Karsten et al. [Karsten, W. E., and Cook, P. F. (1994) *Biochemistry 33*, 2096−2103], a conclusion recently shown to be correct by Edens et al. [Edens, W. A., Urbauer, J. L., and Cleland, W. W. (1997) *Biochemistry 36*, 1141−1147]. To explain the effect of deuteration on the ^{13}C isotope effect with APADP, it is necessary to assume a secondary ^{13}C isotope effect at C-4 on the hydride transfer step of ∼1.0064 (assuming 5.7 as the intrinsic primary deuterium isotope effect and 1.054 as the product of the ^{13}C equilibrium isotope effect on hydride transfer and the intrinsic ^{13}C isotope effect on decarboxylation). The secondary ^{13}C isotope effect on hydride transfer is thought to result from hyperconjugation between the carbonyl group and C-4 of the enzyme-bound fluorooxaloacetate intermediate.

In 1947 it was reported that pigeon liver malic enzyme catalyzes the reversible oxidative decarboxylation of L-malate in the presence of manganese ions (*1*). Thirty-five years later the first convincing experimental evidence was obtained that malic enzyme catalyzes the oxidative decarboxylation of L-malate in a stepwise manner, with hydride transfer preceding decarboxylation. Using multiple isotope effect methodology, in which the ^{13}C isotope effect at C-4 of malate (the carbon which is released as CO_2) was determined with protium ($^{13}(V/K)_H$) or deuterium ($^{13}(V/K)_D$) at C-2 (the hydrogen which is transferred as a hydride to NADP to produce the tightly bound oxaloacetate intermediate), it was demonstrated that $^{13}(V/K)_D$ is less than $^{13}(V/K)_H$, indicating a stepwise mechanism (*2*). These results, along with those from intermediate partitioning studies, allowed the calculation of the intrinsic isotope effects for the system (*3*). The intrinsic ^{13}C isotope effect (^{13}k) on decarboxylation was found to be 1.045 (*3*). By an independent method using values obtained from multiple isotope effect studies with secondary dideuteration at C-3 of L-malate, ^{13}k was determined to be 1.0448 (*4*). The consistent and reasonable results of these additional isotope effect studies further supported the original conclusion (*2*) that the mechanism for chicken liver malic enzyme was stepwise.

However, when 3-acetylpyridine adenine dinucleotide phosphate (3-APADP) is used as the oxidant in the malic enzyme-catalyzed oxidative decarboxylation of L-malate, $^{13}(V/K)_D$ is larger than $^{13}(V/K)_H$, suggesting the possibility that hydride transfer and decarboxylation are concerted (*5*, *6*). The same results are obtained when other NADP analogues with redox potentials more positive than NADP are used as the oxidant for this reaction (*5*). Another explanation for these results is that there is a secondary ^{13}C isotope effect at C-4 of L-malate on hydride transfer which is more effectively expressed when 3-APADP is used as the oxidant, since hydride transfer is more rate-determining with this nucleotide than with NADP (*5*). Such a secondary ^{13}C isotope effect would result from the inductive effect of the carbonyl group at C-2 (especially when polarized by the enzyme), which would reduce the bond order between C-3 and C-4 and lower the fractionation factor of C-4, but not C-3. Recent determinations of ^{13}C isotope effects at C-3 of malate, however, have shown that the reaction does become concerted when the overall equilibrium constant becomes more favorable, although these results do not rule out a possible ^{13}C isotope effect at C-4 during hydride transfer in the cases where the reaction is stepwise (*7*).

An invaluable tool in distinguishing between these possibilities would be a slow, alternate substrate for L-malate, preferably one less easily oxidized. This substrate would presumably have the effect of decreasing the forward commitment to catalysis (*8*) for hydride transfer, allowing a more full expression of not only the primary deuterium

† Supported by NIH grant GM 18938.

* To whom correspondence should be addressed: 1710 University Ave., Madison, WI 53705.

‡ Present address: Dept. of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19104.

10.1021/bi981820f CCC: $15.00
Published on Web 12/03/1998

isotope effect on hydride transfer, but also any secondary ^{13}C isotope effect at C-4 of L-malate on hydride transfer. If the reaction is stepwise, and if with this substrate hydride transfer is predominantly rate limiting, no primary ^{13}C isotope effect on decarboxylation would be observed, and the total ^{13}C isotope effect that is observed would simply be the putative secondary isotope effect on hydride transfer.

In the present work we report the kinetics and isotope effects with (2*R*,3*R*)-*erythro*-fluoromalate, the only malate analogue currently known to undergo oxidative decarboxylation catalyzed by chicken liver malic enzyme. The electron-withdrawing effect of the fluorine atom makes (2*R*,3*R*)-*erythro*-fluoromalate less easily oxidized than L-malate. With both NADP and 3-APADP as the oxidants, the experiments reported here clearly demonstrate that the kinetic mechanism is sequential and rapid equilibrium random, and that the chemical mechanism is stepwise with hydride transfer preceding decarboxylation. Further, by determining the effect of deuteration on the ^{13}C isotope effect at C-4 with APADP as substrate, we have demonstrated that there *is* a secondary ^{13}C isotope effect on hydride transfer.

MATERIALS AND METHODS

Materials. Malic enzyme from chicken liver, malate dehydrogenase from porcine heart, pyruvate kinase from rabbit muscle, glutamate dehydrogenase from bovine liver, and lactate dehydrogenase from rabbit muscle were from Sigma. NAD and 3-APADP were from Sigma, and NADP was from Boehringer Mannheim and Sigma. The (2*R*,3*R*)-*erythro* isomer of fluoromalic acid, the C-2 deuterated analogue, and fluoroxaloacetate were synthesized as described in the previous paper (*9*). Metal-chelating resin was from Sigma. All other compounds were of reagent grade and used without further purification. ^{19}F NMR spectra were recorded with a Bruker AM-400 spectrometer. The ^{19}F chemical shifts were externally referenced to $CFCl_3$ (0 ppm). The ratio of $^{13}C/^{12}C$ in the CO_2 samples was measured with a Finnigan Delta E isotope ratio mass spectrometer. Kinetic measurements were made with either a Cary 118 spectrophotometer or a Hewlett-Packard diode array spectrophotometer, each equipped with a thermostated cell compartment.

Initial Velocity Studies with (2R,3R)-erythro-Fluoromalate, NADP, and 3-Acetylpyridine Adenine Dinucleotide Phosphate. Initial velocity studies were performed at pH 7.25 in 150 mM HEPES (NaOH) at 25 °C by monitoring the absorbance changes at 340 and 363 nm for NADP reduction and 3-APADP reduction, respectively. The extinction coefficients used were 6220 M^{-1} for NADPH and 9100 M^{-1} for 3-APADPH. As shown by Grissom and Cleland (*10*), the substrates for malic enzyme are the free nucleotide and the diacid (as opposed to the metal chelate complexes) and in all cases the true concentrations of the reactants were calculated from the stability constants for the complexes. The dissociation constant used for the (2*R*,3*R*)-*erythro*-fluoromalate−Mg^{2+} complex was 11.9 mM (*9*), and the metal nucleotide dissociation constant used was 19.5 mM (*11*). NADP and 3-APADP concentrations were determined using an enzymatic endpoint assay with malic enzyme and excess L-malate. The concentration of (2*R*,3*R*)-*erythro*-fluoromalate was determined by measurement of fluoride released by strong base (*9*). The concentration of free (2*R*,3*R*)-*erythro*-fluoromalate in solution was varied between 0.981 and 0.098 mM (total (2*R*,3*R*)-*erythro*-fluoromalate varied between 1.81 and 0.181 mM). The concentration of free nucleotide was varied between 0.502 and 0.0099 mM (total nucleotide concentration varied between 0.76 and 0.015 mM). The concentration of free magnesium ion (from $MgSO_4$) was maintained constant at 10.04 ± 0.05 mM (total concentration varied between 11.15 and 10.09 mM). Dithiothreitol was 0.1 mM. Before use in kinetic studies, chicken liver malic enzyme was dialyzed against a solution containing 50% glycerol, 100 mM HEPES, and 100 μM dithiothreitol, pH 8.0, and the activity was normalized using an activity assay consisting of 5 mM L-malate, 2.0 mM NADP, 10 mM magnesium sulfate, and 200 mM HEPES, pH 7.25.

Comparative Initial Velocity Studies with L-Malate and (2R,3R)-erythro-Fluoromalate. Reactions were performed at pH 7.25 in 100 mM HEPES−NaOH at 25 °C and contained 0.5 mM free NADP, 4.9 mM free Mg^{2+} ($MgSO_4$), and 100 μM dithiothreitol. The concentration of free (2*R*,3*R*)-*erythro*-fluoromalate was varied between 0.2 and 2.0 mM and that of free L-malate between 0.1 and 1.0 mM.

Inhibition of Malic Enzyme by (2R,3R)-erythro-Fluoromalate. Oxidative decarboxylation of malic acid catalyzed by malic enzyme is inhibited by (2*R*,3*R*)-*erythro*-fluoromalate. Reactions were performed at pH 7.25 in 100 mM HEPES−NaOH at 25 °C and contained 1.0 mM free NADP, 5.0 mM free Mg^{2+} ($MgSO_4$), and 100 μM dithiothreitol. The concentration of free malate varied from 0.1 to 1.0 mM and that of free (2*R*,3*R*)-*erythro*-fluoromalate from 0.1 to 1.0 mM. The appropriate stability constants (described above) were used to calculate the concentrations of all reactants. Because of the very large difference between the rates of malic enzyme catalysis of the oxidative decarboxylation of malate and the oxidative decarboxylation of (2*R*,3*R*)-*erythro*-fluoromalate (see Results), reaction of the latter could be safely ignored in calculating the appropriate initial rates.

Primary Deuterium Isotope Effects. Primary deuterium isotope effects for chicken liver malic enzyme with (2*R*,3*R*)-*erythro*-fluoromalate were obtained by direct comparison of initial velocities. The values for ^{D}V and $^{D}V/K$ with NADP and 3-APADP were measured by varying the concentration of C-2 deuterated and protiated (2*R*,3*R*)-*erythro*-fluoromalate at saturating concentrations of free metal ion and nucleotide. Reactions were performed at pH 7.25 in 100 mM HEPES−NaOH at 27 °C. The concentration of free nucleotide was 1.0 mM, free Mg^{2+} was 10 mM, and dithiothreitol was 100 μM. The concentration of free (2*R*,3*R*)-*erythro*-fluoromalate was varied between 0.1 and 1.0 mM. As in the kinetic studies outlined above, the concentrations of free nucleotide, Mg^{2+}, and (2*R*,3*R*)-*erythro*-fluoromalate were determined using the appropriate stability constants.

^{13}C Isotope Effects at C-4 of (2R,3R)-erythro-Fluoromalic Acid for Malic Enzyme Catalysis. For the determination of the ratio of $^{13}C/^{12}C$ at the C-4 carbon of both C-2 protiated and C-2 deuterated (2*R*,3*R*)-*erythro*-fluoromalate (total conversion samples), the C-4 carboxyl group was completely removed by the coupled reactions of malate dehydrogenase (to produce fluorooxaloacetate) and pyruvate kinase (which catalyzes the decarboxylation of fluorooxaloacetate (Urbauer and Cleland, unpublished results)), while glutamate dehydrogenase served to recycle the nucleotide. In a total volume

of 5.5 mL, the final concentrations of the reactants in 100 mM HEPES were 4 mM (2*R*,3*R*)-*erythro*-fluoromalate, 10 mM $MgSO_4$, 0.8 mM NAD, 100 mM $(NH_4)_2SO_4$, 50 mM α-ketoglutarate, 1100 units/mL malic dehydrogenase, 130 units/mL glutamate dehydrogenase, and 1000 units/mL pyruvate kinase. All components in the reaction mixture, except the enzymes, were combined and sparged with N_2 (CO_2-free) for 2 h at pH 5.3. The pH was raised to 7.5 with saturated NaOH and sparging continued for another 3 h before the addition of the enzymes. A 500 μL aliquot was withdrawn immediately after enzyme addition to be used to monitor (by ^{19}F NMR) the reaction progress. When the reactions were complete, the mixtures were chilled on ice and then acidified with 2 mL of cold, 18 N H_2SO_4. The CO_2 was isolated and the $^{13}C/^{12}C$ ratio determined as described previously (*2*, *5*).

The malic dehydrogenase and glutamate dehydrogenase used were supplied as ammonium sulfate suspensions. Before use, the suspensions were centrifuged and the pellets resuspended in cold, CO_2-free buffer (100 mM HEPES−NaOH, pH 7.5). To this mixture was added pyruvate kinase (lyophilyzed powder), and this enzyme solution was dialyzed versus CO_2-free buffer (100 mM HEPES−NaOH, pH 7.5) at 4 °C with continuous sparging by N_2 (CO_2-free).

Some concern has been expressed that, in previous total conversion experiments with malic acid, excess nucleotide, and malic enzyme, complete decarboxylation may not have been achieved (*5*). We have performed these total conversion experiments in three ways in this set of experiments: (1) using malic dehydrogenase, pyruvate kinase, and glutamate dehydrogenase as described above; (2) using malic enzyme and excess nucleotide as previously reported (*5*); and (3) using malic enzyme, a lower concentration of nucleotide (1.0 mM), and lactate dehydrogenase to reduce the pyruvate to lactate and recycle the nucleotide. In all cases the $^{13}C/^{12}C$ ratio of the CO_2 produced was identical within experimental error.

For the low-conversion samples, the final concentrations of reactants in 5.5 mL were 10 mM (2*R*,3*R*)-*erythro*-fluoromalate, 10 mM $MgSO_4$, 1.0 mM NADP or 3-APADP, 1600 units/mL of lactate dehydrogenase, and 100 units of malic enzyme in 100 mM HEPES, pH 7.1. Preliminary experiments indicated that, with very high 3-APADP concentrations, the reactions would not proceed to a large enough percentage of the total to permit adequate CO_2 production. Addition of lactate dehydrogenase alleviated this problem, serving to reduce the fluoropyruvate product to fluorolactate and recycle the nucleotide, thus allowing a relatively low concentration of total 3-APADP or NADP to be used. Before enzyme addition, the reaction mixtures were sparged with N_2 (CO_2-free) at low pH and then at the final pH as described for the total conversion samples. The malic enzyme−ammonium sulfate suspension was treated as the malic dehydrogenase and glutamate dehydrogenase suspensions were for the total conversion samples, and both it and the lactate dehydrogenase were dialyzed and sparged as described for the enzymes used in the total conversion samples. After enzyme addition to the reaction mixtures, a 500 μL aliquot was withdrawn from each sample and used to follow the progress of that particular reaction (by ^{19}F NMR). After an appropriate fractional conversion of substrate to product, the reactions were chilled on ice and quenched with 1 mL of cold, 18 N H_2SO_4. The CO_2 was isolated and the $^{13}C/^{12}C$ ratio determined as described above.

^{13}C Isotope Effects at C-4 for the Decarboxylation of Dianionic Fluorooxaloacetate. This isotope effect was determined in a manner similar to that for the dianion of oxaloacetate (*12*). A concentrated solution of fluorooxaloacetate was prepared by the dissolution of dry, lyophilized fluorooxaloacetate (lyophilized twice from metal-free H_2O) into a cold (on ice) buffer solution consisting of metal-free H_2O (prepared by passing H_2O over metal ion-chelating resin), 50 mM HEPES, and 0.1 mM EDTA. The pH was adjusted to 6.0 with saturated, CO_2-free NaOH, and the solution was sparged on ice with cold CO_2-free N_2 (cooled with ice using a cooling coil) for 2 h. The pH of this stock fluorooxaloacetate solution was raised to 7.5 with CO_2-free NaOH just before use.

The total and low-conversion samples consisted of 50 mM HEPES, 0.1 mM EDTA (final concentrations), and metal-free H_2O. The total conversion samples also contained 100 mM $MgSO_4$ to speed the decarboxylation of the fluorooxaloacetate. The pH of each of these solutions was initially ∼5.5. Each of these solutions was sparged with CO_2-free N_2 for 2−3 h. Subsequently the pH of each of the low-conversion samples was raised to 7.5 with sparging continued for another hour. At this point, fluorooxaloacetate from the sparged stock solution was added to each of the total conversion (2 mM final concentration) and low-conversion (20 mM final concentration) flasks. The low-conversion samples were allowed to react until ∼10% of the fluorooxaloacetate had decarboxylated, while the total conversion samples were allowed to proceed until all fluorooxaloacetate was decarboxylated. For all samples, the reactions were quenched with cold, 18N H_2SO_4 and immediately placed on ice until the CO_2 was isolated and the $^{13}C/^{12}C$ ratio determined as described above.

A group of samples identical to the low-conversion samples was also prepared and treated identically to the low-conversion samples except that the cold, 18 N H_2SO_4 was added just before the fluorooxaloacetate was added. We attempted to isolate CO_2 from these samples, but there was no detectable amount present. These controls ensured that the only CO_2 present in the low-conversion samples was due to the decarboxylation of the dianion of fluorooxaloacetate.

Data Analysis. Data were fitted by the least-squares method using the programs of Cleland (*13*). For the initial velocity studies, reciprocal initial velocities were plotted versus the reciprocal of substrate concentration, and the data were fitted to eq 1.

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

For the study directly comparing the malic enzyme-catalyzed oxidative decarboxylation of L-malate and (2*R*,3*R*)-*erythro*-fluoromalate, the data were fitted to eq 2:

$$v = \frac{VA}{(K + A)} \quad (2)$$

For the study of the inhibition of malic enzyme by (2*R*,3*R*)-*erythro*-fluoromalate, the data were fitted to equations

Table 1: Kinetic Parameters for Chicken Liver Malic Enzyme Catalysis of the Oxidative Decarboxylation of (2*R*,3*R*)-*erythro*-Fluoromalate[a]

parameter[b]	NADP	3-APADP
V (nmol/min)	0.069 ± 0.002	0.28 ± 0.02
K_a (mM)	0.0107 ± 0.0015	0.013 ± 0.002
K_b (mM)	0.14 ± 0.02	0.37 ± 0.05
V/K_a	6.4 ± 0.8	22 ± 4
V/K_b	0.49 ± 0.04	0.73 ± 0.08

[a] pH 7.25, 10 mM Mg^{2+}. [b] $K_a = K_{NADP}$ or $K_{3-APADP}$ and $K_b = K_{FMAL}$. The values of K_{ia} were poorly defined (0.003 ± 0.003 mM in each case).

assuming competitive or noncompetitive inhibition. The best fits were to eq 3 for competitive inhibition:

$$v = \frac{VA}{[K(1 + I/K_i) + A]} \quad (3)$$

For the primary deuterium isotope effect studies, the data were fitted to four different equations. The first equation assumed an isotope effect on V only, the second assumed an isotope effect on V/K only, the third allowed for isotope effects on V and V/K (eq 4), while the fourth assumed equal isotope effects on V and V/K. The best fits were obtained in each instance to eq 4:

$$v = \frac{VA}{[K_a(1 + FE_{V/K}) + A(1 + FE_V)]} \quad (4)$$

In eq 4, F is the fraction of deuterium label in the labeled substrate, and $E_{V/K}$ and E_V are the isotope effects minus 1 for V/K and V, respectively.

For the ^{13}C isotope effect studies, the data were fitted to eq 5:

$$^{13}(V/K) = \frac{\log(1 - f)}{\log[1 - f(R_p/R_o)]} \quad (5)$$

where f is the fractional conversion of substrate to product, R_p is the $^{13}C/^{12}C$ ratio in the CO_2 product, and R_o is the $^{13}C/^{12}C$ ratio in the C-4 carbon of (2*R*,3*R*)-*erythro*-fluoromalate determined from the total conversion experiments.

RESULTS

Initial Velocity Studies with (2R,3R)-erythro-Fluoromalate, NADP, and 3-APADP. Kinetic parameters for the malic enzyme-catalyzed decarboxylation of (2*R*,3*R*)-*erythro*-fluoromalate are shown in Table 1. Double-reciprocal plots were intersecting, indicating that the kinetic mechanism is sequential and no product is released before formation of the ternary EAB complex. Since the velocity of the reaction with (2*R*,3*R*)-*erythro*-fluoromalate is approximately 3000 times slower than the corresponding malic enzyme-catalyzed oxidative decarboxylation of L-malate (see below), the binding of the substrates comes to equilibrium. The reciprocal plots of velocity versus (2*R*,3*R*)-*erythro*-fluoromalate concentration show the lines intersecting left of the ordinate, which rules out a rapid equilibrium ordered mechanism. Thus the kinetic mechanism for the oxidative decarboxylation of (2*R*,3*R*)-*erythro*-fluoromalate catalyzed by malic enzyme must be rapid equilibrium random. Since the redox potential of 3-APADP ($E_o' = -0.258$, pH 7) is more positive than that of NADP ($E_o' = -0.32$), it is not surprising that the maximum velocity of the reaction with 3-APADP as the oxidant is greater than the corresponding reaction with NADP.

Table 2: Kinetic Parameters for Chicken Liver Malic Enzyme Catalysis of the Oxidative Decarboxylation of L-Malate and (2*R*,3*R*)-*erythro*-Fluoromalate with NADP as the Oxidant[a]

parameter	L-malate	(2*R*,3*R*)-*erythro*-fluoromalate
V	6.3 ± 0.2	$1.9 \pm 0.1 \times 10^{-3}$
K_m	0.21 ± 0.01	0.25 ± 0.03
V/K	30.5 ± 0.9	$7.5 \pm 0.7 \times 10^{-3}$

[a] Units are μmol min^{-1} mg^{-1} of enzyme for V, and mM for K_m. pH 7.25, 4.9 mM Mg^{2+}, 0.5 mM NADP.

Table 3: Primary Deuterium Isotope Effects for Chicken Liver Malic Enzyme Catalysis of the Oxidative Decarboxylation of L-Malate and (2*R*,3*R*)-*erythro*-Fluoromalate with NADP and 3-APADP as the Oxidants[a]

	NADP	3-APADP
L-malate		
$^D(V/K_{malate})$	1.47 ± 0.03[b]	2.9 ± 0.1[c]
^DV	1.47 + 0.03[b]	2.0 ± 0.1[c]
(2*R*,3*R*)-*erythro*-fluoromalate		
$^D(V/K_{fluoromalate})$	1.39 ± 0.06	3.32 ± 0.14
^DV	1.68 + 0.07	2.83 ± 0.12

[a] pH 7.25, 10 mM Mg^{2+}, 1 mM nucleotide. [b] Reference *2*. [c] Reference *5*.

Comparative Initial Velocity Studies with L-Malate and (2R,3R)-erythro-Fluoromalate and NADP as the Oxidant. The results of initial velocity studies with L-malate and (2*R*,3*R*)-*erythro*-fluoromalate with NADP as the oxidant are shown in Table 2. The maximum velocity of the malic enzyme-catalyzed reaction with L-malate is 3300 times greater than the corresponding reaction with (2*R*,3*R*)-*erythro*-fluoromalate. The apparent K_m in each case is very similar, 0.21 mM for L-malate and 0.25 mM for (2*R*,3*R*)-*erythro*-fluoromalate.

Inhibition of Malic Enzyme by (2R,3R)-erythro-Fluoromalate. As expected, (2*R*,3*R*)-*erythro*-fluoromalate is a competitive inhibitor of malic enzyme-catalyzed oxidative decarboxylation of malate. The K_i for this inhibition was 0.35 ± 0.02 mM, which is similar to the K_m of 0.25 mM.

Primary Deuterium Isotope Effects. Primary deuterium isotope effects for chicken liver malic enzyme and (2*R*,3*R*)-*erythro*-fluoromalate are shown in Table 3. For comparison, primary deuterium isotope effects for chicken liver malic enzyme and L-malate obtained by previous workers (*2*, *5*) are also shown.

^{13}C Isotope Effects at C-4 of (2R,3R)-erythro-Fluoromalic Acid for Malic Enzyme Catalysis. ^{13}C isotope effects at C-4 of fully protiated (2*R*,3*R*)-*erythro*-fluoromalate and C-2 deuterated (2*R*,3*R*)-*erythro*-fluoromalate are shown in Table 4. Also shown are results of ^{13}C isotope effect experiments with protiated and deuterated L-malate.

^{13}C Isotope Effects at C-4 for the Decarboxylation of Dianionic Fluorooxaloacetate. This isotope effect was determined to be 1.0522 ± 0.0001 at pH 7.5, which is identical to the value reported for the dianion of oxaloacetate (*12*). The decarboxylation of fluorooxaloacetate is ~2.5 times faster than the decarboxylation of oxaloacetate at this pH (*14*).

Table 4: ^{13}C Isotope Effects for Chicken Liver Malic Enzyme Catalysis of the Oxidative Decarboxylation of L-Malate and (2*R*,3*R*)-*erythro*-Fluoromalate and Their 2-Deuterated Isomers with NADP and 3-APADP as the Oxidants

	NADP	3-APADP
L-malate		
$^{13}(V/K_{malate})_H$	1.0336 ± 0.0003[a]	1.0051 ± 0.0001[b]
$^{13}(V/K_{malate})_D$	1.0250 ± 0.0005[b]	1.0084 ± 0.0001[b]
(2*R*,3*R*)-*erythro*-fluoromalate[c]		
$^{13}(V/K_{malate})_H$	1.0490 ± 0.0001	1.0138 ± 0.0001
$^{13}(V/K_{malate})_D$	1.0364 ± 0.0003	1.0087 ± 0.0001

[a] Reference *10*. [b] Reference *5*. [c] pH 7.1, 10 mM Mg^{2+}, 10 mM fluoromalate, 1 mM nucleotide (with recycling).

DISCUSSION

Since (2*R*,3*R*)-*erythro*-fluoromalate is the first reported alternate substrate for chicken liver malic enzyme, the mechanism of catalysis is of particular interest. Because previous studies with nucleotide substrates with more positive redox potentials have shown that the reaction which is stepwise with NADP becomes concerted (*5*, *7*), we were curious whether the same would happen with *erythro*-fluoromalate. The presence of fluorine in the 3-position makes the oxidation less favorable and, if hydride transfer became totally rate limiting, we would expect to see ^{13}C isotope effects only on that step. If the reaction were stepwise, any secondary ^{13}C isotope effect on hydride transfer would thus become apparent and deuteration of the substrate at C-2 should enhance the size of the ^{13}C isotope effect if there were a forward commitment to hydride transfer, or have no effect if there were no commitments.

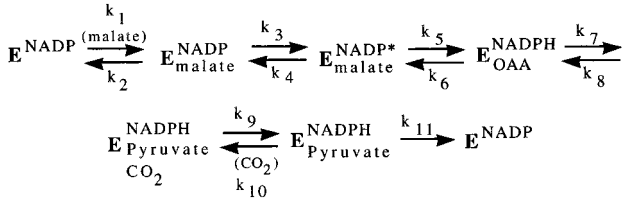
While the intrinsic deuterium isotope effect with L-malate and NADP is 5.7 (*3*), the observed primary deuterium isotope effect for (2*R*,3*R*)-*erythro*-fluoromalate with NADP as the oxidant is 1.39 and with 3-APADP is 3.32 (Table 3). These results clearly indicate that hydride transfer is not completely rate limiting, as least with NADP. The nearly full expression of the ^{13}C isotope effect (with NADP) and the fact that deuteration *decreases*, rather than increases, the isotope effect with both nucleotides show that the reaction is clearly stepwise with both. The reason given by Edens et al. (*7*) for the reaction becoming concerted with APADP was that the equilibrium constant for oxidation was more favorable, so that the free-energy level of the E-APADPH-pyruvate-CO_2 complex was decreased relative to that of E-APADP-malate. This decrease caused no intermediate to be formed in the reaction. The fluorine substitution in *erythro*-fluoromalate *decreases* the equilibrium constant by an order of magnitude (*9*), and thus preserves the stepwise mechanism, even with APADP.

Hermes et al. (*2*) showed that, in a stepwise mechanism where the deuterium-sensitive step came first, the various isotope effects were related by the following:

$$\frac{^{13}(V/K)_H - 1}{^{13}(V/K)_D - 1} = \frac{^{D}(V/K)}{^{D}K_{eq}} \quad (6)$$

With the values from Tables 3 and 4 and $^{D}K_{eq}$ values from the previous paper (*9*), we get 1.35 vs 1.28 with NADP as substrate and 1.59 vs 2.94 with APADP. The isotope effects are in reasonable agreement with NADP as substrate, but not with APADP. The discrepancy in this case may represent the elusive secondary ^{13}C isotope effect on hydride transfer which can be seen only when hydride transfer is the main rate-limiting step and the mechanism is stepwise. The predicted $^{13}(V/K)_D$ value from eq 6 is only 1.0047, so the remaining factor of ~1.004 may correspond to the secondary ^{13}C isotope effect. This isotope effect arises because of hyperconjugation between the carbonyl oxygen of the fluorooxalacetate intermediate and the C-3 to C-4 bond (C-4 being held out of plane by the enzyme to permit subsequent decarboxylation). Such hyperconjugation weakens the C-3 to C-4 bond, but the bond order to C-4 cannot be made up by increasing C−O bond order in the carboxyl group because the geometry does not permit a resonance form with two double bonds until the atoms are nearly collinear during decarboxylation.

We can analyze the observed isotope effects quantitatively in terms of the following mechanism:



In this mechanism, k_3 and k_4 are for the conformation change that sets up catalysis (NADP* is nucleotide activated for reduction), k_5 and k_6 are for hydride transfer, and k_7 is for decarboxylation. For consideration of *V*/*K* isotope effects, we can ignore rate constants k_8−k_{11}, since CO_2 will be released much faster from the enzyme (k_9) than it will react backward so that k_7 will represent an irreversible step. In addition, with a slow substrate such as *erythro*-fluoromalate, k_3 will be much less than k_2 and there will be no external forward commitment. Allowing for ^{13}C isotope effects on both hydride transfer and decarboxylation, the equations for the isotope effects then become the following:

$$^{D}(V/K) = \frac{^{D}k_5 + k_5/k_4 + {}^{D}K_{eq}k_6/k_7}{1 + k_5/k_4 + k_6/k_7} \quad (7)$$

$$^{13}(V/K)_H = \frac{^{13}K_{eq5}{}^{13}k_7 + {}^{13}k_5k_7/k_6 + (k_5/k_4)(k_7/k_6)}{1 + k_7/k_6 + (k_5/k_4)(k_7/k_6)} \quad (8)$$

$$^{13}(V/K)_D = \frac{^{13}K_{eq5}{}^{13}k_7 + {}^{13}k_5k_7{}^{D}k_6/k_6 + {}^{D}K_{eq}(k_5/k_4)(k_7/k_6)}{1 + k_7{}^{D}k_6/k_6 + {}^{D}K_{eq}(k_5/k_4)(k_7/k_6)} \quad (9)$$

Because of the very slow turnover number, k_3, k_5, or k_7 must be much smaller with *erythro*-fluoromalate than with malate. The change in $^{13}(V/K)$ values with deuteration and the low $^{D}(V/K)$ value with NADP show that there are still finite internal commitments in the system. With NADP the ratio of k_6/k_7 must have a large, but finite value, but k_5 could be considerably smaller than k_4. We have already assumed that k_3 is much smaller than k_2, so there is no external commitment. Since the oxidation of fluoromalate is less favored than that of malate by an order of magnitude (*9*), but the ratio of k_7/k_6 appears similar to that with malate, k_5 could be an order of magnitude less than with malate. The

remaining factor of 330 probably comes from a decrease in k_3, perhaps as a result of nonproductive initial binding of *erythro*-fluoromalate. After all, the threo isomer is not a substrate at all!

With NADP as substrate, it is clear that $k_6 > k_7$, and a reasonable fit to the isotope effects is obtained by assuming that $k_6/k_7 = 10$, $k_5 \ll k_4$, ${}^{D}k_5 = 5.7$, and ${}^{13}K_{eq5}{}^{13}k_7 = 1.054$. The value of ${}^{13}k_5$ has little influence on this calculation because of the small value of k_7/k_6.

With APADP as substrate, the ratios of k_7/k_6 and k_5/k_4 are different and we need to have a value for ${}^{13}k_5$ in order to get a suitable fit. If ${}^{13}K_{eq5}{}^{13}k_7$ is still 1.054 and ${}^{D}k_5$ is 5.7, the solution of eqs 7−9, using the ${}^{D}K_{eq}$ value of 1.1 from the previous paper (*9*), gives 1.0064 for ${}^{13}k_5$, 2.61 for k_7/k_6, and 0.58 for k_5/k_4. Compared to those with NADP, these values reflect the more favorable hydride transfer with the higher redox potential nucleotide.

Thus there *does* appear to be a secondary ^{13}C isotope effect on the hydride transfer step, although it is only detected when (1) the mechanism is stepwise, and (2) when hydride transfer is a major rate-limiting step. Because k_7/k_6 is so small with NADP, this effect is not seen. Since ${}^{13}k_5$ is not unity, what has always been assumed to be the ^{13}C isotope effect on decarboxylation is actually ${}^{13}K_{eq5}{}^{13}k_7$. We have no way of knowing how much larger ${}^{13}K_{eq5}$ is than ${}^{13}k_5$, but it is probably not over 1.01. In that case, ${}^{13}k_7$ would be ∼1.044, which is a typical value for a decarboxylation.

REFERENCES

1. Ochoa, S., Mehler, A. H., and Kornberg, A. (1947) *J. Biol. Chem. 167*, 871−872.
2. Hermes, J. D., Roeske, C. A., O'Leary, M. H., and Cleland, W. W. (1982) *Biochemistry 21*, 5106−5114.
3. Grissom, C. B., and Cleland, W. W. (1985) *Biochemistry 24*, 944−948.
4. Urbauer, J. L., and Cleland, W. W. (1989) *J. Cell Biol. 107*, 401a.
5. Weiss, P. M., Gavva, S. R., Harris, B. G., Urbauer, J. L., Cleland, W. W., and Cook, P. F. (1991) *Biochemistry 30*, 5755−5763.
6. Karsten, W. E., and Cook, P. F. (1994) *Biochemistry 33*, 2096−2103.
7. Edens, W. A., Urbauer, J. L., and Cleland, W. W. (1997) *Biochemistry 36*, 1141−1147.
8. Cook, P. F., and Cleland, W. W. (1981) *Biochemistry 20*, 1790−1796.
9. Urbauer, J. L., Bradshaw, D. E., and Cleland, W. W. (1998) *Biochemistry 37*, xxxx−xxxx.
10. Grissom, C. B., and Cleland, W. W. (1988) *Biochemistry 27*, 2927−2934.
11. Park, S.-H., Kiick, D. M., Harris, B. G., and Cook, P. F. (1984) *Biochemistry 23*, 5446−5453.
12. Grissom, C. B., and Cleland, W. W. (1986) *J. Am. Chem. Soc. 108*, 5582−5583.
13. Cleland, W. W. (1979) *Methods Enzymol. 63*, 103−138.
14. Dummel, R. J., Berry, M. N., and Kun, E. (1971) *Mol. Pharmacol. 7*, 367−374.

BI981820F