Isotope Effects on the Crotonase Reaction†

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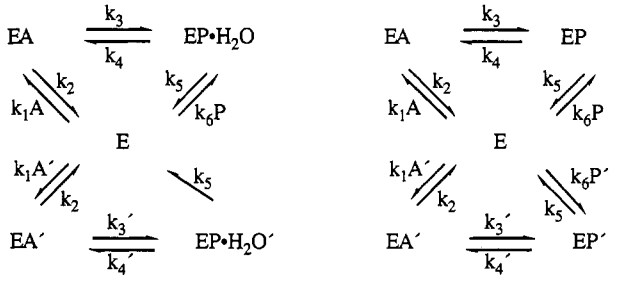
ABSTRACT: The primary, α-secondary, β-secondary, and β'-secondary deuterium and primary ^{18}O kinetic isotope effects on V/K for the dehydration of [(3*S*)-3-hydroxybutyryl]pantetheine by bovine liver crotonase (enoyl-CoA hydratase, EC 4.2.1.17) have been determined by the equilibrium perturbation method. The primary deuterium and ^{18}O kinetic isotope effects are 1.61 and 1.051, respectively. The secondary deuterium effects at C-2, C-3, and C-4 are 1.12, 1.13, and 1.00 per H, respectively. The large ^{18}O isotope effect suggests C–O bond cleavage is largely rate determining but is consistent with either an E1cb or E2 mechanism with a large amount of carbanion character. The β-secondary effect is a factor of 1.05 greater than the equilibrium isotope effect, indicating that this C–H bond is less stiff in the affected transition state or that its motion is coupled to the reaction coordinate motion. Analytical solutions to the differential equations describing uni-uni equilibrium perturbations are presented.

Crotonase (enoyl-CoA hydratase, EC 4.2.1.17) catalyzes the syn addition of water across the double bond of α–β-unsaturated CoA thioesters. The enzyme accepts a wide variety of unsaturated thioesters including acryloyl-CoA and *p*-substituted cinammoyl-CoAs (Person, 1981) besides the normal straight-chain thioesters. The requirement for the CoA portion is far more restricted, the pantetheine esters being utilized with less than 0.1% of the efficiency of the CoA thioesters (Waterson et al., 1972). Some of this efficiency may be returned by use of analogues of the adenosine portion of CoA as a cosubstrate with the pantetheine thioesters.

Far less is known about the chemical mechanisms of enzymatic syn elimination or addition than about the corresponding anti additions and eliminations. It is apparent that those substrates for enzyme-catalyzed elimination reactions that have more acidic hydrogens prefer the syn pathway. The α-hydrogens of CoA thioesters are deprotonated nearly as fast as the α-hydrogens of acetone (Lienhard & Wang, 1968) and fall into this class. The enhanced acidity of the α-hydrogens is believed to stabilize an enolate form of the carbanion at the enzymes active site and result in an E1cb mechanism. The tight binding of the enolate form of acetoacetyl-CoA (Acac-CoA)[1] to the enzyme (Waterson & Hill, 1972) suggests there may be an enolate intermediate. Thibblin and Jencks (1979) have made the contrary suggestion that the kinetic difficulty of deprotonating weak acids such as CoA thioesters may lead to a concerted elimination.

The crotonase-catalyzed elimination is an ideal system to examine the mechanism of syn eliminations. The reaction has an equilibrium constant near unity, and the α–β-unsaturated thioester provides a chromophoric substrate, which makes the equilibrium perturbation methodology (Schimerlik et al., 1975) available for the precise determination of small isotope effects. The structure of the substrate permits not only the primary kinetic isotope effects (KIEs) to be measured, but three separate secondary ^2H KIEs are available to aid in characterizing the hybridization state of the carbon atoms in the transition state (Cleland, 1987). The very large V/K of 5×10^7 M^{-1} s^{-1} and small primary ^2H KIE on dehydration of 3-hydroxybutyryl-CoA (Person, 1981) suggested that to prevent rate-determining diffusion from masking the KIEs, a slower alternative substrate should be employed in the studies. In this paper we report the primary ^2H, primary ^{18}O, and secondary α, β, and β' KIEs[2] on the dehydration of [(3*S*)-3-hydroxybutyryl]pantetheine (HBP) in the presence of the activator 3′,5′-ADP.

Scheme I



THEORY

Perturbation Derivations. The equilibrium perturbation method for determining isotope effects was introduced by Schimerlik et al. (1975). The derivation presented used an ordered bi-ter reaction as the model. The adaptation to hydratases for primary isotope effects employed solvent at either 55 M for oxygen effects on 110 M for ^2H effects as one of the perturbants. To correct for the commitments[3] introduced by

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[1] Abbreviations: Acac-CoA, acetoacetyl-CoA; KIE, kinetic isotope effect; HBP, [(3*S*)-3-hydroxybutyryl]pantetheine; EDTA, ethylenediaminetetraacetic acid; AcacP, S-(acetoacetyl)pantetheine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TAPS, 3-[[tris(hydroxymethyl)methyl]-amino]propanesulfonic acid; CrP, *trans*-crotonylpantetheine; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HB-CoA, (3*S*)-3-hydroxybutyryl-CoA; Cr-CoA, *trans*-crotonyl-CoA; Tris, tris(hydroxymethyl)-aminomethane.

[2] In elimination reactions the α-secondary deuterium effect is for isotopic substitution on the carbon bonded to the heavy atom leaving group, the β-secondary effect is for isotopic substitution at the carbon whose C–H bond is broken, and the β'-secondary effect is for substitution at the nonreacting β-carbon (Cook, 1976).

[3] Northrop (1977) introduced the terminology of commitments to catalysis to reflect the partitioning of enzyme–substrate complexes between catalysis and dissociation of the substrate. If the commitment is generated by the addition of a second substrate, the commitment is termed external. An internal commitment is generated by nonisotope-dependent steps, e.g., product dissociation, being rate determining.

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nonperturbing substrates, Schimerlik et al. introduced a variable y. The procedure for picking the numerical value of y does not yield correct results when solvent is one of the substrates that changes isotopic composition in the approach to isotopic equilibrium.[4] We have analytically solved two systems for the analysis of equilibrium perturbations of hydratases. Scheme Ia is used for the analysis of primary isotope effects where the isotopically substituted position in the substrate exchanges with solvent. Inherent to the solution is the assumption that loss of label to solvent is irreversible. Scheme Ib is used to analyze secondary perturbations where the heavy isotope remains in the dehydrated product. The derivations follow the general outline presented by Schimerlik et al. (1975) (A) The differential equations for the appearance of labeled and unlabeled chromophore are produced, in our case according to the King–Altman procedure to determine the fraction of enzyme present in each form depicted in parts a and b of Scheme I. (B) The rate equations are expressed in terms of the concentration of P and analytically integrated. (C) The time of maximum deviation from the equilibrium absorbance is determined by setting the derivative with time (dP/dt) of the equation developed in step B to 0 and solving for t_{max}. (D) The value of t_{max} is substituted in the integrated rate equation and $[P]_{tmax}$ determined. (E) Algebraic manipulation has shown the results are most easily interpreted when they are reported as a *fractional perturbation*, the difference between the initial or final product concentration ($[P]_{0/\infty}$) and the value at t_{max} ($[P]_{tmax}$) divided by the maximum possible perturbation. The absolute value of the maximum possible perturbation (if there were an infinite isotope effect) is determined by following the change in absorbance when either the substrate or product is omitted from the assay. Detailed derivations for both schemes are provided as supplementary material. The final result for primary KIEs is given in eq 1,

$$\text{fractional perturbation} = \frac{P_{tmax} - P_{\infty}}{\Delta P_{max}} = \frac{X - \alpha X}{\alpha X - 1}(\alpha X^{-\alpha X/(\alpha X-1)} - \alpha X^{-1/(\alpha X-1)}) \quad (1)$$

where α is the KIE on V/K for the hydrated substrate and $X = (K_{eq} + 1)/K_{eq}$ where K_{eq} is the equilibrium constant for dehydration of the unlabeled substrate. To determine α, the value of X is fixed by the experimental system, and a table of values of α and the calculated fractional perturbation are generated with a digital computer. By use of double-precision variables and an interpolative scheme, any fractional perturbation can be converted to a KIE without significant error.

For the secondary KIEs the experimental procedure and definitions are identical, but because the heavy isotope remains in the substrate or product at isotopic equilibrium, i.e., it does not wash out into solvent, the final form of the solution is different:

$$\text{fractional perturbation} = \frac{P_{tmax} - P_{\infty}}{\Delta P_{max}} = X^{1/(1-X)} - X^{X/(1-X)} \quad (2)$$

where

$$X = \frac{1 + {}^{D}K_{eq}/K_{eq}}{\alpha(1 + 1/K_{eq})} \quad (3)$$

Because of our interest in dehydratases, both of these results assumed that the unlabeled dehydrated product was monitored. If the isotopically labeled dehydrated product is monitored, as in secondary effects with [3-^{2}H]crotonylpantetheine, the sign of the perturbation must be changed; i.e., if $[P]_{tmax} > [P]_{0/\infty}$, the perturbation size used in the analysis should be negative.

[4] This has been demonstrated directly by a numerical solution of the differential equations (Anderson and Cleland, unpublished observation).

Materials and Methods

Enzymes. Crotonase was isolated from fresh bovine liver according to a modified procedure of Steinman and Hill (1975) on a reduced scale. The procedure was started with 40% (300 g) of the amount of liver of the Steinman method. Dialysis following the acetone procedure was omitted so that in 1 day the procedure could be carried up through the ammonium sulfate precipitation where the enzyme is more stable. The protein concentration of crystalline crotonase was determined from $\epsilon_{280} = 5.76 \times 10^6$ M^{-1} cm^{-1} (Hass & Hill, 1969). The crotonase was recrystallized and stored frozen as a crystalline suspension in 20 mM potassium phosphate buffer (pH 7.4, 3 mM EDTA, 10% ethanol). (3*S*)-Hydroxyacyl-CoA dehydrogenase was isolated from bovine liver according to a method by Staack et al. (1978). The enzyme was purified on a phosphocellulose column (0.3 × 15 cm) eluted with a linear gradient from 0.01 to 0.2 M potassium phosphate (pH 6.6). The enzyme was stored frozen in a 20 mM potassium phosphate buffer solution (pH 6.6, 20% glycerol, 1 mM mercaptoethanol, 1 mM EDTA). Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (type XXIII) and hexokinase from bakers' yeast (type C-300) were from Sigma Chemical Co.

Chemicals. Diketene, [2,2,2-$^2H_3$]acetyl chloride (99 atom % ^{2}H), and 2,2-dimethyl-1,3-dioxane-4,6-dione (Meldrum's acid) were from Aldrich Chemical Co. D-Pantethine, coenzyme A, 3′,5′-ADP, NADH, D-glucose 6-phosphate, ATP, NAD$^+$, and pentafluorobenzyl bromide were from Sigma Chemical Co. Deuterium oxide (99.9 atom % ^{2}H) and normalized $H_2{}^{18}O$ (97–98 atom % ^{18}O) were from Cambridge Isotope Laboratories. [1-^{2}H]Glucose (98 atom % ^{2}H) was from Omicron Biochemicals. All other chemicals were of reagent grade and were used without further purification.

Proton Nuclear Magnetic Resonance. ^{1}H NMR samples were prepared for analysis by lyophilizing and resuspending the compounds twice in D_2O to remove solvent-exchangeable protons. All spectra were taken on a Bruker WM 250-MHz NMR spectrometer in D_2O. The chemical shifts reported are with respect to tetramethylsilane; however, the internal standards used were the pantetheine methyl peaks at δ 0.89 and 0.92.

High-Performance Liquid Chromatography. HPLC of pantetheine thioester substrates was performed on a 0.46 × 25.0 cm octadecylsilyl Econosphere reverse-phase column (Alltech) using methanol/water solutions eluted at 1 mL/min. Peaks were detected by ultraviolet absorbance at 232 nm. Multiple injections were necessary to separate the substrates since the HPLC column used was not capable of separating more than 2–3 mg of sample per injection. HPLC of CoA thioester substrates was performed on a 1.0 × 25.0 cm Econosphere octadecylsilyl column eluted with methanol, sodium phosphate, and water solutions at 4 mL/min. The column successfully purified 10 mg of sample per injection. CoA thioester peaks were detected at 259 nm by ultraviolet absorbance.

Mass Spectroscopic Analysis of Isotopically Labeled HBP. Analysis of HBP-labeled substrates was performed with EI$^-$ mass spectrometry of pentafluorobenzyl 3-hydroxybutyrate at low resolution. A Kratos MS80RFA mass spectrometer interfaced to a Data General Eclipse System using Kratos

DS-55 software was tuned with perfluorokeroscene in the EI^- mode. Isotopically labeled HBP was hydrolyzed in 0.5 mL of 0.1 M sodium phosphate, pH 10.5. The solution was titrated to pH 6.5 with 1 M H_3PO_4, and a 5-fold excess of pentafluorobenzyl bromide was added. Ethanol (0.5 mL) was added as a cosolvent, and the reaction was stirred at 60 °C for 30 min. The pentafluorobenzyl ester was extracted into ethyl acetate and brought to dryness under a stream of dry nitrogen. Samples were injected into a gas chromatography fused silica capillary column interfaced to the mass spectrometer. Following the injection of sample, the oven temperature was kept at 60 °C for 3 min; then it was increased to 250 °C in 9.5 min with a linear gradient. 3-Hydroxybutyric acid was derivatized with pentafluorobenzyl bromide and used as a standard. The mass spectrum showed an intense fragment peak at 103 amu and a peak 4% the size at 104 amu corresponding to ^{13}C natural abundance of the four-carbon fragment. No other fragment peaks were observed. The isotopic composition of the labeled substrates was determined by comparison of the m through $m + 3$ peak intensities, which were corrected for ^{13}C natural abundance.

S-(Acetoacetyl)pantetheine (AcacP). Pantethine (3.6 mmol) was reduced to pantetheine with an excess of sodium borohydride (10 mmol) in 0.2 M sodium phosphate, pH 9. The solution was acidified to pH 4.0 with 1 M H_3PO_4 to quench unreacted sodium borohydride and then titrated to pH 8.5 with 1 M NaOH and reacted with an excess of diketene (10 mmol). The reaction was stirred at 25 °C until there was no detectable free thiol as assayed by 1.0 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in 0.1 M 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid (TAPS), pH 8.5 (Ellman, 1959). The solution was titrated to pH 5 with 1 M H_3PO_4, saturated with NaCl, and then extracted five times with equal volumes of ethyl acetate. The ethyl acetate was removed by rotary evaporation, and AcacP was purified on a silica gel column (3 × 15 cm) eluted with chloroform/methanol (90/10 v/v). AcacP concentration was assayed by use of $\epsilon_{302nm} = 16\,900\ M^{-1}\ cm^{-1}$ in 0.1 M TAPS, pH 8.5, with 25 mM Mg^{2+} (Middleton, 1972). ^1H NMR: δ 0.89 (s, 3 H), 0.92 (s, 3 H), 2.30 (s, 3 H), 2.47 (t, 2 H), 3.06 (t, 2 H), 3.45 (m, 6 H), 3.99 (s, 1 H).

HBP. AcacP (140 μmol), glucose 6-phosphate (150 μmol), and NADH (catalytic amount) were added to 1 mL of 0.1 M sodium phosphate, pH 7.5. The absorbance at 380 nm was monitored during the following two steps to determine when the reaction had been driven to completion by following the NADH concentration ($\epsilon_{380} = 900\ M^{-1}\ cm^{-1}$). (3*S*)-3-Hydroxyacyl-CoA dehydrogenase, which catalyzes the stereospecific reduction of (3-ketoacyl)pantetheine to [(3*S*)-3-hydroxyacyl]pantetheine (Wakil, 1963), was added, which resulted in the rapid decrease of the measured absorbance until equilibrium was reached. Glucose-6-phosphate dehydrogenase, which regenerates NADH by catalyzing the reduction of NAD^+ by glucose 6-phosphate, was added, which resulted in a gradual increase to a steady-state absorbance. The reaction was determined 100% complete when the absorbance increased rapidly from the steady-state absorbance to an absorbance corresponding to the complete regeneration of NADH. HBP was purified by HPLC eluted isocratically with 25% methanol. The HBP peak was collected at 14 min following the pantetheine peak at 9 min. ^1H NMR: δ 0.89 (s, 3 H), 0.92 (s, 3 H), 1.23 (d, 3 H), 2.47 (t, 2 H), 2.79 (d, 2 H), 3.06 (t, 2 H), 3.45 (m, 6 H), 3.99 (s, 1 H), 4.27 (m, 1 H).

trans-Crotonylpantetheine (CrP). HBP was dehydrated to CrP by crotonase. The equilibrium was attained in 1 mL of 0.1 M 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.0, with 100 μM 3′,5′-ADP and 20 nM crotonase. HPLC eluted isocratically with 35% methanol gave the CrP peak at 20 min. ^1H NMR: δ 0.89 (s, 3 H), 0.92 (s, 3 H), 1.90 (d, 3 H), 2.47 (t, 2 H), 3.06 (t, 2 H), 3.45 (m, 6 H), 3.99 (s, 1 H), 6.31 (d, 1 H), 7.04 (m, 1 H). Crotonylpantetheine that was synthesized by the acylation of pantetheine with crotonic anhydride contained contaminating cis isomer. The ^1H NMR spectrum of crotonic anhydride from Aldrich Chemical Co. indicated the presence of 7% cis isomer. CrP that was synthesized enzymatically from AcacP showed no cis isomer by ^1H NMR.

(2R)-[2-^2H]HBP. Deuterium oxide (0.5 mL) was added to pure HBP to remove residual water. The solution was evaporated and then resuspended in 1 mL of D_2O to which 20 nM crotonase and 100 μM 3′,5′-ADP were added. (2*R*)-[2-^{2}H]HBP was separated from CrP by HPLC after 3 h was allowed for the exchange of deuterium into the C-2 primary position. The ^1H NMR spectrum of (2*R*)-[2-^{2}H]HBP differed from the spectrum of HBP at 2.79 ppm where the doublet corresponding to the C-2 protons integrated to 1 h due to the ^2H incorporation at that position. The spin–spin coupling constant of the doublet was reduced to 4.3 Hz, which is consistent with an erythro coupling constant (Mohrig et al., 1980; Anderson & Hammes, 1984). The multiplet peak at 4.27 ppm corresponding to the C-3 proton was noticeably different due to the loss of the threo spin–spin coupling upon primary deuteration of HBP. The extent of crotonase-catalyzed deuterium exchange into the C-2 primary position was determined to be 91 ± 1 atom % ^2H by low-resolution mass spectrometry.

(3S)-3-Hydroxybutyryl-CoA (HB-CoA), trans-Crotonyl-CoA (Cr-CoA), and (2R)-[2-^2H]HB-CoA. Coenzyme A (50 μmol) was reacted with an excess of diketene (150 μmol) in 1 mL of sodium phosphate (100 mM, pH 8.0), until the absence of free thiol was detected (Ellman, 1959). The solution was titrated to pH 5 with 1 M H_3PO_4 and extracted two times with 1-mL aliquots of ethyl acetate. The solution was titrated to pH 7 with 1 M NaOH. The Acac-CoA was enzymatically reduced to HB-CoA (see above). HB-CoA was purified by HPLC eluted isocratically with an 11% methanol and 10 mM sodium phosphate solution (pH 4.5). The HB-CoA peak was collected at 8–12 min. HB-CoA was equilibrated with crotonase, and the product Cr-CoA was purified by HPLC eluted isocratically with a 16% methanol and 10 mM sodium phosphate solution (pH 4.5). The Cr-CoA peak was collected at 17–20 min. HB-CoA was equilibrated in 1 mL of D_2O with crotonase to exchange ^2H into the primary C-2 position. (2*R*)-[2-^{2}H]HB-CoA was purified from Cr-CoA by HPLC. The ^1H NMR spectrum of (2*R*)-[2-^{2}H]HB-CoA indicated >95 atom % ^2H incorporation by integration of the C-2 proton peak at 2.7 ppm.

[3-^{18}O]HBP. AcacP (140 μmol) was dissolved and evaporated twice from 1 mL of anhydrous ethyl acetate to azeotrope away water. $H_2{}^{18}O$ (400 μL, 97–98 atom % ^{18}O) and 1 μL of concentrated HCl were combined, which made the solution acidic (pH 2) in order to exchange the 3-keto oxygen of AcacP. After 3 days was allowed for the exchange, the solution was titrated to pH 7.0 with tris(hydroxymethyl)aminomethane (Tris). The [3-^{18}O]AcacP was reduced to [3-^{18}O]HBP enzymatically as was done for the unlabeled AcacP to HBP reduction except the reaction was run in $H_2{}^{18}O$. The $H_2{}^{18}O$ was recovered following the reduction by lyophilizing the sample under high vacuum and capturing the $H_2{}^{18}O$ in a trap cooled with liquid nitrogen. Mass spectroscopic analysis in-

dicated 93.0 ± 1.0 atom % ^{18}O incorporation.

[2,2-$^2H_2$]HBP. The C-2 protons of AcacP (140 μmol) were exchanged with deuterium in less than 5 min when dissolved in 1.0 mL of D_2O. The [2,2-$^2H_2$]AcacP was enzymatically reduced to [2,2-$^2H_2$]HBP (as above). The ^1H NMR spectrum of [2,2-$^2H_2$]HBP was used to determine an enrichment of 97 ± 1 atom % ^2H at C-2 by integration of the C-2 proton peak at 2.79 ppm. Mass spectroscopic analysis of the [2,2-$^2H_2$]HBP indicated that during prolonged (2 days) exchange of the C-2 protons of AcacP there was partial exchange of the C-4 methyl protons (>10 atom % ^2H), by the presence of $m + 3$, $m + 4$, and $m + 5$ peaks in addition to the expected m, $m + 1$, and $m + 2$ peaks.

[2-^2H]CrP and (2S)-[2-^2H]HBP. [2-^2H]CrP was obtained by the crotonase-catalyzed dehydration of [2,2-$^2H_2$]HBP to [2-^2H]CrP followed by HPLC isolation. Subsequent hydration of [2-^2H]CrP by crotonase followed by HPLC isolation yielded (2S)-[2-^2H]HBP. The ^1H NMR spectrum of [2-^2H]CrP differed from the spectrum of CrP by the lack of a peak at 6.31 ppm, which corresponds to a deuterated secondary C-2 position, and the multiplet at 7.04 ppm became a broadened singlet. The ^1H NMR spectrum of (2S)-[2-^2H]HBP differed from the spectrum of (2R)-[2-^2H]HBP only by the value of the spin–spin coupling constant. A J_{HCCH} of 8.3 Hz for the C-2 proton peak at 2.79 ppm is consistent with a threo coupling constant (Mohrig, 1980; Anderson & Hammes, 1984).

[3-^2H]HBP and [3-^2H]CrP. AcacP (140 μmol) was reduced stereospecifically with (4R)-[4-^2H]NADH. [1-^2H]Glucose (98 atom % ^2H, 150 μmol), ATP (150 μmol), 5 mg of magnesium acetate, hexokinase, and a catalytic amount of NAD^+ (5 μmol) were added instead of the glucose 6-phosphate and NADH which were used for the unlabeled AcacP to HBP reduction. A rapid increase in absorbance at 380 nm indicated the reaction was complete. [3-^2H]HBP was obtained pure by HPLC. [3-^2H]CrP was obtained by HPLC following equilibration of the [3-^2H]HBP with crotonase. In the ^1H NMR spectrum of [3-^2H]HBP there was no observable peak at 4.27 ppm, corresponding to >98 atom % ^2H incorporation. ^1H NMR peaks at 1.23 and 2.79 ppm, which correspond to C-4 and C-2 protons, respectively, were singlets due to ^2H incorporation at C-3.

[4,4,4-$^2H_3$]HBP and [4,4,4-$^2H_3$]CrP. A solution of [2,2,2-$^2H_3$]acetyl chloride (13.75 mmol) in 10 mL of methylene chloride was added dropwise over 30 min to a mixture of Meldrum's acid (12.5 mmol) and pyridine (25 mmol) in 20 mL of methylene chloride stirring at 0 °C. The solution was stirred an additional 30 min at 25 °C. Rotary evaporation of the solvent gave acylated Meldrum's acid in 70% yield. Pantetheine (1 mmol), from the reduction of pantethine by sodium borohydride, was extracted into ethyl acetate. The solution was dissolved and evaporated from anhydrous ethyl acetate under a stream of dry N_2 twice to azeotrope away residual water. The anhydrous pantetheine was added to acylated Meldrum's acid (1 mmol) in 10 mL of anhydrous THF and refluxed for 2 h. [4,4,4-$^2H_3$]AcacP (20% yield) was separated on a silica gel column (3 × 10 cm) eluted with chloroform/methanol (90/10 v/v). [4,4,4-$^2H_3$]HBP and [4,4,4-$^2H_3$]CrP were obtained by the same route described for the unlabeled substrates, starting with [4,4,4-$^2H_3$]AcacP. The ^1H NMR spectra of [4,4,4-$^2H_3$]HBP and [4,4,4-$^2H_3$]CrP indicated 60 ± 3 atom % ^2H by integration of the C-4 methyl peak relative to the pantetheine methyl peaks. Mass spectroscopic analysis of [4,4,4-$^2H_3$]HBP indicated 57 ± 1 atom % ^2H.

Kinetic Isotope Effects. KIEs reported were measured according to the equilibrium perturbation technique of Schimerlik et al. (1975). A Perkin-Elmer λ-3B UV/vis spectrophotometer thermostated at 25 ± 0.1 °C interfaced to an IBM PC computer using ASYST software was used for data acquisition. Substrate concentrations were measured enzymatically by measuring absorbance changes at 280 nm where the ΔA_{280} corresponding to the hydration of α,β-unsaturated CrP has an extinction coefficient of 3600 M^{-1} cm^{-1} (Lynen & Ochoa, 1953). Typically, equilibrium perturbations were run in 0.1 M MOPS (pH 7.00, 0.1 M ionic strength with potassium salt, 1 mM EDTA) with 100 μM 3′,5′-ADP and 20 nM crotonase in a total volume of 600 μL unless otherwise noted. Under these conditions equilibrium perturbations reached their maximum or minimum absorbance in 2–5 min, and final equilibrium was reached within 20–50 min. Typical substrate concentrations resulted in a ΔA_{280} of +0.2 OD for HBP and −0.2 OD for CrP upon addition of crotonase. By use of the K_{eq} value of 0.29 for the dehydration reaction, ΔA_{280} of 0.2 OD, and the extinction coefficient for the ΔA_{280}, substrate concentrations are calculated to be approximately 250 μM HBP and 70 μM CrP. Initial substrate concentrations were fine tuned so that the final absorbance was equal to the initial absorbance of the perturbation (Cleland, 1980).

HBP and CrP, with one substrate labeled and one substrate unlabeled, were added to a 1-mL quartz cuvette from separate stock solutions so that each substrate had an equal and opposite ΔA_{280}. The perturbation solution was temperature equilibrated to 25 °C for 5 min in the spectrophotometer. Prior to starting the perturbation, the initial absorbance was recorded, and the absence of a background change in absorbance was confirmed. At higher pH or over long storage times (days), HBP is prone to hydrolysis to produce pantetheine. The product thiol forms a Michael adduct by attacking CrP at the C-3 position. The presence of free thiol consequently results in a constant decrease in absorbance, which makes measurement of the equilibrium perturbations less precise. To avoid this problem, it was necessary to purify the substrates by HPLC immediately before use. The equilibrium perturbation was initiated by the addition of a small volume of crotonase (25 μL, 25 °C) followed by three inversions of the cuvette to mix the solution.

The fractional perturbation is reported as the ΔA_{280} of the perturbation (ΔA_{pert}) divided by the maximum ΔA_{280} possible (ΔA_{max}). The ΔA_{pert} is the difference between the perturbation maximum or minimum and the initial or final absorbance. In cases when the initial and the final absorbance of a perturbation were not equal and the difference was not large compared to the ΔA_{pert}, the initial and final absorbance values were averaged. The ΔA_{max} is defined as the ΔA_{280} of either substrate from its initial to its equilibrium absorbance upon addition of crotonase since each substrate should have an equal and opposite ΔA_{280}. The estimated errors are derived from an estimated error of ±0.001 absorbance unit in the perturbation size. Duplicate perturbations were always obtained within the indicated error limits. Differences between this paper and our preliminary results (Bahnson & Anderson, 1988) came from systematic errors, the most important being a failure to exclude all free thiols from the reaction mixture.

For the interpretation of secondary ^2H kinetic isotope effects it was necessary to accurately estimate the ^2H equilibrium isotope effect ($^DK_{eq}$)[5] due to the dependence of the calculated

[5] The notation for isotope effects is from Cleland (1982). A leading superscript of the heavier isotope indicates an isotope effect on the following parameter. The nomenclature for discussing isotope effects on enzyme reactions is from the same source.

$^{D}V/K$ on $^{D}K_{eq}$ (eq 2 and 3). The $^{D}K_{eq}$ used was estimated from the ratio of $^{D}K_{eq}$ values for the transfer of deuterium from molecules resembling HBP and CrP at the secondary labeled position to water (Cleland, 1980).

Results

Equilibrium Perturbation Control Experiment. To ensure that observed equilibrium perturbations were the result of an isotope effect of the labeled substrate, a control was run with unlabeled substrates. HBP and CrP, which were at concentrations that correspond to a ΔA_{max} of 0.2 OD, were added together. There was no equilibrium perturbation detectable ($\Delta A_{pert} < 0.002$). This rules out the possibility that something other than an isotope effect of the labeled substrate is responsible for the equilibrium perturbations reported below. One potential problem that was discovered by this control was a 7% cis contaminant in CrP which had been synthesized from crotonic anhydride. Crotonase catalyzes the hydration of *cis*-crotonyl-CoA at about $^1/_3$ of the rate of *trans*-crotonyl-CoA (Stern, 1961). Using CrP that had contaminating cis isomer in the equilibrium perturbation control experiment gave a positive perturbation as expected for a slower alternative substrate (Anderson, unpublished observation). This problem was corrected by synthesizing CrP enzymatically starting from AcacP.

Primary $^{D}V/K$ with (2R)-[2-^{2}H]HBP. The primary $^{D}V/K$ was measured with a mixture of (2*R*)-[2-^{2}H]HBP and CrP which were at equilibrium concentrations prior to and following the equilibrium perturbation. Equilibrium perturbations measured at pH 7.00 had a fractional perturbation of −25.0 ± 0.5% with a ΔA_{max} of 0.226 OD. The mass spectroscopic analysis of (2*R*)-[2-^{2}H]HBP, which indicated 91.0 ± 1.0 atom % ^{2}H, was used to correct the fractional perturbation to −27.5 ± 0.9%. From eq 1, which relates the $^{D}V/K$ to the fractional perturbation for a primary KIE, the primary $^{D}V/K$ was calculated to be 1.61 ± 0.03. The equilibrium perturbation was repeated at pH 5.92 with the same substrate solutions, and an identical fractional perturbation was measured, showing the lack of a pH dependence on the $^{D}V/K$ between pH 5.92 and pH 7.00.

Primary $^{D}V/K$ with (2R)-[2-^{2}H]HB-CoA. The primary $^{D}V/K$ was measured with a mixture of (2*R*)-[2-^{2}H]HB-CoA and Cr-CoA. Equilibrium perturbations measured at pH 7.00 with no added 3′,5′-ADP and a crotonase concentration of 10 pM resulted in a fractional perturbation of 8.8 ± 1.7%, where the ΔA_{max} was 0.1 OD. From eq 1, the primary $^{D}V/K$ was calculated to be 1.15 ± 0.03.

Primary $^{18}(V/K)$. The primary $^{18}(V/K)$ was measured with a mixture of [3-^{18}O]HBP and CrP. A typical perturbation with [3-^{18}O]HBP as perturbant is shown in Figure 1. Equilibrium perturbations measured at pH 7.00 had a fractional perturbation of −3.0 ± 0.2%. The equilibrium perturbations were measured at λ = 263 nm to give a larger value of ΔA_{max} (0.5270 OD) with all other conditions of the experiments being standard. The mass spectroscopic analysis of [3-^{18}O]HBP, which indicated 93.0 ± 1.0 atom % ^{18}O, was used to correct the fractional perturbation to −3.23 ± 0.24%. From eq 1, which relates the $^{18}(V/K)$ to the fractional perturbation for a primary KIE, the primary $^{18}(V/K)$ was calculated to be 1.051 ± 0.004. In preliminary experiments slightly larger values (1.060) were obtained but with greater uncertainty due to the smaller absolute perturbation size and less ^{18}O enrichment in the [3-^{18}O]HBP.

β-Secondary $^{D}V/K$. During the dehydration reaction, the substituted hydrogen changes from being on an sp^3 carbon α to a carbonyl to being on an sp^2 carbon. The $^{D}K_{eq}$ value for this reaction was estimated to be 1.07 from the ratio of the $^{D}K_{eq}$ values of 0.93 for the transfer of ^{2}H from [3-^{2}H]ketoglutarate to water and 0.87 for the transfer of ^{2}H from [2-^{2}H]fumarate to water (Cleland, 1980).

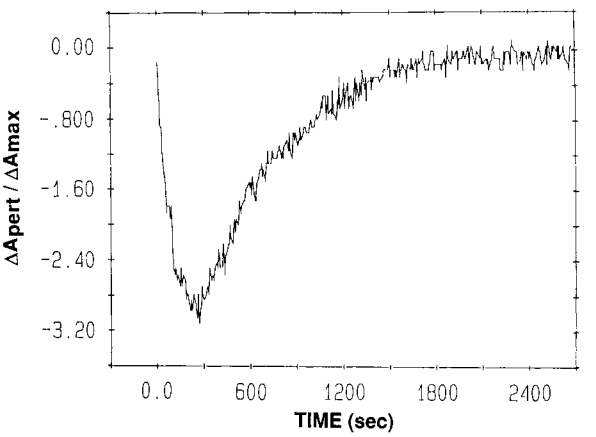


FIGURE 1: Spectrophotometric time course of an equilibrium perturbation. Crotonase is added at time = 0 to a temperature-equilibrated assay mix containing equilibrium concentrations of CrP and [3-^{18}O]HBP in the presence of 100 μM 3′,5′-ADP. The absorbance of the CrP has been normalized by the maximum possible perturbation size (ΔA_{max}) and is reported as a percentage. The perturbation size (ΔA_{pert}) was 0.016 absorbance unit.

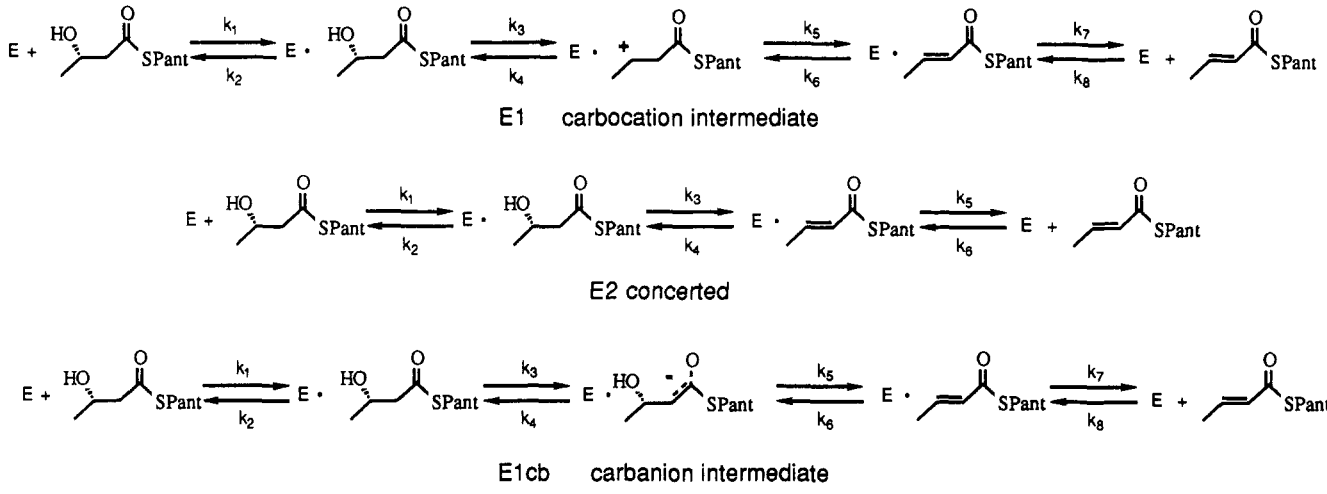
The β-secondary $^{D}V/K$ was measured with a mixture of [2-^{2}H]CrP and HBP that were at concentrations that correspond to a ΔA_{max} of 0.300 OD. The fractional perturbation measured at pH 7.00 was 2.10 ± 0.33%. From the ^{1}H NMR analysis of 97 ± 1 atom % ^{2}H at C-2, the fractional perturbation was corrected to 2.17 ± 0.36%. By use of the $^{D}K_{eq}$ value of 1.07 in eq 2, which relates the $^{D}V/K$ to the fractional perturbation measured for a secondary $^{D}V/K$, the $^{D}V/K$ for the dehydration reaction was calculated to be 1.12 ± 0.01. The equilibrium perturbation was repeated at one-fifth the activator concentration (20 μM 3′,5′-ADP) in the same buffer, enzyme, and substrate solutions. An identical fractional perturbation of 2.10% was measured, showing a lack of an activator concentration dependence on the β-secondary $^{D}V/K$ between 20 and 100 μM 3′,5′-ADP.

Alternatively, the β-secondary $^{D}V/K$ was measured with a mixture of (2*S*)-[2-^{2}H]HBP and CrP that were at concentrations that correspond to a ΔA_{max} of 0.187 OD. The fractional perturbation measured at pH 7.00 was −1.93 ± 0.53%. The fractional perturbation was −2.02 ± 0.55% after correction for the ^{2}H enrichment at C-2. By use of the $^{D}K_{eq}$ value of 1.07 in eq 2, the $^{D}V/K$ for the dehydration reaction was calculated to be 1.11 ± 0.02. Errors in the estimate of $^{D}K_{eq}$ will be reflected proportionately in the measured isotope effect (see below) leaving the more important ratio, $(^{D}V/K)/^{D}K_{eq}$, unchanged. These two complementary perturbations are shown in Figure 2.

α-Secondary $^{D}V/K$. During dehydration the substituted carbon–hydrogen bond is converted from an sp^3 bond with an α C–O bond to an sp^2 bond. The $^{D}K_{eq}$ value for this reaction was estimated to be 1.33 from the ratio of the $^{D}K_{eq}$ values of 1.16 for the transfer of ^{2}H from [2-^{2}H]-2-propanol or [1-^{2}H]cyclohexanol to water and 0.87 for the transfer of [2-^{2}H]fumarate to water (Cleland, 1980).

The α-secondary $^{D}V/K$ was measured with a mixture of [3-^{2}H]HBP and CrP that were at concentrations that correspond to a ΔA_{max} of 0.276 OD. The fractional perturbation measured at pH 7.00 with 100 μM 3′,5′-ADP was 3.99 ± 0.36%. By use of the $^{D}K_{eq}$ value of 1.33 in eq 2, the $^{D}V/K$ for the dehydration reaction was calculated to be 1.13 ± 0.01.

Scheme II



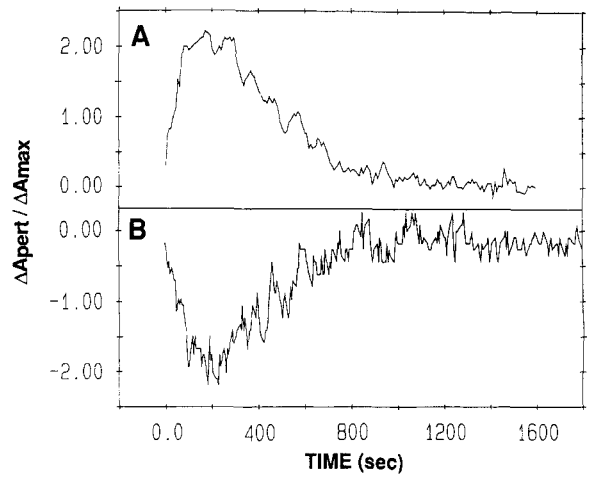


FIGURE 2: β-Secondary $^{D}V/K$ determination by equilibrium perturbation. Same conditions as in Figure 1 except the isotopic label was introduced as [2-^{2}H]CrP (A) or (2*S*)-[2-^{2}H]HBP (B). The perturbations were +0.0063 and −0.0036 absorbance unit, respectively.

The equilibrium perturbation was repeated at 20 μM 3′,5′-ADP in the same buffer, enzyme, and substrate solutions. An identical fractional perturbation of 3.99% was measured, showing the lack of an activator concentration dependence on the $^{D}V/K$ between 20 and 100 μM 3′,5′-ADP.

The α-secondary $^{D}V/K$ was additionally measured with a mixture of [3-^{2}H]CrP and HBP that were at concentrations that correspond to a ΔA_{max} of 0.204 OD. The fractional perturbation measured at pH 7.00 was −4.12 ± 0.49%. By use of the $^{D}K_{eq}$ value of 1.33 in eq 2, the $^{D}V/K$ for the dehydration reaction was calculated to be 1.12 ± 0.02. The ratio of $(^{D}V/K)/^{D}K_{eq}$ of 0.846 ± 0.015 is independent of the estimated value for $^{D}K_{eq}$.

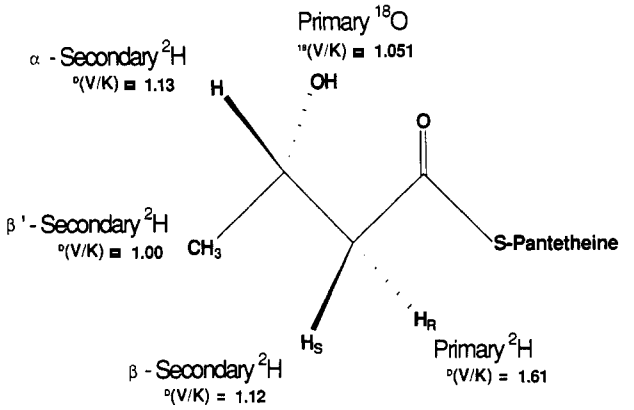
β′-Secondary $^{D}V/K$. The $^{D}K_{eq}$ value for the reaction between [4,4,4-$^{2}H_3$]HBP and [4,4,4-$^{2}H_3$]CrP was estimated to be 1.00, which means that ^{2}H at that position has no effect on the equilibrium constant. Equilibrium perturbations were measured first with [4,4,4-$^{2}H_3$]HBP and CrP and then with [4,4,4-$^{2}H_3$]CrP and HBP that were at concentrations that correspond to a ΔA_{max} of 0.200 absorbance unit. In no case was a fractional perturbation measured that was greater than 0.50%. After correction for 57 ± 1 atom % ^{2}H, the fractional perturbation became 0.00 ± 0.88%. By use of the $^{D}K_{eq}$ value of 1.00 in eq 2, this range in the fractional perturbation corresponds to a 4,4,4-$^{2}H_3$ secondary $^{D}V/K$ of 1.00 ± 0.03 or (1.00 ± 0.01)/^{2}H.

Discussion

A priori the C–H and C–X bond cleavages in α–β elimination reactions can occur in either order or in a concerted fashion as shown in Scheme II. When a carbanion can be stabilized β to the leaving group, base-promoted E2 and E1cb reactions predominate. Gandler and Jencks (1982) have argued that there is a distinct *transformation* from an E2 mechanism to an E1cb mechanism as the stability of the carbanion is enhanced within a fixed substrate structure and that the two mechanisms cannot both be operative for the same molecule. The argument is physically rationalized on the basis of the similar location in More O'Ferrall diagrams (More O'Ferrall, 1970) of the two different transition states and the assumption that if there is an accessible well for the carbanion intermediate, the E2 reaction pathway could not avoid it. Mayer et al. (1984) studied α–β elimination reactions in β-decalone systems and concluded that the strained lactone ring of 11-oxatricyclo[4.4.3.0^{1,6}]tridecan-3-one promotes an E2 elimination despite the activation of the eliminated β-hydrogen by the ketone. These two arguments frame the mechanistic questions for the crotonase-promoted α–β elimination from 3-hydroxy thioesters: Has the enzyme stabilized the enol(ate) of the thioester sufficiently for an E1cb-type mechanism to be operative, or has the leaving group been activated to a greater extent, resulting in a concerted elimination?

For crotonase the thioester functionality has been invoked as providing the necessary stabilization for the formation of a carbanion intermediate. The submicromolar binding of the carbanionic form of Acac-CoA (Waterson & Hill, 1972) is the only experimental evidence for such an enolate intermediate in the crotonase-catalyzed reaction. However, the affinity of crotonase for this inhibitor may arise from stabilization of the negative charge at the C-3 oxygen where OH^- is eliminated in the normal reaction. Additional support for enol(ate) intermediates in reactions of coenzyme A thioesters has come from theoretical calculations on the condensation of malonyl-CoA with thioesters (Dewar & Dieter, 1988), from double-isotope fractionation studies on malate synthase (Clark et al., 1988) and from direct observation of the enethiol(ate) of acetyldithio-CoA bound to citrate synthase (Wlassics & Anderson, 1989). As discussed below individually, the KIEs reported here and summarized in Scheme III are consistent with both the E1cb and E2 mechanisms but confirm the carbanionic character of the transition state and provide the necessary background for the unequivocal demonstration of the concerted or stepwise nature of the reaction by a subsequent double-isotope fractionation study.

Scheme III



Lack of External Commitments to Catalysis. The small primary $^DV/K$ effects observed with [2-^{2}H]HB-CoA in this study and by Person (1981) may only indicate a diffusion-limited reaction. To be able to interpret the KIEs in terms of transition-state structure, it must be established that there are no external commitments from slow dissociation of substrate and product. CrP in the presence of 100 μM 3′,5′-ADP as activator is hydrated with a relative V/K of 0.05% of the rate for the CoA thioesters but at least 20-fold faster than that in the absence of activator. This suggests that the dissociation of the pantetheine thioesters has become faster than the catalyzed hydration/dehydration reaction. External commitments may also arise from a compulsory ordered addition of the pantetheine ester followed by the activator. The activator concentration chosen is less than 10% of the half-saturation concentration (data not shown), and consequently, an ordered addition cannot contribute significantly to an external commitment. The invariance of the experimentally determined secondary α and β KIEs with different activator concentration confirms this conclusion. Finally, the primary ^{18}O effect of 1.051 requires commitments of less than 1.0, or the calculated intrinsic effect would be unreasonably larger than the maximum calculated value of 1.09 for primary ^{18}O KIEs (O'Leary & Marlier, 1979).

Primary Kinetic Isotope Effects. The primary ^{18}O and ^{2}H KIEs on the crotonase-catalyzed dehydration of HBP are consistent with any of the three mechanisms shown in Scheme II. In the carbocation and carbanion intermediate mechanisms the carbon–oxygen and carbon–hydrogen bond cleavage steps will generate internal commitments for each other, thereby reducing the observed KIEs from their intrinsic values. For the E1cb mechanism of Scheme II the exact relationships are given by eq 4 and 5, where $^DK_{eq}$ is for the exchange of the 2R proton of HBP with the protonated active site base.

$$^DV/K = \frac{^Dk_3 + (k_4/k_5)^DK_{eq}}{1 + k_4/k_5} \quad (4)$$

$$^{18}V/K = \frac{^{18}k_5 + k_5/k_4}{1 + k_5/k_4} \quad (5)$$

The size of the commitments (k_4/k_5 and k_5/k_4) can be estimated if an intrinsic ^{18}O effect on C–O bond cleavage is assumed. The largest intrinsic ^{18}O effects observed are 1.072 for the fumarase-catalyzed dehydration of [2-^{18}O]malate (Blanchard & Cleland, 1980) and 1.062 for the hydrazinolysis of HC(O)^{18}OCH$_3$ (Sawyer & Kirsch, 1973). In the fumarase-catalyzed reaction the observed primary ^{2}H KIE is inverse, indicating that the C–H bond cleavage has equilibrated and the C–O bond cleavage is solely rate limiting (Blanchard & Cleland, 1980). By substitution of the $^{18}V/K$ of 1.072 for the dehydration of malate as the largest possible intrinsic ^{18}O KIE in eq 5, the largest commitment derived from C–H bond cleavage (k_5/k_4) is 0.41. Because the internal commitment factor generated by the C–O bond cleavage for C–H bond cleavage is the reciprocal, the smallest value of k_4/k_5 is 2.4. With this minimal value, a minimum intrinsic primary ^{2}H KIE ($^Dk_3$) of 3.1 can be calculated. Qualitatively, the large observed ^{18}O primary KIE requires that the C–O bond cleavage step be predominantly rate determining in a stepwise mechanism, and consequently, the primary ^{2}H KIE is reduced considerably from its intrinsic value. If the intrinsic ^{18}O KIE is smaller than 1.072, it results in a larger k_5/k_4 and a larger calculated intrinsic primary ^{2}H KIE. This minimum primary ^{2}H KIE of 3.1 is on the low side of experimentally determined KIEs for deprotonating carbon acids (Bell, 1973). These calculations depend on a speculative value for the intrinsic ^{18}O KIE and assume a stepwise mechanism. What is unequivocally demonstrated is that for an E1cb mechanism C–O bond cleavage would be rate limiting and the intrinsic deuterium isotope effect would be significantly larger than the observed effect.

The primary KIEs are consistent with a concerted elimination as well. In a concerted reaction all of the KIEs would be intrinsic effects. Although the primary ^{2}H KIE of 1.6 is significantly smaller than the intrinsic effect on most proton transfer reactions, values as low as 1.7 have been observed for the deprotonation of diethyl malonate by water (Bell & Crooks, 1965). These small KIEs have been attributed to asymmetrical transition states for the proton transfer (Westheimer, 1961). In syn E2 elimination reactions primary ^{2}H KIEs of less than 2.0 have been both observed (Cooke & Coke, 1968; Brown & Saunders, 1970; Subramanian & Saunders, 1984) and calculated (Saunders, 1974, 1985). Besides asymmetrical transition states, it is possible to speculate that a coupling of the motions of the primary proton with the β-secondary proton results in a restoring force along the reaction coordinate that reduces the intrinsic primary effect. This possibility was initially suggested for hydride transfer reactions (Kurz & Frieden, 1981) and has been theoretically considered for elimination reactions (Saunders, 1985).

The unequivocal distinction between a concerted or stepwise reaction will come from a determination of the ^{18}O KIE with ^{2}H present at C-2 of HBP. If the presence of ^{2}H reduces the measured ^{18}O effect, it will indicate the increased commitment of the prior and, because of the ^{2}H substitution, slower C–H bond cleavage (Belasco et al. 1983; Hermes et al., 1982). To accomplish this without artifact by the equilibrium perturbation methodology presented here would require the atom percent ^{2}H in the solvent to identically match that in the [2-^{2}H,3-^{18}O]HBP; a formidable technical task that is currently being addressed.

Secondary ^{2}H KIEs. The α-secondary $^DV/K$ value was determined to be 1.13 compared to the $^DK_{eq}$ value of 1.33. In the rate-limiting transition state the vibrational motion for the nontransferred α-hydrogen, which gives rise to the KIE, has a force constant that is intermediate between the sp^3 substrate and sp^2 product. In an E2 transition state the C_α–H bond is slightly more sp^3 like since the KIE is closer to 1.0 than to 1.33. The conclusion of a slightly more sp^3-like C–H bond is consistent with carbanion character in the rate-limiting transition state. If the reaction is stepwise and there is a carbanion intermediate, the analysis is more complex. However by assuming that the only isotope-sensitive step occurs when the hybridization of the C-3 carbon changes, then the observed α $^DV/K$ is slightly reduced from the intrinsic value by the

internal commitment introduced by the C–H bond cleavage step. As noted above, an estimate of the largest commitment possible is 0.41, allowing us to estimate the largest intrinsic α-secondary ^{2}H KIE to be 1.18. The magnitude of this effect together with the large primary ^{18}O KIE is not consistent with a carbocation intermediate. In the S_N1 solvolysis of isopropyl tosylate where C–O bond cleavage is rate limiting, an α-secondary $^DV/K$ of 1.22 is observed (Streitweiser & Dafforn, 1969).

The β-secondary $^DV/K$ value of 1.12 ± 0.01 compared to the $^DK_{eq}$ value of 1.07 is the most intriguing KIE measured in this study. A $^DV/K$ in excess of the $^DK_{eq}$ for the dehydration indicates that in the rate-limiting transition state there is a vibrational mode for the nontransferred β-hydrogen which has a reduced force constant compared to either substrate or product. This phenomenon cannot be explained solely by the extent of rehybridization of the β-carbon in the transition state. Errors in the estimated $^DK_{eq}$ value also cannot account for this difference. Equations 2 and 3 can be simplified to eq 6 and 7 when $K_{eq} \ll 1.0$, indicating that the observed perturbation size really determines the ratio of $^DV/K$ and $^DK_{eq}$. For the

$$X = {}^DK_{eq}/\alpha \quad (6)$$

$$\text{fractional perturbation} = \frac{P_{tmax} - P_\infty}{\Delta P_{max}} = ({}^DK_{eq}/\alpha)^{1/(1-{}^DK_{eq}/\alpha)} - ({}^DK_{eq}/\alpha)^{({}^DK_{eq}/\alpha)/(1-{}^DK_{eq}/\alpha)} \quad (7)$$

crotonase-catalyzed dehydration of HBP, $K_{eq} = 0.29$, and the relationships in eq 6 and 7 are not exact. Assuming $^DK_{eq} = 1.12$ (instead of 1.07) yielded a $^DV/K$ of 1.17, numerically indicating that the perturbation has established $(^DV/K)/^DK_{eq} = 1.05 \pm 0.01$. The equal and opposite perturbations shown in Figure 2 are unequivocal evidence that $^DV/K$ is greater than $^DK_{eq}$. If $^DV/K$ were equal to $^DK_{eq}$, the absolute value of both fractional perturbations would be less than 0.5%. Any error present is limited to the magnitude that $^DV/K$ exceeds $^DK_{eq}$.

If the reaction is stepwise, the C–O bond cleavage step will generate a significant reverse internal commitment. Using the minimum k_4/k_5 of 2.4 estimated above and $^DK_{eq} = 1.07$[6] in eq 4 yields a minimum intrinsic β-secondary KIE of 1.24. This value is not too large to be explained by a coupled motion in the transition state. In formate dehydrogenase the ratio of $(^DV/K)/^DK_{eq}$ for the secondary effect at C-4 of the nicotinamide ring is 1.38 (Hermes et al., 1984).

Subramanian and Saunders (1984) reported β-secondary KIEs that exceed the equilibrium isotope effect in the elimination reactions of 2-arylethyl derivatives. They observed that an increase of carbanion character in the transition state resulted in the measured β-secondary KIE being larger than $^DK_{eq}$. Saunders (1984, 1985) calculated KIEs for E2 elimination reactions which predict a tunneling correction is necessary when the bending motion of the nontransferred β-hydrogen is coupled to the stretching motion of the transferred hydrogen. These calculations also predict small primary ^{2}H KIEs. This is consistent with the small primary ^{2}H KIE of 1.61 measured in this study, which would be an intrinsic effect if an E2 mechanism were operative. The β-secondary $^DV/K$ measured in the crotonase reaction could be explained by coupling of the nontransferred β-hydrogen with the transfer of the primary hydrogen in the rate-limiting transition state. The accepted method to study coupled motions of protons in the transition state (Kurz & Frieden, 1980; Hermes et al., 1984) is to determine if deuteration at one of the coupled sites reduces the deuterium KIE at the second site. These studies are in progress in our laboratory.

The β'-secondary $^DV/K$ value of unity unequivocally eliminates the possibility of a partial positive charge developing on C-3 in the rate-limiting transition state. A carbocation stepwise mechanism or concerted mechanism with carbocation character would be expected to have a β'-secondary $^DV/K$ of $1.1/^2$H due to hyperconjugation resonance effects that preferentially stabilize the undeuterated transition state (Westaway, 1987). The lack of positive charge in the rate-limiting transition state indicates that the increase in electron density due to C–C double bond formation must nearly balance the loss of electron density associated with C–O bond cleavage that occurs in the rate-determining transition state.

Supplementary Material Available

Derivation of eq 1–3 from Scheme I (9 pages). Ordering information is given on any current masthead page.

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[6] $^DK_{eq}$ should ideally be for the conversion of HBP to the enolate. We were unable to locate any suitable fractionation factors for hydrogens on enolate carbanions. A crystal structure of a model thioester enolate (P. W. Willard and M. Hintze, personal communication) indicates that the α-carbon–carbonyl carbon bond distance is 1.348 Å and the α-carbon substituents are nearly coplanar with the C(O^-)S; i.e., the α-carbon is crystallographically indistinguishable from a normal sp^2 carbon, supporting the validity of using a $^DK_{eq}$ for the overall reaction.

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Phospholipid Asymmetry in Large Unilamellar Vesicles Induced by Transmembrane pH Gradients†

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ABSTRACT: The influence of membrane pH gradients on the transbilayer distribution of some common phospholipids has been investigated. We demonstrate that the transbilayer equilibrium of the acidic phospholipids egg phosphatidylglycerol (EPG) and egg phosphatidic acid (EPA) can be manipulated by membrane proton gradients, whereas phosphatidylethanolamine, a zwitterionic phospholipid, remains equally distributed between the inner and outer monolayers of large unilamellar vesicles (LUVs). Asymmetry of EPG is examined in detail and demonstrated by employing three independent techniques: ion-exchange chromatography, ^{13}C NMR, and periodic acid oxidation of the (exterior) EPG headgroup. In the absence of a transmembrane pH gradient (ΔpH) EPG is equally distributed between the outer and inner monolayers of LUVs. When vesicles composed of either egg phosphatidylcholine (EPC) or DOPC together with 5 mol % EPG are prepared with a transmembrane ΔpH (inside basic, outside acidic), EPG equilibrates across the bilayer until 80–90% of the EPG is located in the inner monolayer. Reversing the pH gradient (inside acidic, outside basic) results in the opposite asymmetry. The rate at which EPG equilibrates across the membrane is temperature dependent. These observations are consistent with a mechanism in which the protonated (neutral) species of EPG is able to traverse the bilayer. Under these circumstances EPG would be expected to equilibrate across the bilayer in a manner that reflects the transmembrane proton gradient. A similar mechanism has been demonstrated to apply to simple lipids that exhibit weak acid or base characteristics [Hope, M. J., & Cullis, P. R. (1987) *J. Biol. Chem. 262*, 4360–4366].

Phospholipid asymmetry is now well established for many biological membranes (Op den Kamp, 1979; Zwaal, 1978; Michaelson et al., 1983; Higgins & Pigott, 1982; Herbette et al., 1984; Houslay & Stanley, 1982). The most commonly studied systems are mammalian plasma membranes in which the amino-containing phospholipids phosphatidylethanolamine (PE) and phosphatidylserine (PS) are observed to be predominantly located in the cytoplasmic side of the bilayer [for review see Op den Kamp (1979)].

The mechanism whereby phospholipid asymmetry is generated and maintained is not understood. However, several recent papers provide compelling evidence for the existence of a transport mechanism that exhibits specificity for PE and

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