Synthesis of (2*R*,3*R*)-*erythro*- and (2*R*,3*S*)-*threo*-Fluoromalate Using Malic Dehydrogenase; Stereoselectivity of Malic Dehydrogenase†

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

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

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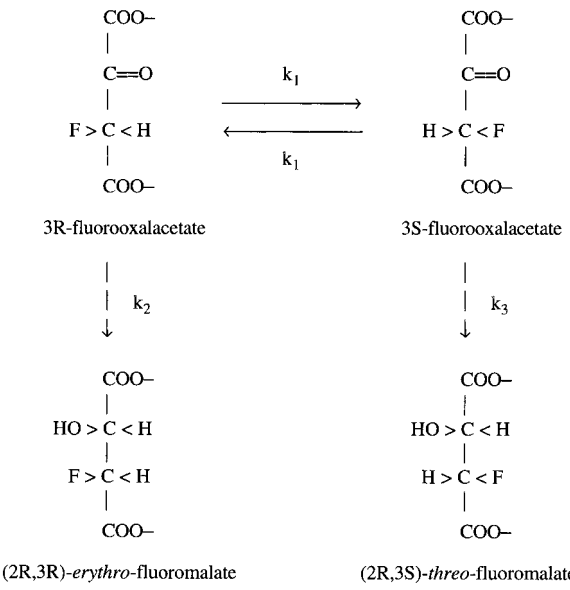
ABSTRACT: 3-Fluorooxalacetate is a substrate for malic dehydrogenase. When enzymatic reduction is slower than the rate of epimerization of the two enantiomers, only (2*R*,3*R*)-*erythro*-fluoromalate is formed. Conversely, when a high enzyme level and excess of NADH lead to reduction that is fast relative to the epimerization rate, equal amounts of (2*R*,3*R*)-*erythro*- and (2*R*,3*S*)-*threo*-fluoromalate are formed. These data suggest that the *V*/*K* value for reduction of the R enantiomer to give the erythro isomer is ∼100 times greater than for reduction of the S enantiomer to give the threo isomer. The equilibrium constant for the oxidation of fluoromalate is an order of magnitude less favorable than for oxidation of malate, while the equilibrium deuterium isotope effect from deuteration at C-2 of the substrate is 1.09 for fluoromalate versus 1.18 for malate. These effects reflect the inductive effect of fluorine at the 3-position.

Malic dehydrogenase is an α-hydroxydicarboxylic acid dehydrogenase, active with acids containing three to five carbons and possessing the same stereochemical arrangement about the α-carbon atom as L-malate (*1*). Kinetic experiments indicate that pig heart malic dehydrogenase catalyzes the reduction of oxalacetate to L-malate and concomitant oxidation of NADH with a compulsory order substrate binding mechanism in which NADH binds before oxalacetate in the forward direction and NAD before L-malate in the reverse direction (*1, 2*).

Malic dehydrogenase from bovine heart also catalyzes the reduction of fluorooxalacetate, producing fluoromalate with concomitant oxidation of NADH. Even though malic dehydrogenase is stereospecific at C-2, producing only the R isomer of fluoromalate (which is the same configuration as the S isomer of malate), at C-3 the conclusions of previous studies have been conflicting. Skilleter et al. (*3*) synthesized fluoromalate on a preparative scale using fluorooxalacetate and malic dehydrogenase, with recycling of the nucleotide using alcohol dehydrogenase and ethanol, and identified the single product as (2*R*,3*R*)-*erythro*-fluoromalate (see Scheme 1). The half time for the reaction was about 1.2 h, which is 6−8 times the half-life for isomerization of the fluroooxalacetate enantiomers at pD 8.5 via enolization (*4*). Under much different conditions (high enzyme concentration; reaction for 3 min), and on an analytical scale, fluoromalate has also been synthesized from fluorooxalacetate and NADH using malic dehydrogenase (*4, 5*). In these cases the products were reported to be *both* (2*R*,3*R*)-*erythro*- and (2*R*,3*S*)-*threo*-fluoromalate (Scheme 1). On the basis of these results, both Goldstein et al. (*4*) and Hwang and Nowak (*5*) concluded that malic dehydrogenase was not stereoselective. It was

Scheme 1



speculated by Goldstein et al. (*4*) that perhaps both isomers of fluoromalate *were* produced in the synthesis reported by Skilleter et al. (*3*) but the threo isomer of fluoromalate was lost during the purification procedure in which fluoromalate was precipitated as the lead salt. As we shall show, the apparent stereoselectivity depends on the speed with which the reduction of fluorooxalacetate is carried out relative to the rate of epimerization of the two enantiomers of fluroooxalacetate. The apparent conflict surrounding the stereoselectivity of malic dehydrogenase for reduction of fluorooxalacetate has thus now been resolved.

MATERIALS AND METHODS

Materials. Malic dehydrogenase from bovine heart, malic enzyme from chicken liver, and alcohol dehydrogenase from Bakers yeast were from Sigma. The fluoride ion standard

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* 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.

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was from Orion. Ethyl fluoroacetate (98%) and diethyl oxalate (99%+) used for the synthesis of fluorooxalacetate and ethanol-d_6 (99.5 + atom % D) were from Aldrich. Diethyl oxalate and ethyl ether (Mallinckrodt) were distilled before use. All other compounds were of reagent grade and used without further purification. The ^{19}F NMR spectra were acquired using a Bruker AM-400 spectrometer at a probe temperature of 25 °C. The chemical shifts are reported with respect to $CFCl_3$ (0 ppm).

Synthesis and Purification of Fluorooxalacetic Acid. A racemic mixture of diethyl fluorooxalacetate was prepared from diethyl oxalate, sodium ethoxide, and ethyl fluoroacetate (*6*). The crude mixture of diethyl fluorooxalacetate was distilled in vacuo, and fractions boiling from 120 to 122 °C at 9 mm (*6*) were collected and checked for the presence of diethyl fluorooxalacetate by HPLC (reverse-phase, Spherisorb, 250 × 4.6 mm, S10 ODS). The purest fractions were deprotected using a 2:1 mixture of glacial acetic acid and concentrated hydrochloric acid (*7*) to give a mixture of (3*R*)- and (3*S*)-fluorooxalacetic acids. The remaining fractions were combined and further purified using a reverse-phase silica gel column (total bed volume, 314 mL). The oily diethyl fluorooxalacetate mixture was added directly to the column, and the column was eluted with 10% methanol, 25% methanol, 35% methanol, and 50% methanol. The presence of diethyl fluorooxalacetate was detected by an increase in the absorbance at 280 nm and by HPLC (reverse-phase, Spherisorb, 250 × 4.6 mm, S10 ODS). The pure diethyl fluorooxalacetate was then deprotected as described above. All structures were confirmed by proton and fluorine NMR.

Stereoselective Enzymatic Synthesis of (2R,3R)-erythro-Fluoromalic Acid and C-2 Deuterated (2R,3R)-erythro-Fluoromalic Acid. The (3*R*)-fluorooxalacetate (K^+ salt) in a racemic mixture of R and S isomers was selectively reduced to (2*R*,3*R*)-*erythro*-fluoromalate (K^+ salt) in a 50 mL reaction mixture consisting of 0.2 mM NAD, 1.3 mM dithiothreitol, 200 units/mL malic dehydrogenase, and 0.44 g (approximately 2 mmol) of fluorooxalacetate. In addition, 200 units/mL alcohol dehydrogenase and 400 mM ethanol (or ethanol-d_6 for the preparation of C-2 deuterated (2*R*,3*R*)-*erythro*-fluoromalate) were added to the reaction mixture to recycle the nucleotide. The enzymes were added to the reaction mixture after the pH was adjusted to 8.0 with potassium hydroxide. The pH of the reaction mixture was monitored and readjusted to 8.0 periodically with potassium hydroxide. The decrease of fluorooxalacetate in the reaction mixture as a function of time was monitored using a malic dehydrogenase endpoint assay which consisted of 5 μL of the reaction mixture (rendered enzyme-free by centrifugation through a 10 000 molecular weight cutoff filter in a Centricon (Amicon) apparatus), 0.2 mM NADH, and 48 units/mL malic dehydrogenase. The approximate concentration of fluorooxalacetate was calculated using the extinction coefficient for NADH, 6220 M^{-1}. When the concentration of fluorooxalacetate was significantly reduced an additional 2 mmol of the fluorooxalacetate was added as well as another 100 units/mL malic dehydrogenase, 200 units/mL alcohol dehydrogenase, and 0.1 mM NAD. When all of the fluorooxalacetate was either converted to fluoromalate or decarboxylated, the reaction mixture was filtered through an Amicon PM 10 membrane (10 000 molecular weight cutoff) to remove the enzymes.

Purification of (2R,3R)-erythro-Fluoromalic Acid and C-2 Deuterated (2R,3R)-erythro-Fluoromalic Acid. (2*R*,3*R*)-*erythro*-Fluoromalic acid was purified by a modification of the procedure of Bulen et al. (*8*). A mixture of 30 g of silica gel (Merck grade 60, 230−400 mesh) and 24 mL of 0.5 N sulfuric acid was dried carefully in an oven at 120 °C. The resulting free-flowing powder was slurried in 300 mL of 0.5 N sulfuric acid-saturated chloroform and added to the chromatographic column in successive portions. The crude K^+ salt of (2*R*,3*R*)-*erythro*-fluoromalate (approximately 5 mL) was acidified to a pH of 1−2 with 18 N sulfuric acid and combined with 2 g of dry silica gel. The fluoromalate−silica gel mixture was dried by evaporation in vacuo and poured directly onto the column. The column was washed with 200 mL of acidified chloroform, and the various components of the reaction mixture were eluted with 500 mL of 15% *n*-butanol in chloroform, 500 mL of 25% *n*-butanol in chloroform, and 1000 mL of 35% *n*-butanol in chloroform. The eluents were equilibrated before use with 0.5 N sulfuric acid, filtered through a Whatman number 2 filter paper to remove small water droplets, and added to the top of the column through a reservoir. Pressure from a nitrogen tank was applied to the top of the column in order to speed elution and avoid back diffusion of the fluoromalic acid as it passed through the column. Acids were identified by titration with base. To a small tube containing 500 μL of H_2O and 20 μL of 0.02% phenol red indicator was added 250 μL of each 25 mL fraction. This mixture was titrated to a pink endpoint with 2 mM potassium hydroxide. The absorbance of each fraction at 260 nm was also determined. In this manner, fractions containing fluoromalic and fluoropyruvic acids could be distinguished from those containing any fluorooxalacetic acid or nucleotides. The acidic fractions which had a low absorbance at 260 nm were subjected to a malic enzyme activity assay to ensure distinction between fractions containing fluoromalic and fluoropyruvic acids. To a cuvette containing 1 mM NADP, 10 mM $MgSO_4$, and 2.8 units/mL of malic enzyme was added 10 μL of the butanol-chloroform-free sample, and the change in absorbance at 340 nm was recorded. Fluoropyruvate was also identified by reduction with NADH catalyzed by lactic dehydrogenase and by proton NMR.

Additional Purification of (2R,3R)-erythro-Fluoromalic Acid. Depending on the purity of the fluorooxalacetate used in the synthesis of fluoromalate, the following purification scheme was performed prior to the chloroform column procedure previously described. The enzyme-free orange−yellow reaction mixture containing (2*R*,3*R*)-*erythro*-fluoromalate was acidified to a pH of 5.0 and carefully added to the top of a DEAE-Sephadex A-25 anion exchange column (total bed volume, 177 mL). The nucleotides present in the reaction mixture were eluted from the column with an acetic acid gradient of 0−3 M and were detected by an increase in absorbance at 254 nm. The (2*R*,3*R*)-*erythro*-fluoromalic acid was eluted with 5 M acetic acid and was detected using a malic enzyme activity assay. The acetic acid was removed from the relatively pure (2*R*,3*R*)-*erythro*-fluoromalic acid in vacuo, and the sample was added to the top of a reverse-phase silica gel column (total bed volume, 177 mL) and eluted with H_2O. The column fractions were checked for the presence of (2*R*,3*R*)-*erythro*-fluoromalic acid using HPLC (Spherisorb HPLC column, S5 ODS1), elution with H_2O,

and detection by refractive index. The fractions containing the product were pooled, and the water was removed by rotary evaporation in vacuo or by lyophilization.

Enzymatic Synthesis of (2R,3R)-erythro- and (2R,3S)-threo-Fluoromalate Diastereomers. An initial reaction mixture containing 60 000 units of desalted malic dehydrogenase, 180.7 μmol of NADH, and 97.3 μmol of fluorooxalacetate (NH_4^+ salt) in 50 mL of 0.1 M ammonium bicarbonate buffer (pH 7.5) was allowed to stand at room temperature for 5 min. The reaction mixture was filtered through an Amicon YM 10 membrane (10 000 molecular weight cutoff) until approximately 2 mL of the solution remained. To the remaining solution, still in the Amicon filter apparatus, was added 97.3 μmol of fluorooxalacetate and a 50 mL aliquot of a stock solution containing 0.1 M ammonium bicarbonate buffer (pH 7.5) and 3.7 mM NADH. The mixture was gently stirred and allowed to stand at room temperature for 5 min before the reaction mixture was filtered down to a volume of 2 mL. This was repeated five times. The enzyme-free mixture was filtered through an Amicon YC 05 membrane (500 molecular weight cutoff) to remove 85−90% of the nucleotide. The mixture was rotary evaporated to dryness rinsing several times with methanol and H_2O. The dry ammonium bicarbonate-free sample was acidified to a pH of 1−2 with 0.5 N sulfuric acid and combined with 0.5 g of dry silica gel. The *erythro*- and *threo*-fluoromalic acid isomers were separated on a silica gel column as described above for (2*R*,3*R*)-*erythro*-fluoromalic and C-2 deuterated *erythro*-fluoromalic acids except that the column was prepared with 20 g of silica gel, washed with 150 mL of chloroform, after which the fluoromalic acid isomers were eluted with 250 mL of 15% *n*-butanol in chloroform, 250 mL of 25% *n*-butanol in chloroform, and 300 mL of 35% *n*-butanol in chloroform. As before, all eluents were equilibrated before use with sulfuric acid and filtered as described above.

Determination of Fluoromalate Concentration. The concentration of (2*R*,3*R*)-*erythro*-fluoromalate was determined using an Orion fluoride electrode to detect fluoride ions generated from fluoromalate by alkaline dehydrofluorination. The 500 μL dehydrofluorination sample contained a 200 μL aliquot of an approximately 10 mM fluoromalate solution and 0.6 M KOH. Complete dehydrofluorination, as determined by ^{19}F NMR, was achieved after heating the solution at 80 °C for 3 h. The sample was prepared for fluoride ion measurement by the addition of 4.5 mL of a 4 M buffered potassium acetate solution, pH 5.0, and 5.0 mL of TISAB buffer containing 1.0 M NaCl, 0.25 M acetic acid, 1.2 M sodium acetate, and 1.0 mM sodium citrate. A standard curve was generated by measuring 10.0 mL samples containing known concentrations of sodium fluoride in 0.03 M KOH, 4.5 mL of acetic acid buffer, and 5.0 mL of TISAB buffer. The acetic acid and TISAB buffers prevent fluoride electrode interference by OH^- ions. The correlation constant for a linear fit of the standard curve data (8 points) was >0.999. The concentration of the threo isomer was determined from digital integration of ^{19}F NMR spectra containing the threo isomer and known concentrations of the erythro isomer.

Determination of the Acid Dissociation Constants for (2R,3R)-erythro- and (2R,3S)-threo-Fluoromalic Acid. A solution of 10 mM (2*R*,3*R*)-*erythro*-fluoromalic acid or (2*R*,3*S*)-*threo*-fluoromalic acid and 100 mM KCl was titrated to pH 1.0 with HCl. The chemical shift of the single fluorine resonance relative to that in an external standard (neat $CFCl_3$) at a probe temperature of 25 °C was determined. The ^{19}F chemical shift was then determined as a function of pH as the solution was titrated with KOH, to a final pH of 6.55. The data were fitted to eq 1

$$\delta_{obs} = \frac{\delta_{min}([H^+]^2/K_1/K_2) + \delta_{med}([H^+]/K_2) + \delta_{max}}{[H^+]^2/K_1/K_2 + [H^+]/K_2 + 1} \quad (1)$$

where δ_{obs} is the observed chemical shift, δ_{med} is the intermediate chemical shift, δ_{min} and δ_{max} are the limiting chemical shifts at the pH extremes, and K_1 and K_2 are the acid dissociation constants.

Determination of the Stability Constant for the Mg^{2+}−(2R,3R)-erythro-Fluoromalate Complex. The effective stability constant for the Mg^{2+}−(2*R*,3*R*)-*erythro*-fluoromalate complex was determined at a pH of 7.25 in 100 mM HEPES−NaOH and 25 °C, conditions similar to those under which kinetic studies were to be performed. Even though binding of Mg^{2+} to HEPES is reported to be negligible (*9*, *10*), a concentration of 100 mM HEPES may of course bind a significant proportion of the added Mg^{2+}, especially at low Mg^{2+} concentrations. Under the conditions chosen, a known concentration and volume of a fluoromalate solution was titrated with a standard $MgCl_2$ solution (also in 100 mM HEPES, pH 7.25), and using ^{19}F NMR, the chemical shift of the fluorine resonance after each addition was determined (external reference $CFCl_3$). The concentrations of total fluoromalate and total Mg^{2+} were calculated after each addition of Mg^{2+}, with compensation for the dilution. The data (chemical shift, total fluoromalate, total Mg^{2+}) were fitted to eq 2

$$\ln \delta_{obs} = \ln \frac{\delta_{min} + \delta_{max} K/[Mg^{2+}]_{free}}{1 + K/[Mg^{2+}]_{free}} \quad (2)$$

where

$$[Mg^{2+}]_{free} = [-(K + [Fmal]_T - [Mg^{2+}]_T) + \{(K + [Fmal]_T - [Mg^{2+}]_T)^2 + 4[Mg^{2+}]_T\}^{1/2}]/2$$

and δ_{obs} is the observed chemical shift, δ_{min} and δ_{max} are the limiting chemical shifts at 0 and infinite concentrations of metal ion, respectively, K is the dissociation constant, $[Mg^{2+}]_{free}$ is the concentration of Mg^{2+} free in solution, $[Mg^{2+}]_T$ is the total Mg^{2+} concentration, and $[Fmal]_T$ is the total fluoromalate concentration.

Stereoselectivity of Malic Dehydrogenase. The first set of reactions was performed to demonstrate the substrate dependence of the stereoselectivity of malic dehydrogenase-catalyzed reduction of the isomers of fluorooxalacetate. The reaction mixtures contained malic dehydrogenase (1200 units/mL), NADH (1.0−10 mM), fluorooxalacetate (1.0−10.0 mM), 0.5 mM EDTA, and 100 mM HEPES−NaOH, pH 8.0. Reactions were initiated by the addition of malic dehydrogenase, and after 30 min at 25 °C, the protein was removed by ultrafiltration through 30 000 molecular weight cutoff filters in Centricon 30 (Amicon) centrifugal ultrafiltration units. The process of protein removal took 15 min for each

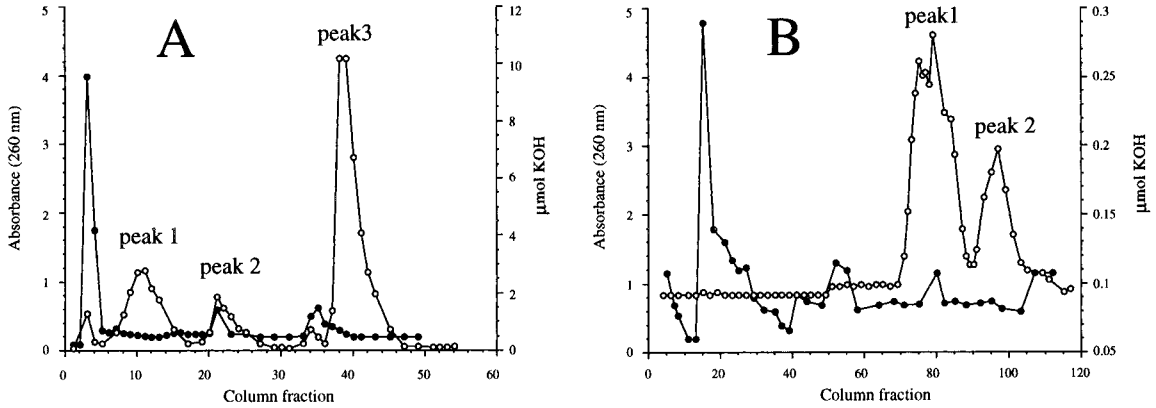

FIGURE 1: Profiles for elution of the components of the preparative enzymatic syntheses of (2*R*,3*R*)-*erythro*-fluoromalate (A) and a mixture of (2*R*,3*R*)-*erythro*- and (2*R*,3*S*)-*threo*-fluoromalate (B) from silica gel columns. Closed symbols represent absorbance at 260 nm, and open symbols represent the number of micromoles of KOH necessary to titrate the acid(s) in the indicated fractions. Peaks containing significant quantities of acidic components are labeled. In A, peak 1 contains fluoropyruvate, peak 2 contains fluorooxalacetate, and peak 3 contains (2*R*,3*R*)-*erythro*-fluoromalate. In B, peak 1 contains (2*R*,3*S*)-*threo*-fluoromalate while peak 2 contains (2*R*,3*R*)-*erythro*-fluoromalate.

sample. Each sample was frozen with liquid nitrogen, lyophilized, and resuspended in 0.5 mL of H_2O for ^{19}F NMR analysis. The ratio of *erythro*/*threo*-fluoromalate concentrations produced was determined by digital integration of the fluorine resonances in the ^{19}F NMR spectrum.

The second experiment was performed to elucidate the time dependence of the stereoselectivity of fluorooxalacetate reduction catalyzed by malic dehydrogenase. These reaction mixtures contained initially 10 mM fluorooxalacetate, 0.2 mM NAD, 60 mM glucose-6-phosphate, 200 units/mL glucose-6-phosphate dehydrogenase, and 20 units/mL of malic dehydrogenase in 200 mM HEPES, pH 8.0. The temperature was kept constant at 25 °C. The glucose-6-phosphate and glucose-6-phosphate dehydrogenase served to recycle the nucleotide and keep its concentration constant. The reaction was initiated by the addition of malic dehydrogenase, and aliquots were withdrawn as a function of time. The protein was removed from each aliquot using Centricon 30 ultrafiltration units as described above, and the samples were further handled and analyzed as described above.

Determination of Equilibrium Constants and Equilibrium Deuterium Isotope Effects. The equilibrium constants and equilibrium isotope effects for the oxidation of L-malate or (2*R*,3*R*)-*erythro*-fluoromalate by both NAD and APAD were accomplished essentially as described by Cook et al. (*11*). Nucleotide (NAD or APAD) and malate (or malate analogue) were added to a cuvette along with buffer solution and allowed to equilibrate to 25 °C. Enough malate dehydrogenase was then added to quickly establish the equilibrium. The change in absorbance (at 340 nm for NAD, and 363 nm for APAD) was used to determine the concentrations of the reactants and products at equilibrium. In all cases, the buffer used was 200 mM HEPES at pH ~8.0. The malate dehydrogenase suspension was centrifuged and resuspended in the HEPES buffer before use. The final pH of each determination of a given equilibrium constant was determined by direct measurement of the pH of the solution in the cuvette after equilibrium was reached. In all cases, it ranged from 7.85 to 8.0. Several different starting concentrations of reactants were used in determining the equilibrium constants, and each reported equilibrium constant is the result of up to 17 determinations.

The concentrations of L-malate and L-malate-2-*d* were determined by enzymatic endpoint assay using malic enzyme and excess NADP (*11*). The concentrations of (2*R*,3*R*)-*erythro*-fluoromalate and (2*R*,3*R*)-*erythro*-fluoromalate-2-*d* were determined as described above. The concentrations of NAD and APAD were determined by enzymatic endpoint using glucose-6-phosphate dehydrogenase and excess glucose-6-phosphate.

RESULTS

Enzymatic Synthesis and Purification of (2R,3R)-erythro-Fluoromalic Acid. A typical profile (Figure1A) for elution of the components of the preparative enzymatic synthesis of (2*R*,3*R*)-*erythro*-fluoromalic acid from a silica gel column shows three acidic peaks. The first and third to elute show low absorbances (at 260 nm) while the second has a distinctly higher absorbance. The first acidic peak to elute (low absorbance) was inactive with the malic enzyme activity assay, was active with a lactate dehydrogenase enzymatic assay, and was confirmed using ^1H NMR to be fluoropyruvic acid. The second acidic peak to elute (higher absorbance) was active with a malic dehydrogenase enzymatic assay and confirmed to be fluorooxalacetic acid using NMR. The third acidic peak to elute (low absorbance) was active with the malic enzyme activity assay and was confirmed using ^1H and ^{19}F NMR to be (2*R*,3*R*)-*erythro*-fluoromalic acid.

Enzymatic Synthesis and Purification of (2R,3R)-erythro- and (2R,3S)-threo-Fluoromalic Acid Diastereomers. The elution profile of the components of the preparative enzymatic syntheses of a mixture of (2*R*,3*R*)-*erythro*- and (2*R*,3*S*)-*threo*-fluoromalic acids from a silica gel column is shown in Figure 1B. The first acidic peak to elute contains the (2*R*,3*S*)-*threo*-fluoromalic acid which is not active with the malic enzyme activity assay, while the second acidic peak contains the (2*R*,3*R*)-*erythro*-fluoromalic acid which is active with the malic enzyme activity assay. The assignment of the column peaks to the specific isomers of fluoromalic acid was based upon the results of Goldstein et al. (*4*) and ^1H and ^{19}F NMR.

Fast Atom Bombardment Mass Spectrometry. A sample of (2*R*,3*R*)-*erythro*-fluoromalic acid was subjected to FAB mass spectrometry. In the negative ion mode, the predominant peak was the parent peak at *m*/*e* 151 as expected.

Table 1: Coupling Constants (in Hz) for C-2 Deuterated and Protiated (2*R*,3*R*)-*erythro*-Fluoromalic and (2*R*,3*S*)-*threo*-Fluoromalic Acids

acid	$J_{F\text{-}H}$, geminal	$J_{F\text{-}H}$, vicinal
(2*R*,3*R*)-*erythro*-fluoromalic acid		
pH 2.0[a]	47.6	23.3
pH 8.0[b]	49.9	25.0
C-2 deuterated (2*R*,3*R*)-*erythro*-fluoromalic acid		
pH 2.0[a]	47.4	NR[c]
pH 8.0[b]	49.7	2[d]
(2*R*,3*S*)-*threo*-fluoromalic acid		
pH 2.0[a]	46	33

[a] Sample dissolved in H_2O, pH approximate. [b] Sample in 100 mM HEPES−NaOH. [c] At acidic pH and the field strength used herein, this coupling is not resolved. [d] This coupling is poorly resolved and is approximate.

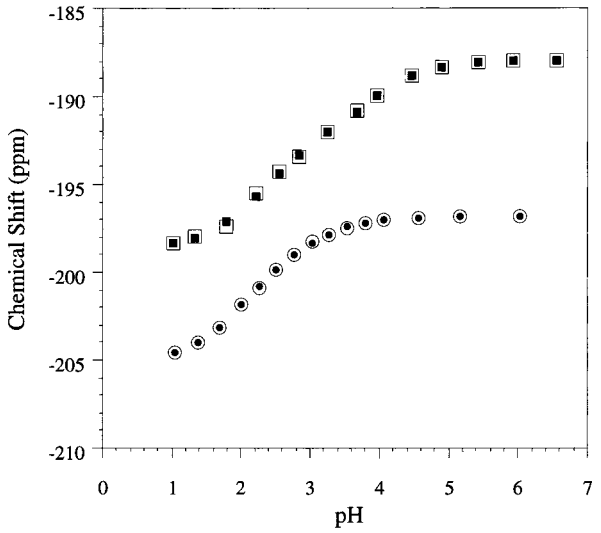

FIGURE 2: pK_a titration curve used to determine pK_{a1} and pK_{a2} for (2*R*,3*R*)-*erythro*-fluoromalic acid and (2*R*,3*S*)-*threo*-fluoromalic acid. ^{19}F chemical shifts were referenced to $CFCl_3$ (0 ppm). Open symbols represent experimental data. Closed symbols represent calculated values. Squares correspond to the erythro isomer while circles correspond to the threo isomer of (2*R*,3*R*)-fluoromalic acid.

Fluorine NMR of the Isomers of Fluoromalic Acid. We have reported previously the ^{19}F NMR spectra and coupling constants of (2*R*,3*R*)-*erythro*- and (2*R*,3*R*)-*threo*-fluoromalic acid at neutral pH as these compounds are products of the coupled reactions of phosphoenolpyruvate carboxylase and malate dehydrogenase using (*Z*)- and (*E*)-3-fluoro-phosphoenolpyruvate as substrates (*12*). The coupling constants for these compounds, as well as C-2 deuterated (2*R*,3*R*)-*eythro*-fluoromalic acid at pH 2.0 and 8.0, are shown in Table 1. The C-2 deuterated (2*R*,3*R*)-*eythro*-fluoromalic acid shows a doublet of doublets which resonates upfield of the protiated analogue by about 0.25 ppm, with a very small vicinal coupling.

Acid Dissociation Constants for (2R,3R)-erythro- and (2R,3S)-threo-Fluoromalic Acid. Figure 2 shows a titration curve resulting from the titration of (2*R*,3*R*)-*erythro*- and (2*R*,3*S*)-*threo*-fluoromalic acid with KOH. The pK_a values of (2*R*,3*R*)-*erythro*-fluoromalic acid were found to be 2.27 ± 0.13 and 3.86 ± 0.16 while those for the threo isomer were found to be 1.98 ± 0.05 and 2.99 + 0.10 (Table 2). The pK_a values of DL mixtures of threo and erythro fluoromalic acids produced by sodium borohydride reduction of dimethyl fluorooxalacetate and deprotection in acid have been determined previously (Table 2). The pK_a values for malic acid are included in Table 2 for comparison.

Table 2: pK_a Values for Malic and Fluoromalic Acids

acid	pK_{a1}	pK_{a2}
DL-3-fluoromalic acid A[a]	2.6	3.6
DL-3-fluoromalic acid B[b]	2.7	4.3
(2*R*,3*R*)-*erythro*-fluoromalic acid[c]	2.27 ± 0.13	3.86 ± 0.16
(2*R*,3*S*)-*threo*-fluoromalic acid[c]	1.98 ± 0.05	2.99 ± 0.10
malic acid[d]	3.46	5.10

[a] Reference *13*; mixture of (2*R*,3*S*)-*threo* and (2*S*,3*R*)-*threo* isomers. "A" refers to the fact that these isomers elute first (and together) during silica gel chromatography. [b] Reference *13*; mixture of (2*R*,3*R*)-*erythro* and (2*S*,3*S*)-*erythro* isomers. "B" refers to the fact that these isomers elute second (and together) during silica gel chromatography. [c] This work; error limits are standard errors from the fit to eq 1. [d] Reference *14*.

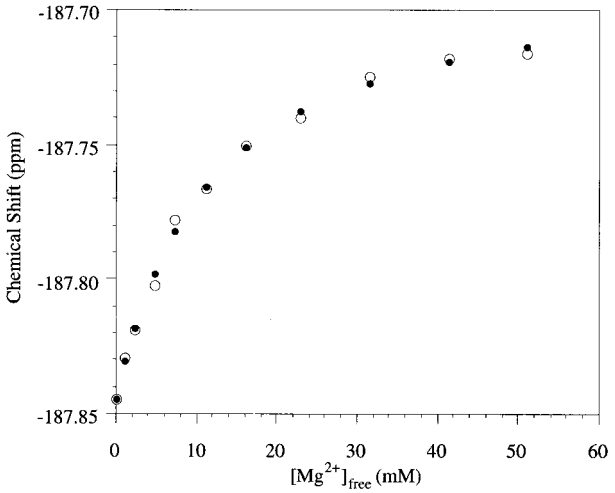

FIGURE 3: Titration curve used to determine the stability constant for the Mg^{2+}−(2*R*,3*R*)-*erythro*-fluoromalate complex (pH 7.25, 25 °C). ^{19}F chemical shifts were referenced to $CFCl_3$ (0 ppm). Open symbols represent experimental data. Closed symbols represent calculated values.

Stability Constant for the Mg^{2+}−(2R,3R)-erythro-Fluoromalate Complex. Figure 3 shows a titration curve used to determine the stability constant for the Mg^{2+}−(2*R*,3*R*)-*erythro*-fluoromalate complex. The dissociation constant for the Mg^{2+}−(2*R*,3*R*)-*erythro*-fluoromalate complex was determined to be 11.9 ± 1.4 mM from a fit of the data to eq 2.

Stereoselectivity of Malic Dehydrogenase Catalysis of Fluorooxalacetate Reduction. Table 3 shows the results of a study of the substrate dependence of the stereoselectivity of malic dehydrogenase-catalyzed reduction of fluorooxalacetate. The results indicate that a profound range of stereoselectivity is exhibited by malic dehydrogenase, depending on the concentrations of the reactants. The results shown in Figure 4 show the time course for the reduction of the isomers of fluorooxalacetate catalyzed by malic dehydrogenase under conditions known to favor the production of the (2*R*,3*R*)-*erythro*-fluoromalate isomer. Since the initial concentration of fluoroxalacetate was ∼10 mM, it is clear that not only is the 3S isomer of fluorooxalacetate not reduced initially, but also epimerization allows production essentially of only the (2*R*,3*R*)-*erythro*-fluoromalate isomer as the 3S isomer epimerizes to the 3R isomer (the unaccounted for fluorooxalacetate has decarboxylated). (2*R*,3*S*)-

Table 3: Stereoselective Production of Fluoromalate Isomers from Fluorooxalacetate Catalyzed by Malic Dehydrogenase[a]

[Fluorooxalacetate] (mM)	[NADH] (mM)	[*erythro*]/[*threo*]
1	1	1.4
1	5	1.2
1	10	0.9
5	1	>10[b]
5	5	4.6
5	10	1.6
10	1	∞[c]
10	5	∞
10	10	∞

[a] Reaction for 30 min at 25 °C in 100 mM HEPES, pH 8.0, with 1200 units/mL malic dehydrogenase. See Methods. [b] This value is approximate since accurate integration of the extremely small threo resonance was not possible. [c] No threo detected.

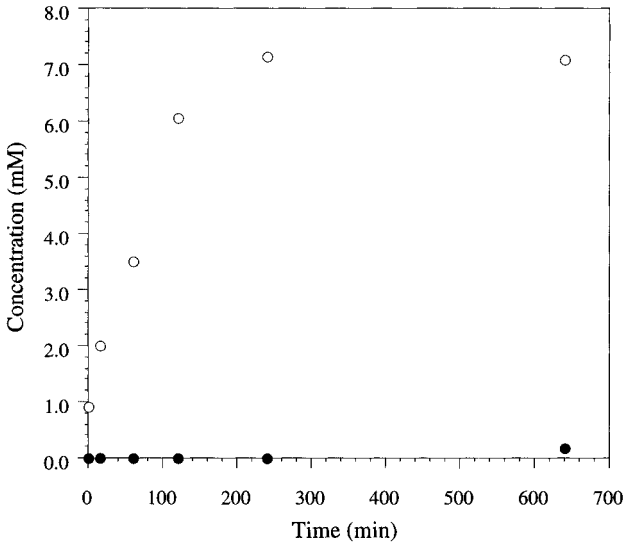

FIGURE 4: Time course for the production of (2*R*,3*R*)-*erythro*- and (2*R*,3*S*)-*threo*-fluoromalate from fluorooxalacetate and NADH catalyzed by malic dehydrogenase. Open circles mark the erythro isomer concentrations while closed circles mark the threo isomer concentrations. Some (2*R*,3*R*)-*erythro*-fluoromalate is produced at $t = 0$ because of the finite time necessary to remove the enzymes. The reaction mixture (pH 8) contained 10 mM fluorooxalacetate, 20 units/mL malic dehydrogenase, and 0.2 mM NADH maintained by recycling (see methods for details).

threo-Fluoromalate is only detected near the end of the experiment when the concentration of fluorooxalacetate remaining is small.

Equilibrium Constants and Deuterium Isotope Effects. Table 4 shows equilibrium constants for the oxidation of the various malates by either NAD or APAD catalyzed by malic dehydrogenase, as well as the equilibirum deuterium isotope effects calculated from these data. The observed values are corrected for the presence of 81% ketone in oxalacetate or 13% ketone in fluorooxalacetate. The values for malate or malate-2-d and NAD are similar to those determined by Cook et al. (*11*), while the reaction is more spontaneous by a factor of ~80 with APAD (the factor calculated from the difference in redox potentials of NAD and APAD is 125). The presence of fluorine at C-3 favors reduction and thus decreases the equilibrium constant by a factor of 10. An accurate value for the equilibrium constant for reaction of APAD and fluoromalate was not obtained, but on the basis of the redox potentials, the value should be about 5×10^{-12} M.

Table 4: Equilibrium Constants and Equilibrium Isotope Effects for Malic Dehydrogenase

substrates	K_{eqH} (M)	K_{eqD} (M)	$^{D}K_{eq}$
malate, NAD	$6.65 \pm 0.03 \times 10^{-13}$	$5.59 \pm 0.08 \times 10^{-13}$	1.18 ± 0.01
malate, APAD	$5.36 \pm 0.02 \times 10^{-11}$	$4.63 \pm 0.02 \times 10^{-11}$	1.16 ± 0.01
F-malate, NAD	$0.63 \pm 0.02 \times 10^{-13}$	$0.58 \pm 0.02 \times 10^{-13}$	1.09 ± 0.01

DISCUSSION

The primary goal of these experiments was to synthesize, purify, and characterize the fluoromalate isomers in preparation for kinetic and isotope effect experiments with malic enzyme. Depending on the reaction conditions chosen, either (2*R*,3*R*)-*erythro*-fluoromalate or both (2*R*,3*R*)-*erythro*- and (2*R*,3*S*)-*threo*-fluoromalate, as well as their deuterated counterparts, can be synthesized enzymatically on a preparative scale. When the purification schemes described are used, the fluoromalate isomers can be separated from all components present in the reaction mixture as well as from each other, as judged from the elution profiles from the silica columns and as confirmed by NMR. The results of these syntheses also confirm the results of Skilleter et al. (*3*) in that the conditions they chose were very similar to those that we used for stereoselectively producing only the (2*R*,3*R*)-*erythro*-fluoromalate isomer. In contrast to their conclusions, however, malic dehydrogenase catalysis is not totally stereospecific with respect to fluorooxalacetate reduction. We have shown that, on a preparative scale, both (2*R*,3*R*)-*erythro*- and (2*R*,3*S*)-*threo*-fluoromalate may be produced, consistent with the results of Goldstein et al. (*4*). Goldstein et al. (*4*) concluded incorrectly, however, that malic dehydrogenase catalysis is not stereoselective. Our results from the preparative syntheses clearly show that the stereoselectivity of this enzyme is dependent on the rate of the reduction relative to the rate of epimerization of fluorooxalacetate enantiomers (see below).

The coupling constant between the fluorine and the vicinal proton for (2*R*,3*S*)-*threo*-fluoromalate is larger than for (2*R*,3*R*)-*erythro*-fluoromalate because trans couplings are larger than cis couplings (*15*). The fluorine does couple with the C-2 deuterium, but the splitting is small enough so that the spectrum of C-2 deuterated (2*R*,3*R*)-*erythro*-fluoromalate appears as a very broad (line width) doublet rather than a doublet of doublets.

Until now, malic acid has been the only compound to have been confirmed to undergo oxidative decarboxylation catalyzed by malic enzyme. We have determined that the chicken liver enzyme catalyzes the oxidative decarboxylation of (2*R*,3*R*)-*erythro*-fluoromalate, but not of the (2*R*,3*S*)-*threo* isomer (following paper). Interestingly, malic enzyme from *Ascaris suum* will not use either fluoromalate isomer as a substrate at concentrations similar to those used for the chicken enzyme (Urbauer and Cleland, unpublished results). Even though the chicken liver enzyme will catalyze the oxidative decarboxylation of (2*R*,3*R*)-*erythro*-fluoromalate, and this reaction may be used for the detection of (2*R*,3*R*)-*erythro*-fluoromalate, the slow rate of this catalysis prohibits the use of malic enzyme endpoint assays for routine determinations of concentration. Of course malic enzyme endpoint assays are useless for quantitation of the (2*R*,3*S*)-*threo* isomer of fluoromalate. These facts dictated the necessity for a simple, efficient, and reproducible method

for the quantitation of fluoromalate. The method described herein using a fluoride electrode to detect fluoride ions generated from fluoromalate by alkaline dehydrofluorination meets this demand. The fluoromalate concentration can also be determined using NMR, but with slightly less accuracy, and with less convenience.

As shown in Table 2, the pK_a values for the fluoromalic acid isomers are significantly lower than those reported for malic acid, as expected from the inductive effect of the fluorine atom. The discrepancies in the values determined in the present work and those reported by Krasna (*13*) for the corresponding DL mixtures are about 0.4−0.6 pK_a units, although the separation of the two pK_a values for a particular isomer determined by the two methods is identical (1.6 pH units). The NMR procedure we used eliminates the necessity of standard solutions, and knowledge of the concentrations of any of the components (other than pH) should be accurate. Krasna (*13*) reported that "pK_a values were determined from titration curves produced on a Beckman pH meter".

Examination of the titration curves in Figure 2 and the fit of the data to eq 1 show that the limiting chemical shifts for (2*R*,3*R*)-*erythro*-fluoromalic acid at low and high pH are −198.6 and −187.9, respectively, and the chemical shifts at the pH values corresponding to the low and high pK_a values are −195.3 and −190.2, respectively. The difference between the limiting chemical shift at high pH and the chemical shift at the high pK_a value is 2.3, while the difference between the limiting chemical shift at low pH and the chemical shift at the low pK_a value is 3.3. It is expected that the change in chemical shift of the fluorine atom will be larger for the titration of the carboxyl group closest to the fluorine atom. In this instance, the fluorine atom is one carbon closer to the C-4 carboxyl than to the C-1 carboxyl, indicating that the pK_a value of the C-4 carboxyl is actually the lower of the two pK_a values for (2*R*,3*R*)-*erythro*-fluoromalic acid. The situation is the same for the threo isomer. This of course is in contrast to the case of malic acid where the C-4 carboxyl is that with the highest pK_a.

It can be expected that this reversal of high and low pK_a values can be extrapolated to the case of fluorooxalacetate, as compared to oxalacetate. In support of this, molecular orbital calculations (albeit in the gas phase) indicate that fluorooxalacetate with the C-4 carboxyl protonated is at least 1 kcal less stable than that with the C-1 carboxyl protonated, while the reverse is true for oxalacetate (Urbauer, unpublished results). With oxalacetate, at low pH the C-4 carboxyl is preferentially protonated, and this species cannot decarboxylate. The rate of decarboxylation is then dependent upon the very small proportion present with C-1 protonated, with this species decarboxylating rapidly. With fluorooxalacetate, under similar conditions, the C-1 carboxyl will be protonated preferentially, and since this is the species whose decarboxylation is most facile, this will result in the apparent increase in the rate of decarboxylation of this compound compared to oxalacetate. The high extent of hydration of fluorooxalacetate (87% hydrate, 13% ketone, no detectable enol at neutral pH), however, masks this increase so that the actual rate of decarboxylation is only 40% higher than that of oxalacetate at pH 3 (16).

The dissociation constant for the Mg^{2+}−malate complex is reported to be 25.1 mM (*17*). The value of 11.9 ± 1.4 mM for the Mg^{2+}−(2*R*,3*R*)-*erythro*-fluoromalate complex (Figure 3) refects the greater stability of this complex as compared to the Mg^{2+}−malate complex.

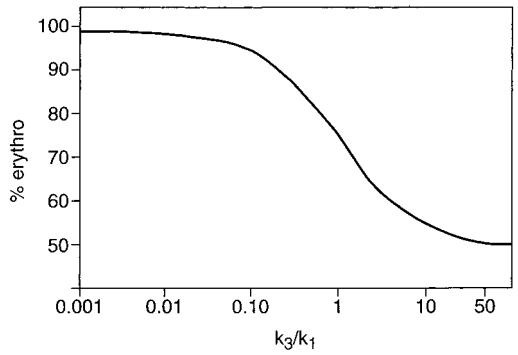

FIGURE 5: The percent of erythro isomer produced during the reduction of fluorooxalacetate. The *V*/*K* for the production of the erythro isomer is assumed to be 100 times that for the production of the threo isomer. The rate constant k_1 is for epimerization of fluorooxalacetate enantiomers, while k_3 is the *V*/*K* for the production of the threo isomer. Calculated from the model in Scheme 1. The percent plotted is not an integration over time, but rather the result for a given set of conditions.

One of the most intriguing results of this research is the striking range of stereoselectivity exhibited by malic dehydrogenase in the catalysis of fluorooxalacetate reduction. The results of the preparative syntheses of the fluoromalate isomers reported herein, along with the results shown in Table 3, clearly illustrate why the conclusions of previous investigators have been at odds. A slow reduction favors the production of the erythro isomer, while rapid reduction leads to equal production of erythro and threo isomers.

The reason for this behavior can be made clear by the model in Scheme 1. In this model, k_1 is the rate constant for the epimerization of fluorooxalacetate and k_2 and k_3 are the *V*/*K* values for the reduction by NADH catalyzed by malic dehydrogenase. Figure 5 is a plot of % *erythro*-fluoromalate produced by the reduction of fluorooxalacetate as a function of the ratio of k_3/k_1, when $k_2/k_3 = 100$. While the experiments we have carried out do not define k_2/k_3 precisely, it is clear from graphs similar to Figure 5 for other ratios that the true *V*/*K* ratio is not far from 100. When fluorooxalacetate concentration is high, the first-order equilibration of the two enantiomers is relatively fast, and slow reduction gives almost entirely the erythro isomer of fluoromalate. Conversely, when large amounts of enzyme are used so that k_3 is large and only low concentrations of fluorooxalacetate are present along with excess NADH, one approaches a 50−50 mixture of fluoromalate isomers. The reaction will be biphasic, with the erythro isomer produced rapidly, and the remaining 3S-fluorooxalacetate reduced to the threo isomer more slowly, although still faster than epimerization.

The slow epimerization of fluorooxalacetate isomers (half time of at least 9−12 min (*4*)) is a consequence of the inhibitory effect of fluorine on enolization. This effect is also shown by the low level of enol present at neutral pH (not detectable by NMR), compared to the 12% enol present in oxalacetate (*18*).

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