Crotonase-Catalyzed \( \beta \)-Elimination Is Concerted: A Double Isotope Effect Study

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ABSTRACT: Determining the sequence of bond cleavages, and consequently the nature of intermediates, in enzyme-catalyzed reactions is a major goal of mechanistic enzymology. When significant primary isotope effects on \( V/K \) are observed for two different bond cleavages, both bonds may be broken in the same transition state or they can reflect two different transition states that are of nearly identical energy and consequently both are partially rate limiting. For the crotonase-catalyzed dehydration of 3-hydroxybutyrylpantetheine, the primary \( ^{1}V/K \) and \( ^{18}V/K \) are 1.60 and 1.053 [Bahnsen, B. J., & Anderson, V. E. (1989) Biochemistry 28, 4173–4181], respectively. In this case, double isotope effects can discriminate between the two possibilities [Hermes, J. D., Roeseke, C. A., O'Leary, M. H., & Cleland, W. W. (1982) Biochemistry 21, 5106–5114; Belasco, J. G., Albery, W. J., & Knowles, J. R. (1983) J. Am. Chem. Soc. 105, 2475–2477]. The ratio of the \( \alpha \)-secondary \( ^{1}V/K \) for the hydration of crotonylpantetheine catalyzed by crotonase in \( H_2O \) and \( D_2O \) has been determined to be 1.003 ± 0.006. The invariance of the \( \alpha \)-secondary effect where the chemical reaction is completely rate determining requires that both bond cleavages be concerted or that the substitution of \( ^{2}H \) at the primary position does not significantly alter the partitioning of a hypothetical carbanion. The observation of a solvent discrimination isotope effect determined from the relative incorporation of \( ^{2}H \) from 50% \( D_2O \) of 1.60 ± 0.03, identical with the primary \( ^{1}V/K \), and the determination that the rate of exchange of the abstracted proton with solvent proceeds at less than 3% of the overall reaction rate also fail to provide evidence for a carbanion intermediate and are consistent with a concerted reaction. Identical primary \( ^{1}V/K \) 's determined in \( H_2O \) and \( D_2O \) indicate that there is not a significant solvent isotope effect on C-O bond cleavage. The isotope ratios determined in these studies were performed by negative ion chemical ionization whole molecule mass spectrometry of the pentafluorobenzyl esters, a new method whose validity is established by comparison with previously determined kinetic and equilibrium isotope effects.

Crotonase (enoyl-CoA hydratase, EC 4.2.1.17) catalyzes the syn addition of water across the double bond of \( \alpha-\beta \) unsaturated CoA thioesters (Willadsen & Eggerer, 1975). The nearly diffusion limited \( V/K \) of \( 5 \times 10^{-7} M^{-1} s^{-1} \) and the small \( ^{18}V/K \) on the dehydration of 3-hydroxybutyryl-CoA (Person, 1981) showed that large external commitments to catalysis would complicate a detailed mechanistic study of the transition state using CoA thioester substrates. Pantetheine thioesters, which lack the adenosine portion of CoA, have a \( V/K \) that is less than 0.1% that of the CoA thioesters (Waterson et al., 1972) but are ideal for an isotope effect study since they have external commitments of zero, resulting in a rate-limiting chemical step (Bahnsen & Anderson, 1989).

The primary \( ^{1}V/K \), \( ^{18}V/K \), and secondary \( ^{1}V/K \) on the dehydration of (S)-3-hydroxybutyrylpantetheine (HBp) in the presence of the activator, 3',5'-ADP, were measured by the equilibrium perturbation technique (Bahnsen & Anderson, 1989). These isotope effects could not distinguish between a carbanion intermediate mechanism, where abstraction of the proton precedes elimination of \( OH^- \), or a concerted mechanism with carbanion character. A mechanism that has carbanion character was ruled out. The measurement of the individual isotope effects reported in the previous study are essential for defining the mechanism of the crotonase-catalyzed reaction, because they provide the necessary background for the unequivocal demonstration of the concerted/stepwise nature of the reaction by a double isotope effect study.

There is greater precedent for the crotonase-catalyzed elimination to be stepwise. The tight binding of the enol(ate) form of acetocacetyl-CoA (AcAc-CoA) to crotonase (Watson & Hill, 1972) suggests that there may be an enolate intermediate. All well-characterized enzyme-catalyzed \( \beta \)-eliminations are suggestive of proceeding through a carbanion intermediate (Anderson, 1991). The enzyme systems studied by both primary and secondary isotope effects, fumarase (Blanchard & Cleland, 1980), enolase (Anderson, 1981; Stubbe & Abeles, 1980), aspartase (Nuiry et al., 1984), and arginine succinate lyase (Kim & Raushel, 1986) all catalyze

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1 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.
2 Northrop (1977) introduced the terminology of commitments to catalysis to reflect the partitioning of enzyme–substrate complexes between catalysis and dissociation of the substrate. Internal and external commitments are obtained by arbitrarily assuming an infinite dissociation rate for all the substrates. The remaining commitment is the internal commitment, while the portion that is lost is the external commitment.
3 In elimination reactions, the secondary \( ^{18}V/K \) is for \( ^{2}H \) substitution at the carbon that is bonded to the heavy atom leaving group, the secondary \( ^{1}V/K \) is for \( ^{1}H \) substitution at the carbon whose \( C-H \) bond is broken, and the secondary \( ^{1}V/K \) is for \( ^{1}H \) substitution at the nonreacting \( \beta \)-carbon (Cook, 1976).
4 Abbreviations: AcCoA, acetocacetyl-CoA; AcA-CoA, P, S-acetoacetyl-pantetheine; 3',5'-ADP, adenosine 3',5'-diphosphate; CrP, trans-crotonylpantetheine; Cr-CoA, trans-crotonyl-CoA; HB-CoA, (S)-3-hydroxybutyryl-CoA; HBP, (S)-3-hydroxybutyrylpantetheine; KIE, kinetic isotope effect; MOPS, 3-(N-morpholino)propanesulfonic acid; m/z, mass/unit charge; NCI, negative chemical ionization; PFTBA, perfluorotributylamine; SDIE, solvent discrimination isotope effect; SIM, selected ion monitoring.

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anti eliminations that proceed through discrete carbaniion intermediates. However, precedent for a concerted elimination may be taken from the concerted α-β dehydrogenation of acyl-CoAs catalyzed by acyl-CoA dehydrogenase (Murfin, 1974; Reinsch et al., 1980; Pohl et al., 1986). Nonenzymatic elimination reactions with protons activated by adjacent carbonyl functionalities have been shown to react through carbaniion intermediates (Fedor, 1967, 1969; Robert et al., 1984) or in other systems with resonance stabilized carbanions when the leaving group was changed from OH- to Br- (Marshall et al., 1977; More-O’Ferral & Warren, 1975). As the first enzymatic syn elimination to be examined by multiple isotope effects, the crotonase system can help define whether the syn stereochemistry is related to a change in mechanism from carbaniion stepwise to concerted.

In the present study, four experimental approaches are reported that are designed to detect the presence of a carbaniion intermediate in the crotonase-catalyzed hydration of trans-crotonyl-CoA (Cr-CoA). (1) A double isotope effect study is reported in which the secondary D(K)/V(K) on the hydration of CrP was measured in H2O and D2O. If deuterium substitution in the solvent alters the partitioning of a carbaniion intermediate, which will occur if there is a significant primary D(K)/V(K) in H2O should be closer to the measured secondary equilibrium isotope effect, D(K)/V(K). (2) The distribution of ([(R)-2-3H]- and [(R)-2-3H]-HBP following the hydration of CrP in 50:50 H2O:D2O was determined. If the proton donated to C-2 of a carbaniion intermediate exchanges rapidly with solvent, then a solvent discrimination isotope effect (SDIE) larger than the primary D(K)/V(K) for the hydration reaction would be measured. (3) Exchange of the abstracted proton from an enzyme-carbanion intermediate was investigated by a complementary method of isotope exchange in D218O. During the elimination of HBP, the exchange rate of the proton abstracted from the primary position compared to the rate of reaction can place limits on the solvent accessibility of the potential carbaniion intermediate. (4) The solvent D(K)/V(K) was measured by the equilibrium overshoot technique to probe for a solvent isotope effect in a stepwise mechanism on the C-O bond cleavage step. We conclude that the crotonase-catalyzed β-elimination of water from HBP is concerted.

**Materials and Methods**

**Enzymes.** Crotonase was isolated from fresh bovine liver by a modified procedure of Steinman and Hill (1975). Dialysis following the acetone precipitation was omitted so that in one day the procedure could be carried out through the ammonium sulfate precipitation where the enzyme is more stable. The crotonase was recrystallized and stored frozen as a crystalline suspension in 20 mM potassium phosphate buffer (pH 7.4, 3 mM EDTA, 10% EtOH). The protein concentration of crystalline crotonase was estimated by UV absorbance from ε280 = 97 000 M⁻¹ cm⁻¹ (Hass & Hill, 1969). The purity of crotonase was tested by polyacrylamide gel electrophoresis. The gel was loaded with 5 μL of a 0.35 mg/mL solution of crotonase electrophoresed on a 15% acrylamide gel. The silver-stained gel showed only very faint impurities.

**Thioester Substrates.** S-Acetoacetyl-pantetheine (AcAc-P), HBP, CrP, [3-3H]HBP, [3-3H]CrP, and AcAc-CoA were synthesized as described by Bahnson and Anderson (1989). HBP, Cr-CoA, and 3'-5'-ADP were synthesized as described by Ochoa, 1953]. The high concentration of 3'-5'-ADP used in kinetic measurements required that assays be monitored at 280 nm where the absorbance from the adenine of the activator 3',5'-ADP varied from 0.5 to 6.08 mM and the activator 3',5'-ADP varied from 0.5 to 7 mM. Initial velocities were measured with the substrate Cr-CoA. Initial velocities were measured with the substrate Cr-CoA at 0.76 to 2.35% molar substrate concentration with a 50 μM solution of crotonase. The reagents were followed at 280 nm from ε280 = 3600 M⁻¹ cm⁻¹, ε660 = 6600 M⁻¹ cm⁻¹ (Lynen & Ochoa, 1953). The high concentration of 3',5'-ADP used in kinetic measurements required that assays be monitored at 280 nm where the absorbance from the adenine of the activator 3',5'-ADP varied from 0.5 to 6.08 mM and the activator 3',5'-ADP varied from 0.5 to 7 mM. Initial velocities were measured with the substrate Cr-CoA at 0.76 to 2.35% molar substrate concentration with a 50 μM solution of crotonase. The reagents were followed at 280 nm from ε280 = 3600 M⁻¹ cm⁻¹, ε660 = 6600 M⁻¹ cm⁻¹ (Lynen & Ochoa, 1953). The high concentration of 3',5'-ADP used in kinetic measurements required that assays be monitored at 280 nm where the absorbance from the adenine of the activator 3',5'-ADP varied from 0.5 to 6.08 mM and the activator 3',5'-ADP varied from 0.5 to 7 mM. Initial velocities were measured with the substrate Cr-CoA at 0.76 to 2.35% molar substrate concentration with a 50 μM solution of crotonase. The reagents were followed at 280 nm from ε280 = 3600 M⁻¹ cm⁻¹, ε660 = 6600 M⁻¹ cm⁻¹ (Lynen & Ochoa, 1953). The high concentration of 3',5'-ADP used in kinetic measurements required that assays be monitored at 280 nm where the absorbance from the adenine of the activator 3',5'-ADP varied from 0.5 to 6.08 mM and the activator 3',5'-ADP varied from 0.5 to 7 mM. Initial velocities were measured with the substrate Cr-CoA at 0.76 to 2.35% molar substrate concentration with a 50 μM solution of crotonase. The reagents were followed at 280 nm from ε280 = 3600 M⁻¹ cm⁻¹, ε660 = 6600 M⁻¹ cm⁻¹ (Lynen & Ochoa, 1953). The high concentration of 3',5'-ADP used in kinetic measurements required that assays be monitored at 280 nm where the absorbance from the adenine of the activator 3',5'-ADP varied from 0.5 to 6.08 mM and the activator 3',5'-ADP varied from 0.5 to 7 mM. Initial velocities were measured with the substrate Cr-CoA at 0.76 to 2.35% molar substrate concentration with a 50 μM solution of crotonase. The reagents were followed at 280 nm from ε280 = 3600 M⁻¹ cm⁻¹, ε660 = 6600 M⁻¹ cm⁻¹ (Lynen & Ochoa, 1953).
concentrations in order to directly compare the \( V_{\text{max}} \) values of HB-CoA relative to Cr-CoA. The reactions, monitored by UV absorbance at 280 nm, were run with 165 \( \mu \)M HB-CoA, 175 \( \mu \)M Cr-CoA, and a crotonase concentration of 0.7 nM.

**Quench Experiments.** Three different types of quench experiments were performed to explore for the presence of a carbanion intermediate: (1) The secondary \( \alpha-D(\text{V/K}) \) was measured for the hydration reaction in \( \text{H}_2\text{O} \) and \( \text{D}_2\text{O} \) with an approximately 50:50 mixture of \([3-\text{H}]\)- and \([3-\text{H}]\text{CrP} \) (\([3-\text{H}]\text{HBP} \)). The secondary \( \alpha-D(\text{V/K}) \) for the hydration reaction run in \( \text{H}_2\text{O} \) is abbreviated as \( \alpha-D(\text{V/K})_{\text{H}_2\text{O}} \) and the secondary \( \alpha-D(\text{V/K}) \) measured in \( \text{D}_2\text{O} \) is abbreviated as \( \alpha-D(\text{V/K})_{\text{D}_2\text{O}} \). (2) The product distribution of \([\text{R}]-2-'\text{H}/\text{H}\text{HBP} \) for the hydration of CrP in 50:50 \( \text{H}_2\text{O}:\text{D}_2\text{O} \) was measured. (3) The isotope exchange of the 2-\( \text{R} \) proton and 3-hydroxy group of HBP with solvent was measured in \( \text{D}_2\text{O} \). Typically quench reactions were run in 0.1 M MOPS (pH 7.00, 0.1 M ionic strength with potassium acetate, 1 mM EDTA) with 40-50 \( \mu \)M \( 3',5'\text{-ADP} \) as an activator in a total volume of 1.0 mL. Reactions were quenched by the addition of 100 \( \mu \)L of acetic acid.

**Pentafluorobenzyl Ester Derivatization.** The carboxylic acid portions of HBP or CrP were derivatized to form their pentafluorobenzyl esters following pig liver esterase catalyzed hydrolysis at pH 7.0. The HPLC-purified thioesters were dissolved in 1 mL of pH 7.00 MOPS (0.1 M) with 60 units of esterase. The hydrolysis, monitored by UV absorbance at 240 nm, was complete in less than 10 h. No exchange of deuterium was detected by mass spectrometry in a control experiment, except in \( \text{D}_2\text{O} \) at pH 7.0. Preliminary experiments indicated that base hydrolysis of HBP at pH 10.5 showed a 1-5% exchange of the \( \alpha \)-protons when performed in \( \text{D}_2\text{O} \). The carboxylic acids were derivatized by adding a 10-fold excess of pentafluorobenzyl 3-hydroxybutyrate was protected by reaction with dihydropyran. The pentafluorobenzyl 3-hydroxybutyrate was routinely injected into the GC and analyzed hydration of \([3-\text{H}]/2\text{H}\text{CrP}, which was quenched 5% of the way toward equilibrium with HBP (3.9% reaction). The --V/K) was measured from the crotonase-catalyzed hydration of \([3-\text{H}]/2\text{H}\text{HBP} \). The ion range 103.05-103.50 corresponds to the \( m \) peak and is shown in A. The ion range 104.05-104.50 corresponds to the \( m+1 \) peak and is shown in B. The ion abundances were integrated over the entire GC peak as shown in A and B from 5.25 to 5.95 min.

**Secondary \( \alpha-D(\text{V/K}) \) Measured in \( \text{H}_2\text{O} \) and \( \text{D}_2\text{O} \).** The secondary \( \alpha-D(\text{V/K}) \) was measured from the crotonase-catalyzed hydration of \([3-\text{H}]/2\text{H}\text{CrP} \), which was quenched 5% of the way toward equilibrium with HBP (3.9% reaction). The hydration of CrP to give HBP has a \( K_{\text{eq}} \) of 3.5 (Stern & del Campillo, 1956). To compare the measured secondary \( \alpha-D(\text{V/K})_{\text{H}_2\text{O}} \) when a proton is transferred to the primary 2R position, to the secondary \( \alpha-D(\text{V/K})_{\text{D}_2\text{O}} \), when a deuteron is transferred to the primary 2R position, the hydration reaction was run under identical conditions first in \( \text{H}_2\text{O} \) and then in \( \text{D}_2\text{O} \). In a given experiment, the same batch of \([3-\text{H}]/2\text{H}\text{CrP} \) was used to ensure that an identical isotope ratio was present in the initial substrate. The concentration of \([3-\text{H}]\text{CrP} \) was
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matched to [3-2H]CrP by a UV absorbance assay at 263 nm, although an exact 50:50 mixture is not essential to the accuracy of the experiment. The reactions were run with 5–10 mg of [3-1H/2H]CrP and monitored by UV spectroscopy at 308–312 nm where the extinction coefficient of CrP is significantly reduced so that there was a maximum absorbance of 2.3 absorbance units. The reactions run in D2O were adjusted to a pH of 7.00 in order to compare the reactions run in H2O at pH 7.00. The buffer was prepared by drying pH 6.37 MOPS (0.1 M, 1 mM EDTA) and resuspending it in D2O. This solution was dried again to remove residual protons and reconstituted with D2O (glass vials, 99.96 atom % 2H). Due to an isotope effect on the glass electrode, the measured pH of 6.69 corresponds to a pH of 7.00. The correction is given by eq 1 (Schowen & Schowen, 1982), where XH2O is the mole fraction of deuterium in water.

\[
pL = pH_{\text{app}} + XH_2O(0.4)
\]

The mass spectrometrically determined isotope ratios of the initial substrate and quenched product were performed following derivatization to their pentafluorobenzyl esters. The initial substrate [3-1H/2H]CrP was derivatized and the [3-1H/2H] ratio was determined by mass spectrometric scanning from 80–90 m/z. Following HPLC separation from the remaining CrP, the product [3-1H/2H]HBP was derivatized to its pentafluorobenzyl ester, and the mass spectrometric isotope ratio was determined. For the secondary 2D(V/K) measured in H2O, the m/z ion ranges 103.05–103.50 and 104.05–104.50 were scanned. For the secondary 2D(V/K) measurement performed in D2O, the m/z ion ranges 104.05–104.50 and 105.05–105.50 were scanned since the product will have either one or two deuterium atoms. The HBP formed in D2O was also scanned over the m/z ion ranges 103.05–103.50 and 104.05–104.50 in order to correct for contaminating protons present at C-2 in the primary position of the product [2-(R)-3,3-2H/3-2H]HBP.

**Solvation Discrimination Isotope Effect in 50:50 H2O:D2O.** The SDIE was measured by determining the m/m+1 ratio of HBP formed by reacting CrP 5% of the way to equilibrium in 50:50 H2O:D2O at pH 7.00. The reactions were run with 5–10 mM CrP and monitored by UV spectroscopy at 308–312 nm. The pH 7.00 buffer was prepared by twice drying pH 6.69 MOPS (0.1 M, 1 mM EDTA) and resuspending in D2O. This solution was dried again to remove residual protons and reconstituted with 50:50 H2O:D2O. The pH measured with a glass electrode was 6.80, which corresponds to a pH of 7.00 by eq 1. The pH measured in D2O (99.96 atom % 2H) was determined at 25 °C and correcting by mass for the ratio of H2O to D2O. Following HPLC separation of the substrate CrP, the isotopic composition of the perfluorobenzyl 3-hydroxybutyrate obtained from the product [2-(R)-3,3-2H/3-2H]HBP was determined. The sample was measured over the m/z ion ranges 102.25–102.45, 103.25–103.45, 104.25–104.45, and 105.25–105.45, which correspond to the m-1, m, m+1, and m+2 peaks, respectively. The hydroxyl group was then protected with dihydropyran and scanned over the m/z ion ranges 186.15–186.35, 187.15–187.35, 188.15–188.35, and 189.15–189.35, which correspond to the m-1, m, m+1, and m+2 peaks of dihydroxypropylated 3-hydroxybutyrate, respectively.

**Isotope Exchange of HBP in D2O.** The crotonase-catalyzed dehydration of HBP (5 mg) in D218O (98 atom % 18O, unnormalized) was performed to measure the relative rates of exchange of solvent with the CrP and the 3-hydroxy group. D218O was added to an evaporated buffer that was prepared identically to the buffer used to achieve a pH of 7.00 for the secondary 2D(V/K) measurement in D2O (see above). Half of the reaction was quenched at approximately 90% of the way to equilibrium with CrP (20% reaction), and the other half was quenched at equilibrium after >65 half-lives of the reaction to generate an isotopic equilibrium. The remaining HBP was separated from CrP by HPLC. Isotope ratio analysis of pentafluorobenzyl 3-hydroxybutyrate derived from the isolated HBP was performed to detect exchange of 2H and/or 18O into the respective primary isotope positions. The mass spectrometer was set up to scan over the same m/z ion ranges as described in the SDIE experiment (see above). Compared to a standard sample of pentafluorobenzyl 3-hydroxybutyrate, increases of the m+1 peak corresponded to the exchange of only 2H, increases of the m+2 peak corresponded to exchange of only 18O, and increases of the m+3 peak corresponded to exchange of both 2H and 18O into HBP during the reaction. The HBP sample was brought to isotopic equilibrium, and the mass spectrometric isotope ratio analysis was used to correct for 2H and 18O present in the D218O solvent as described in the appendix.

**Solute (V/K) by D2O Equilibrium Overshoot.** An overshoot was measured by UV absorbance at 263 nm for the crotonase-catalyzed dehydration reaction in D2O. HBP (200 μM) and 3',5'-ADP (51 μM) were suspended in buffer D2O (pH 7.00, 0.1 M MOPS, prepared as described above) to a volume of 575 μL in a quartz cuvette. The equilibrium overshoot solution was temperature equilibrated at 25 ± 1 °C in the UV spectrophotometer. Prior to starting the overshoot, the initial absorbance was recorded. The reaction was initiated by the addition of a small volume of crotonase in D2O (25 μL, 100 mM final concentration, 25 °C). To describe the magnitude of the overshoot required the measurement of an initial absorbance (A0), a maximum absorbance Amax, and the final equilibrium absorbance (A). The experimental overshoot will be reported as a fractional overshoot as in

\[
\text{fractional overshoot} = (A_{\text{max}} - A)/(A - A_0)
\]
The ¹H/²H isotope ratio of the initial substrate (R₀) by the following equation (Melander & Saunders, 1980).

\[ D(V/K) = \log \left( \frac{1 - f}{1 - f(R_0/R_p)} \right) \]  \hspace{1cm} (3)

When f is very small, eq 3 simplifies to

\[ D(V/K) = R_p/R_o \]  \hspace{1cm} (4)

Our interest is in the variation of \( a\text{-}D(V/K) \) measured in H₂O and D₂O. The error introduced by using eq 4 in place of eq 3 can be calculated when the extent of reaction and the isotope effect are known (Melander & Saunders, 1980). However, since the secondary \( a\text{-}D(V/K) \) measurements for the hydration reaction in H₂O and D₂O were run to the same fraction of reaction, the small deviation of eq 4 from eq 3 will be nearly identical in both cases and not affect the ratio of \( a\text{-}D(V/K) \) in both H₂O/D₂O. Additionally, this ratio is independent of \( R_o \) since the initial substrate [³H/²H]CrP was made experimentally identical by division of the sample to perform the secondary \( a\text{-}D(V/K) \) measurements in H₂O and then in D₂O.

The effect of substituting deuterium in a primary position on \( a\text{-}D(V/K) \) has been experimentally determined for dehydrogenases (Hermes et al., 1984), on primary ¹³C isotope effects (Hermes et al., 1982; Rendine et al., 1984), on primary ¹⁵N isotope effects (Hermes et al., 1985), and on a primary deuterium isotope effects (Belasco et al., 1986a). To quantitatively interpret the effect of deuterium on a primary position, on the size of a deuterium isotope effect on a stepwise mechanism normally requires the measurement of the fractionation factor of the enzyme involved in the reaction. The measurement of the secondary \( a\text{-}D(V/K) \) for the hydration reaction in H₂O and D₂O was designed to minimize the need to have information on the fractionation factor of the enzyme. To distinguish whether the secondary \( a\text{-}D(V/K) \) and the primary \( D(V/K) \) occur in a concerted or stepwise manner, the effect on the \( a\text{-}D(V/K) \) when deuterium is substituted in the primary position will be explored for both cases.

Secondary \( a\text{-}D(V/K) \) Step Precedes \( D(V/K) \) Step. Equations 6–9 were derived from a kinetic model for a stepwise reaction as described in eq 10, where \( k_3 \) and \( k_4 \) represent the first-order rate constants for the hydration and dehydration of crotonase bound CrP and HBP, respectively.

\[ \frac{E}{k_A} = \frac{EA}{k_1} \times \frac{k_1}{k_2} \times \frac{k_3}{k_2} \times \frac{k_4}{k_5} \times EP \times k_7 \]  \hspace{1cm} (5)

\[ D(V/K)_A = \frac{Dk_3 + k_5/k_4(1 + k_3/k_2) + DK_eqk_6/k_7}{1 + k_5/k_4(1 + k_3/k_2) + k_6/k_7} \]  \hspace{1cm} (6)

\[ a\text{-}D(V/K)_A\text{H}_2O = \frac{k_3/k_2 + \text{DK}_{eq}(k_4/k_3)(1 + k_5/k_2)}{1 + k_5/k_4(1 + k_3/k_2)(1 + k_6/k_7)} \]  \hspace{1cm} (7)

\[ a\text{-}D(V/K)_A\text{D}_2O = \frac{a\text{-}Dk_3 + k_5/k_4 + a\text{-}DK_eq(k_4/k_3)(Dk_5 + k_4/k_6)}{1 + k_5/k_4(1 + k_3/k_2)(Dk_6 + k_5/k_4a\text{-}DK_eq)} \]  \hspace{1cm} (8)

The analysis assumes that \( k_1 \) and \( k_2 \) are the only rates affected by substituting ²H at C-3 because they correspond to the chemical step when the hybridization at C-3 changes and the C-O bond is either formed or broken. Similarly, we assume that ³H substitution at the primary position will only alter \( k_3 \) and \( k_4 \), where the C-H bond at C-2 is broken or formed. For a stepwise reaction, the substitution of ³H at the primary position reduces both \( k_3 \) and \( k_4 \). The reduction in \( k_3 \) increases the reverse commitment \( (c_r) \) as shown in eq 8. In our case, where the dissociation of the pantetheine substrates is rapid, i.e., \( k_r > k_p \), the commitment increases by a factor of \( Dk_5 \). This increase in \( c_r \) makes the observed value of \( a\text{-}D(V/K)\text{D}_2O \) closer to \( a\text{-}DK_{eq} \) than \( a\text{-}D(V/K)\text{H}_2O \). Intuitively, the ²H substitution has made the proton transfer transition state more rate determining. Since C-3 has been rehybridized and the C-O bond formed in the previous step, the chemical phenomena that produce the equilibrium isotope effect, the observed \( a\text{-}D(V/K) \) should reflect this shift in the rate-determining transition state. The predicted reduction of \( a\text{-}D(V/K)\text{D}_2O \) relative to \( a\text{-}D(V/K)\text{H}_2O \) can be calculated with equation 9 and from the known values of \( a\text{-}D(V/K)\text{H}_2O, a\text{-}DK_{eq}, \) and \( D(V/K) \).

\[ \frac{a\text{-}D(V/K)\text{H}_2O - a\text{-}DK_{eq}}{a\text{-}D(V/K)\text{D}_2O - a\text{-}DK_{eq}} = D(V/K) \]  \hspace{1cm} (9)

Secondary \( a\text{-}D(V/K) \) and \( D(V/K) \) Steps Are Consecrated. Equations 11–13 were derived from a kinetic model for a concerted reaction as described in eq 10, where \( k_3 \) and \( k_4 \) represent the first-order rate constants for the hydration and dehydration of crotonase bound CrP and HBP, respectively.

\[ E \times \frac{k_A}{k_1} = \frac{EA}{k_2} \times \frac{k_1}{k_4} \times \frac{k_5}{k_3} \times \frac{k_4}{k_7} \times EP \times k_7 \]  \hspace{1cm} (10)

\[ D(V/K)_A = \frac{Dk_3 + c_r + DK_{eq}}{1 + c_r + k_7} \]  \hspace{1cm} (11)

\[ a\text{-}D(V/K)_A\text{H}_2O = \frac{a-Dk_3 + c_r + a-DK_{eq}}{1 + c_r + k_7} \]  \hspace{1cm} (12)

\[ a\text{-}D(V/K)_A\text{D}_2O = \frac{a-Dk_3 + c_r/Dk_3 + a-DK_{eq}c_r/Dk_3}{1 + c_r/Dk_3 + c_r/DK_{eq}Dk_3} \]  \hspace{1cm} (13)

The forward and reverse commitments, \( c_r \) and \( c_r' \), correspond to \( k_3/k_1 \) and \( k_4/k_3 \), respectively. Deuterium substitution for the transferred proton slows down the chemical step, reducing \( c_r \) and \( c_r' \) by \( Dk_3 \) and \( Dk_3/DK_{eq} \) respectively. For a concerted reaction, the value of \( a\text{-}D(V/K)\text{D}_2O \) will be closer to the intrinsic value, \( a\text{-}Dk_5 \), since the deuterium substitution makes the chemical step more rate limiting; or, for the current case where the commitments are already negligible, the value will not change since the chemical step is completely rate limiting.

Solvent Discrimination Isotope Effect in 50:50 H₂O/D₂O. A SDIE can be determined by running a reaction to a small fractional conversion in 50:50 H₂O/D₂O. To obtain the measured SDIE the proto/deutero product isotope ratio \( (R_p) \) is divided by the mole fraction ratio of H₂O to D₂O.

\[ \text{SDIE} = R_p/(X_{H2O}/X_{D2O}) \]  \hspace{1cm} (14)

The SDIE depends on both a kinetic isotope effect and an equilibrium isotope effect

\[ \text{SDIE} = Dk_5(E_{BH}/E_{BD}) \]  \hspace{1cm} (15)

where \( Dk_5' \) is the isotope effect on the net rate constant (Cleland, 1975) for converting the enzyme form represented by \( E_{BH} \) and \( E_{BD} \) (which are the fraction of enzyme where the protonated base will donate proton or deuterium to the substrate, respectively) to product. The ratio of \( E_{BD} \) to \( E_{BH} \) can only be known if there is free exchange of the proton with solvent and the fractionation factor relative to water is known. This fractionation factor is generally believed to be close to unity but could potentially be much smaller (Weiss et al., 1985).
Crotonase β-Elimination Is Concerted

Table I: Secondary \(^{13}D(V/K)_{\text{H}_2O}/^{13}D(V/K)_{\text{D}_2O}\)

<table>
<thead>
<tr>
<th>sample(^*)</th>
<th>correction</th>
<th>(m/z) isotope ratio</th>
<th>first exp(^t)</th>
<th>second exp(^t)</th>
</tr>
</thead>
<tbody>
<tr>
<td>standard 3-hydroxybutyrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>([(R)-2-H,3-1H/2H]HBP) from (\text{D}_2\text{O}) hydration</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>([(R)-2-H,3-1H/2H]HBP) from (\text{D}_2\text{O}) hydration</td>
<td>contaminating primary protons(^e)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>([3-1H/2H]HBP) from (\text{H}_2\text{O}) hydration</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^*\)The sample was derivatized to its pentafluorobenzyl ester. \(^t\)The number of determinations is in parentheses, and the reported error is the standard deviation. \(^e\)The \(m+1/m+2\) ratio of the sample from the \(\text{D}_2\text{O}\) hydration was corrected for the contaminating primary proton contribution of the \(m+1\) peak as described in the text. \(^t\)The ratio of \(^{13}D(V/K)_{\text{H}_2O}/^{13}D(V/K)_{\text{D}_2O}\), which is the important ratio for the interpretation of the chemical mechanism, was calculated by dividing the \(m/m+1\) ratio of \([3-1H/2H]HBP\) by the \(m+1/m+2\) ratio of \([(R)-2-H,3-1H/2H]HBP\), which has been corrected for protons contaminating the deuterated primary position.

1987). Scheme I represents the SDIE measured, dependent on which enzyme form exchanges rapidly, assuming a fractionation factor of the enzymatic base of unity. If \(k_3\) is fast relative to \(k_5\) and there is free exchange of the proton from a hypothetical carbanion intermediate, then the SDIE yields the intrinsic KIE on \(k_5\). If there is no exchange of the proton from the enzyme–carbanion intermediate but there is rapid exchange from an enzyme-substrate complex, then the isotopic effect on the product ratio will be \(Dk_5'\). Finally, if the proton only exchanges rapidly from free enzyme, then the SDIE will be \(Dk_3'\), which is \(D(V/K)_{\text{D}_2O}\).

**Scheme I**

**Scheme II**

180°. Scheme I represents the SDIE measured, dependent on which enzyme form exchanges rapidly, assuming a fractionation factor of the enzymatic base of unity. If \(k_3\) is fast relative to \(k_5\) and there is free exchange of the proton from a hypothetical carbanion intermediate, then the SDIE yields the intrinsic KIE on \(k_5\). If there is no exchange of the proton from the enzyme–carbanion intermediate but there is rapid exchange from an enzyme-substrate complex, then the isotopic effect on the product ratio will be \(Dk_5'\). Finally, if the proton only exchanges rapidly from free enzyme, then the SDIE will be \(Dk_3'\), which is \(D(V/K)_{\text{D}_2O}\).

Solvent \(D(V/K)_{\text{D}_2O}\) Equilibrium Overshoot. The equilibrium overshoot has been described for epimerases by Cardinale and Abeles (1968) and by Cleland (1977). We have numerically modeled equilibrium overshoots for hydratases starting from a model described in Scheme II where \(A\) represents HBP and \(P\) represents CrP. Inherent to the solution is the assumption that loss of the primary hydrogen to the solvent is irreversible during the elimination reaction. The King-Altman procedure was used to determine the fraction of enzyme present in each form depicted in Scheme II. The differential equations describing unlabeled and labeled substrate concentrations (\(A\) and \(A'\)) as a function of time are

\[
dx = -A'V/K_A \quad 0.0341 \pm 0.0021 \quad (3) \quad 0.0110 \pm 0.0001 \quad (3)
\]

\[
0.0615 \pm 0.0040 \quad (4) \quad 0.0223 \pm 0.0012 \quad (3)
\]

\[
0.8942 \pm 0.0027 \quad (8) \quad 1.0600 \pm 0.0050 \quad (5)
\]

\[
0.8676 \pm 0.0061 \quad 1.0548 \pm 0.0050
\]

where the pseudo-first-order rate constant for labeled substrate is \(v/K_{A'}\), the initial substrate concentration is \(A\), and \(K_{eqD}\) is the equilibrium constant between the labeled substrate and product in \(\text{D}_2\text{O}\). A numerical solution of the differential equations was obtained with an IBM AT computer using ASYNTAT software. By variation of the \(D(V/K)_{\text{D}_2O}\), the theoretical overshoot was generated to match the experimental overshoot. A numerical analysis of the differential eqs 16 and 17 is also used to determine the extent of reaction for the isotope exchange experiment in \(\text{D}_2\text{H}^1\text{O}\).

**RESULTS**

**Initial Velocities.** The relative \(V_{\text{max}}\) values were measured for the crotonase-catalyzed dehydration of HBP in the presence of saturating activator, 3',5'-ADP, and for the physiological substrate Cr-CoA.

The \(V_{\text{max}}\) value with Cr-CoA as the substrate was \(6 \pm 1\) times larger than with HBP, which had saturating 3',5'-ADP. In a separate experiment, the relative \(V_{\text{max}}\) values of HB-CoA and Cr-CoA were measured. The \(V_{\text{max}}\) value with Cr-CoA as the substrate was 7 ± 1 times larger than with HB-CoA. Consequently, there is no significant difference in the \(V_{\text{max}}\) values for the pantetheine or CoA substrates.

Secondary \(^{13}D(V/K)\) in \(\text{H}_2\text{O}\) and \(\text{D}_2\text{O}\). Mass spectrometric isotope ratio measurements were obtained to determine the effect that substituting deuterium for the transferred proton has on the secondary \(^{13}D(V/K)\) and are reported in Table I. In order to make conclusions from this experiment, the isotope ratios of the product \([(R)-2-H,3-1H/2H]HBP\) from the \(\text{D}_2\text{O}\) hydration and \([3-1H/2H]HBP\) from the \(\text{H}_2\text{O}\) hydration reaction are the most important to compare. The critical ratio for interpreting the chemical mechanism is \(^{13}D(V/K)_{\text{H}_2O}/^{13}D(V/K)_{\text{D}_2O}\). and, if life were simple, it would be calculated by dividing the \(m/m+1\) ratio of \([3-1H/2H]HBP\) formed during the hydration in \(\text{D}_2\text{O}\) by the \(m+1/m+2\) ratio of \([(R)-2-H,3-1H/2H]HBP\) formed during the hydration in \(\text{D}_2\text{O}\). Since \(\text{D}_2\text{O}\) is not 100% \(^2\text{H}\), a correction needs to be made. This correction is described in the appendix. The key result shown in the bottom line of Table 1 is that \(^{13}D(V/K)\) is not different in \(\text{H}_2\text{O}\) or \(\text{D}_2\text{O}\), i.e., \(^{13}D(V/K)_{\text{H}_2O}/^{13}D(V/K)_{\text{D}_2O}\) is within experimental error of 1.000. Experiments 1 and 2 in Table I are completely independent experiments. The improved resolution in experiment 2 was obtained by increasing the mass spectrometer repeller voltage and by decreasing the residual \(^1\text{H}\) content in the \(\text{D}_2\text{O}\). These two experimental improvements reduced the magnitude of the corrections required.

In order to calculate absolute values of \(^{13}D(V/K)_{\text{H}_2O}\) and \(^{13}D(V/K)_{\text{D}_2O}\) with eq 3, additional corrections need to be made to the \(m+1/m+2\) ratio of the \(\text{D}_2\text{O}\) hydration product, the \(m/m+1\) ratio of the \(\text{H}_2\text{O}\) hydration product, and the \(m+1/m+2\) ratio of the initial substrate \([3-1H/2H]\text{CrP}\) by subtracting the predicted contributions of the natural abundances of \(^{13}\text{C}\), \(^2\text{H}\), and \(^1\text{O}\) to the \(m+1\) and \(m+2\) peaks. The corrections are made and the values of \(^{13}D(V/K)_{\text{H}_2O}\) and \(^{13}D(V/K)_{\text{D}_2O}\) are reported in Table II for comparison with the value determined previously by equilibrium perturbation.
Table II: Absolute Secondary $^{34}D(V/K)_{H_2O}$ and $^{34}D(V/K)_{D_2O}$

<table>
<thead>
<tr>
<th>Sample</th>
<th>Correction</th>
<th>$m/z$ Isotope Ratio</th>
<th>First Exp $^b$</th>
<th>Second Exp $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3-1H/2H]CrP initial substrate</td>
<td>natural abundance $^c$</td>
<td>$m+1$ (85/86)</td>
<td>1.001 ± 0.01 (2)</td>
<td>1.249 ± 0.01 (2)</td>
</tr>
<tr>
<td>[3-1H/2H]CrP initial substrate</td>
<td>natural abundance $^c$</td>
<td>$m+1$ (85/86)</td>
<td>1.048 ± 0.01</td>
<td>1.323 ± 0.08</td>
</tr>
<tr>
<td>[3-1H/2H]HBP from $D_2O$ hydration</td>
<td>natural abundance $^c$</td>
<td>$m+1$ (104/105)</td>
<td>0.9079 ± 0.0027</td>
<td>1.1112 ± 0.0050</td>
</tr>
<tr>
<td>[3-1H/2H]HBP from $H_2O$ hydration</td>
<td>natural abundance $^c$</td>
<td>$m+1$ (103/104)</td>
<td>0.9073 ± 0.0027</td>
<td>1.1158 ± 0.0038</td>
</tr>
</tbody>
</table>

$^*$The sample was derivatized to its pentafluorobenzyl ester. $^b$The number of determinations is in parentheses. $^c$The reported error is the standard deviation. $^d$The $^{13}C$, $^2H$, and $^{17}O$ natural abundance contribution to the $m+2$ peak was subtracted. $^e$The $^{13}C$, $^2H$, and $^{17}O$ natural abundance contribution to the $m+1$ peak of [3-1H/2H]HBP and the corrected $m/m+1$ ratio of [3-1H/2H]CrP by using eq 3. $^f$The $^{13}C$, $^2H$, and $^{17}O$ natural abundance contribution to the $m+1$ peak of [3-1H/2H]HBP and the corrected $m/m+1$ ratio of [3-1H/2H]CrP by using eq 3.

Table III: Solvent Discrimination Isotope Effect Measured in 50:50 $H_2O:D_2O$

<table>
<thead>
<tr>
<th>Sample</th>
<th>$m/z$</th>
<th>Isotope Abundance Relative to Largest Peak $^b$</th>
<th>Isotope Abundance Corrected for Natural Abundance $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard 3-hydroxybutyrate (2)</td>
<td>$m=1$ (102)</td>
<td>0.0018 ± 0.00004</td>
<td>0.0018 ± 0.00004</td>
</tr>
<tr>
<td></td>
<td>$m=103$</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>$m+1$ (104)</td>
<td>0.0462 ± 0.00025</td>
<td>0.0462 ± 0.00025</td>
</tr>
<tr>
<td></td>
<td>$m+2$ (105)</td>
<td>0.0075 ± 0.00011</td>
<td>0.0075 ± 0.00011</td>
</tr>
<tr>
<td></td>
<td>$m+1$ (102)</td>
<td>0.0040 ± 0.000010</td>
<td>0.0040 ± 0.000010</td>
</tr>
<tr>
<td></td>
<td>$m=103$</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>$m+1$ (104)</td>
<td>0.6612 ± 0.00018</td>
<td>0.6612 ± 0.00018</td>
</tr>
<tr>
<td></td>
<td>$m+2$ (105)</td>
<td>0.0373 ± 0.00022</td>
<td>0.0373 ± 0.00022</td>
</tr>
<tr>
<td>([R]-2-1H/2H]HBP alcohol unprotected (6)</td>
<td>$m=1$ (150)</td>
<td>0.0013 ± 0.000018</td>
<td>0.0013 ± 0.000018</td>
</tr>
<tr>
<td></td>
<td>$m=187$</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>$m+1$ (188)</td>
<td>0.7415 ± 0.00138</td>
<td>0.7415 ± 0.00138</td>
</tr>
<tr>
<td></td>
<td>$m+2$ (189)</td>
<td>0.0703 ± 0.00279</td>
<td>0.0703 ± 0.00279</td>
</tr>
</tbody>
</table>

$^*$The sample was derivatized to its pentafluorobenzyl ester. $^b$The number of determinations is in parentheses. $^c$The reported error is the standard deviation. $^d$The $^{13}C$, $^2H$, and $^{17}O$ natural abundance contribution to the $m+1$ peak was subtracted. $^e$The product distribution KIE was calculated from the corrected $m/m+1$ ratio of the hydration product by using eq 14.

The mass spectrometric analysis of 3-hydroxybutyrate derivatives showed a considerable fragmentation peak that corresponds to the elimination of $H_2O$. There are potentially both an $\alpha$-secondary and primary isotope effect on elimination in the mass spectrometer. We attempted to detect both of these effects. To detect an $\alpha$-secondary effect, the [3-1H/2H]HBP sample was scanned over the 80-120 $m/z$ range. The parent ion peak minus 18 $m/z$ represents elimination of $H_2O$ from the 3-hydroxybutyrate ester. The 85/86 and 103/104 isotope ratios were indistinguishable, which indicates that there is not a detectable $\alpha$-secondary isotope effect in the gas-phase elimination in the mass spectrometer source.

The presence of a large primary $^2H$ KIE on elimination in the mass spectrometer would complicate all of the isotope ratio measurements reported. Our initial plan was to prevent the elimination by protecting the hydroxyl with dihydropyran. The 3-hydroxybutyrate derivative that had the hydroxyl group protected with dihydropyran shows less than 1% of the elimination fragment (data not shown). The unprotected sample shows 20-40% elimination of water. The equivalent value of the SDIE for samples shown in Table III, with and without dihydropyranylation, shows the lack of a primary $^2H$ KIE upon elimination in the mass spectrometer. Furthermore, the 85/86 ($m/m+1$) isotope ratio of the elimination fragment derived from enzymatically synthesized ([R]-2-1H/2H]HBP did not differ significantly from 1.0 after correction for natural abundance $^{13}C$, indicating that the gas-phase elimination has no preference for theo or erythro stereochemistry and a negligible primary isotope effect. Because of the additional derivatization and larger natural abundance isotope corrections required when the pentafluorobenzyl 3-hydroxybutyrate is dihydropyranylated, we returned to analyzing the pentafluorobenzyl 3-hydroxybutyrate.

Solvent Discrimination Isotope Effect in 50:50 $H_2O:D_2O$. The SDIE for the hydration of CrP to ([R]-2-1H/2H]HBP catalyzed by crotonase was measured in 50:50 $H_2O:D_2O$. The product $m-1$, $m+1$, and $m+2$ isotope abundances relative to the $m$ peak of ([R]-2-1H/2H]HBP formed by hydration of CrP to 3.9% of equilibrium are reported in Table III. The $m+1$ peak was corrected by the subtraction of natural abundance $^{13}C$, $^2H$, and $^{17}O$ using the $m/w+1$ ratio of the standard 3-hydroxybutyrate. The $H_2O:D_2O$ mole ratio of the solvent was determined to be 1.005 from mass measurements of $H_2O$ and $D_2O$ during the buffer preparation. The $m-1/m$ ratio of the standard 3-hydroxybutyrate equal to 0.00187 showed that the $m$ peaks were sufficiently resolved under the mass spectrometric conditions of this experiment to obviate the need for bleed-over corrections. The SDIE for the hydration of CrP to ([R]-2-1H/2H]HBP catalyzed by crotonase was calculated to be 1.60 ± 0.03.

Isotope Exchange of HBP in $D_2^{18}O$. HBP was partially dehydrated by crotonase in $D_2^{18}O$. One aliquot of the reaction mixture was quenched after approximately 90% of the net flux to equilibrium had occurred and the remainder quenched after complete isotopic equilibration. After conversion to the pentafluorobenzyl ester, mass spectroscopic analysis revealed the relative $m$, $m+1$, $m+2$, and $m+3$ peaks, which would ideally correlate with unreacted HBP, HBP with just the C-2 proton exchanged, HBP with just the C-3 hydroxyl exchanged, and HBP that has exchanged both the proton and hydroxyl, respectively. These values and the corrections for the nonisotopic purity of the $D_2^{18}O$ are reported in Table IV.

Because the initial isotopic conditions will lead to an overshoot similar to that shown in Figure 3, the extent of reaction was determined by a numerical integration of eqs 16 and 17. Because the $D_2^{18}O$ was not isotopically pure, corrections de-
Table IV: Isotope Exchange of HBP in D$_2$O

| sample | $m/z$ | isotope abundance relative to largest peak | isotope abundance corrected for natural abundance | isotope abundance corrected for rehydration of CrP
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>standard 3-hydroxybyturate (6)</td>
<td>$m-1$ (102)</td>
<td>0.00185 ± 0.00010</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$m$ (103)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$m+1$ (104)</td>
<td>0.04544 ± 0.00033</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$m+2$ (105)</td>
<td>0.00741 ± 0.00009</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBP exchanged in D$_2$H/O to 20% reaction (6)</td>
<td>$m$ (103)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$m+1$ (104)</td>
<td>0.05822 ± 0.00023</td>
<td>0.01278 ± 0.00056</td>
<td>&lt;0.004</td>
</tr>
<tr>
<td></td>
<td>$m+2$ (105)</td>
<td>0.05352 ± 0.00017</td>
<td>0.04543 ± 0.00059</td>
<td>&lt;0.012</td>
</tr>
<tr>
<td></td>
<td>$m+3$ (106)</td>
<td>0.10469 ± 0.00027</td>
<td>0.10253 ± 0.00083</td>
<td>0.000</td>
</tr>
<tr>
<td>HBP exchanged in D$_2$H/O to isotopic equilibrium (6)</td>
<td>$m$ (103)</td>
<td>1</td>
<td>0.06266 ± 0.00051</td>
<td>0.06364 ± 0.00052</td>
</tr>
<tr>
<td></td>
<td>$m+1$ (104)</td>
<td>0.08480 ± 0.00064</td>
<td>0.08323 ± 0.00117</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$m+2$ (105)</td>
<td>0.32963 ± 0.00030</td>
<td>0.33054 ± 0.00178</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$m+3$ (106)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The sample was derivatized to its pentafluorobenzyl ester. The number of determinations is in parentheses. The reported error is the standard deviation. The 3C, 3H, and 17O natural abundance contribution to the $m+1$, $m+2$, and $m+3$ peaks was subtracted. The correction applied is described in the appendix and assumes all of the $m+3$ peak arises from rehydration of CrP.

Table V: $\alpha$-H Equilibrium Isotope Effect

<table>
<thead>
<tr>
<th>sample</th>
<th>$m/z$</th>
<th>isotope abundance relative to $m$ peak</th>
<th>isotope abundance corrected for natural abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>standard</td>
<td>$m-1$ (102)</td>
<td>0.00749 ± 0.00012</td>
<td></td>
</tr>
<tr>
<td>3-hydroxybutyrate (3)</td>
<td>$m$ (103)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$m+1$ (104)</td>
<td>0.04715 ± 0.00057</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$m+2$ (105)</td>
<td>0.00739 ± 0.00009</td>
<td></td>
</tr>
<tr>
<td>[3-$\text{H}/\text{H}$]HBP</td>
<td>$m-1$ (102)</td>
<td>0.00084 ± 0.00031</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$m$ (103)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$m+1$ (104)</td>
<td>1.04326 ± 0.00520</td>
<td>0.9962 ± 0.0058</td>
</tr>
<tr>
<td></td>
<td>$m+2$ (105)</td>
<td>0.0558 ± 0.0004</td>
<td></td>
</tr>
<tr>
<td>[3-$\text{H}/\text{H}$]CrP</td>
<td>$m-1$ (84)</td>
<td>0.00275 ± 0.00003</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$m$ (85)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$m+1$ (86)</td>
<td>0.79156 ± 0.00091</td>
<td>0.7451 ± 0.00105</td>
</tr>
<tr>
<td></td>
<td>$m+2$ (87)</td>
<td>0.0449 ± 0.0006</td>
<td></td>
</tr>
</tbody>
</table>

*Each sample was derivatized to its pentafluorobenzyl ester. The number of determinations is in parentheses. The reported error is the standard deviation. The 3C, 3H, and 17O natural abundance contribution to the $m+1$ peak was subtracted. $\delta^{13}$K$_{eq}$ was calculated from the ratio of the corrected $m+1$ to $m$ of [3-$\text{H}/\text{H}$]HBP and [3-$\text{H}/\text{H}$]CrP.

mental value of 21.2% with a K$_{eq}$ of 0.33 and a D($V/K$) of 1.6. The fractional overshoot is sensitive to differences in the KIE. For example, the theoretical curves generated with a K$_{eq}$ of 0.33 and a D($V/K$) of 1.3 had a fractional overshoot of only 10%.

$\alpha$-H Equilibrium Isotope Effect. The $m/z$ abundances at $m-1$, $m$, $m+1$, and $m+2$ isotopes relative to the $m$ peak for unlabeled 3-hydroxybyturate or crotonate are reported in Table V for the [3-$\text{H}/\text{H}$]HBP and [3-$\text{H}/\text{H}$]CrP substrates that were brought to isotopic equilibrium in the presence of crotonase. The $m/m+1$ isotope ratio of the standard pentafluorobenzyl 3-hydroxybutyrate was used to subtract 3C, 3H, and 17O natural abundance contributions to the $m+1$ peak of the [3-$\text{H}/\text{H}$]HBP sample. The $m+1$ peak of the [3-$\text{H}/\text{H}$]CrP sample was corrected by an estimated $m/m+1$ ratio of crotonate based on the experimental $m/m+1$ isotope ratio of the standard 3-hydroxybyturate. The ratio of the $m$ and $m+1$ corrected isotope abundances of [3-$\text{H}/\text{H}$]HBP and [3-$\text{H}/\text{H}$]CrP gave an $\delta^{13}$K$_{eq}$ of 1.34 ± 0.01. This value is consistent with the estimated $\delta^{13}$K$_{eq}$ value of 1.33, which was obtained from the ratio of the D($V/K$) values of 1.16 for the transfer of 3H from [2-$\text{H}$]-2-propanol or [1-$\text{H}$]-cyclohexanol to water and 0.87 for the transfer of [2-$\text{H}$]malate to water (Cleland, 1980).

The agreement of the measured primary and secondary kinetic isotope effects with the values previously measured by equilibrium perturbation and the agreement of the secondary
With the calculated value serve to validate the described whole molecule isotope ratio experimental protocol. The negative ion chemical ionization analysis of pentafluorobenzyl esters has overcome two common drawbacks to isotope ratio measurements. The electron capture, carboxylate ionization mechanism gives rise to an isotope-independent ionization and minimal fragmentation that precludes isotope effects on fragmentation. The extremely efficient ionization process also permits the analysis to be done on very small amounts of material, allowing product ratios to be determined at very small fractional conversion and many duplicate analyses to be performed.

**Discussion**

β-Elimination reactions can occur with C–H and C–X bond cleavages in either an ordered or a concerted fashion. Concerted (E2) and carbanion intermediate (Elcb) reactions predominate when a carbanion can be stabilized by the leaving group. Gandler and Jencks (1982) have argued that there is a distinct transition from an E2 to an Elcb mechanism as the stability of the carbanion is enhanced. Mayer et al. (1984) studied α-β elimination reactions in β-decalone systems and observed a change in mechanism from Elcb to E2 promoted by the activation of the leaving group by ring strain. The strained lactone ring of 11-oxatricyclo[4.3.3.0]tridec-3-one eliminates by an E2 mechanism despite the activation of the abstracted proton by the ketone. Additionally, the complex structure of a concerted mechanism's transition state may be highly unfavorable entropically for the nonenzymatic β-elimination, whereas precise binding interactions between crotonase and this complex transition state structure may favor a concerted mechanism. The mechanistic question for the crotonase-catalyzed elimination from 3-hydroxy thioester is, Does the enzyme predominantly activate the proton for abstraction resulting in the Elcb mechanism, or has the leaving group been activated as well, resulting in a concerted mechanism?

In a previous study we reported the primary $^{13}$C/H and $^{18}$O/(V/K) and secondary $^{13}$C/D(V/K), $^{15}$N/(V/K), and $^{18}$O/D(V/K) for the crotonase-catalyzed elimination of HBP (Bahnsen & Anderson, 1989). These results are summarized in Scheme III and are consistent with an Elcb stepwise or an E2 concerted mechanism with carbanion character, shown in Scheme IV. A mechanism that has carbocation character was ruled out since hyperconjugation would lead to a $^{13}$C/D(V/K) significantly greater than unity. The micromolar binding to crotonase of the enol(ate) form of AcAcCoA (Waterson & Hill, 1972) is the only experimental evidence for a carbanion intermediate in the crotonase 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 CoA 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), from direct observation of the enethiol(ate) of acetyldithio-CoA bound to citrate synthase (Wlassics & Anderson, 1989), and

**Scheme III**

α-DK$^-$ with the calculated value serve to validate the described whole molecule isotope ratio experimental protocol. The negative ion chemical ionization analysis of pentafluorobenzyl esters has overcome two common drawbacks to isotope ratio measurements. The electron capture, carboxylate ionization mechanism gives rise to an isotope-independent ionization and minimal fragmentation that precludes isotope effects on fragmentation. The extremely efficient ionization process also permits the analysis to be done on very small amounts of material, allowing product ratios to be determined at very small fractional conversion and many duplicate analyses to be performed.

**Scheme IV**

by the reaction and α-proton exchange of acetyldithio-CoA catalyzed by thiolase (Anderson et al., 1990).

**Identity of the Pantetheine and CoA Thioester Transition-State Structures.** The slower alternative pantetheine thioester substrates were used in this isotope effect investigation in order to make the chemical steps in the reaction mechanism completely rate limiting. Presumably the pantetheine thioester substrates, which lack the adenosine portion of CoA, have increased dissociation rates relative to the CoA thioester substrates. The apparent $K_s$ for the pantetheine substrates are over 100-fold greater than the $K_s$ for the analogous CoA thioesters. An alternative explanation of the decreased catalytic efficiency of pantetheine relative to CoA thioesters is that the transition state is significantly different and consequently would exhibit different isotope effects. A comparison of $V_{max}$ values for the pantetheine and CoA substrates addresses whether differences exist in the transition-state structure. Within the experimental uncertainty of the values, the relative $V_{max}$ for HB-CoA is indistinguishable from the $V_{max}$ for HBP with saturating 3',5'-ADP activator. From the equivalent $V_{max}$ values, a conclusion is made that the transition-state structure is effectively identical for the crotonase-catalyzed reaction of CoA thioesters and pantetheine thioesters with 3',5'-ADP as activator and that the lower V/K values for the pantetheine substrates result from enhanced rates of dissociation from the Michaelis complexes.

**Interpretation of an α-D(V/K)$_{H_2O}$/α-D(V/K)$_{D_2O}$ of Unity.** Double isotope effect studies are able to distinguish whether two different isotopic substitutions affect the same or different chemical steps. As argued under Theory, the measurement of the secondary α-D(V/K) for the hydration reaction in H$_2$O or D$_2$O should provide a distinction between a concerted or stepwise reaction.

The isotope effects for the crotonase-catalyzed dehydoration of HBP, shown in Scheme III, are only consistent with a carbanion stepwise or concerted mechanism with carbanion character (Bahnsen & Anderson, 1989). The primary $^{13}$C/H and $^{18}$O/(V/K) values of 1.051 and 1.61, respectively, require that the bond-cleavage step(s) is (are) the most rate limiting. If a stepwise reaction is invoked, the C–O bond-cleavage step is more rate determining than C–H bond cleavage. Figure 4 qualitatively illustrates the predicted relationship between the α-D(V/K)$_{D_2O}$ and the α-D(V/K)$_{H_2O}$ for the carbonyl stepwise (4A) and concerted (4B) mechanism. For the carbanion stepwise mechanism, the α-secondary sensitive transition state is slightly more rate limiting than the C–H bond-forming transition state. The hydration reaction run in D$_2$O has an α-secondary sensitive transition state that is less rate limiting due to the primary $^2$H label, resulting in the α-D(V/K)$_{D_2O}$ < α-D(V/K)$_{H_2O}$. For the concerted mechanism, in which there is only one isotopically sensitive transition state, deuteration

[^4]: If the C–H bond cleavage step were more rate limiting, the intrinsic $^{18}$K$^-$ would have to be greater than 1.10, which is 20% larger than any previously observed $^{18}$K$^-$.
Crotonase β-Elimination is Concerted

Potential Complication by Coupled Proton Motions.
Classically, it was argued that multiple sites in a molecule behave independently in a single transition state with respect to isotopic substitution (Kresge, 1964). However, Kurz and Frieden (1980), Cook et al. (1981), and Hermes et al. (1984) observed α-secondary KIEs larger than the corresponding equilibrium isotope effect with reactions of NADH. The unexpectedly large α-secondary KIEs were postulated to result from the motion of the nontransferred proton being included in the reaction coordinate motion, i.e., there is a coupled motion between the primary and secondary protons of the cofactor in the transition state. This coupling results in large normal α-D(V/K) KIEs when there is significant tunneling (Huskey & Schowen, 1983) that is eliminated when the primary position is deuterated. In formate dehydrogenase, deuterated formate reduced the α-D(V/K)NAD from 1.23 to 1.06 (Hermes et al., 1984).

Saunders has proposed that there is coupling between the abstracted and remaining proton at the β-carbon in model β-eliminations (Saunders, 1985; Amin et al., 1988). In the present study, α-secondary effects were measured with a deuterium substituted in the primary position. Since the α-secondary and primary 2H label are on adjacent carbons, the concern of coupled motion between the two positions can be neglected for the carboxy anion mechanism in which the substitutions apply to separate transition states. For the concerted mechanism with a single transition state, both isotopically substituted sites are in flight and have the possibility to be coupled. Hypothetically, if we had measured a reduced α-D(V/K)D2O for the crotonase-catalyzed reaction, then a concerted mechanism in which the primary and α-secondary protons are coupled could not be ruled out by the double isotope effect study alone. The equivalence of the α-D(V/K)H2O and α-D(V/K)D2O in the crotonase reaction strongly suggests that there is no coupled motion between these isotopically labeled positions in the transition state, and the concerted mechanism is still supported.

Solvent D(V/K) by Equilibrium Overshoot. The double-isotope fractionation results above do not rule out a circumstance in which a stepwise carboxy anion mechanism would still result in equivalent values of α-D(V/K)H2O and α-D(V/K)D2O. Qualitatively, if a primary intrinsic solvent KIE on the C-O bond forming step existed and was comparable to the PK for the C-H bond forming step, then the partitioning of a carboxy anion intermediate would be the same in H2O as in D2O. Physically, the primary solvent isotope effect could arise from a solvent exchangeable proton being donated to a lone pair of electrons on the oxygen while the C-O bond was breaking. Since this proton is "in flight" during the C-O bond cleavage, it would be associated with a primary 2H KIE. This hypothetical situation is shown by a comparison of the dashed and solid lines in Figure 5, where a primary solvent isotope effect of identical magnitude exists on both steps, leaving the partitioning of the carboxy anion intermediate unaffected by the substitution of D2O. Quantitatively this can be shown in eq 18 by first simplifying eq 8 for the case where there are no external commitments, i.e., k2 >> k3 and k1 >> k5.

\[ α-D(V/K)_{D2O} = \frac{α-D(k_2 + k_3) + k_4}{1 + k_5(k_2/k_3)} \]

The c1 for the C-O bond forming step is increased in D2O by a factor of Dk5. An intrinsic solvent KIE on C-O bond
cleavage would reduce the \( c \) by the factor \( D(c\rightarrow 0)k_4 \) as shown by

\[
\alpha-D(V/K)_{D_2O} = \frac{\alpha-Dk_3 + \alpha-DK \cdot k_2(k_4/k_5)/D(c\rightarrow 0)k_4}{1 + Dk_2(k_4/k_5)/D(c\rightarrow 0)k_4}
\]

(19)

where \( \alpha-D(c\rightarrow 0)k_4 \) is the intrinsic \( ^2H \) solvent KIE on the \( C-O \) bond cleavage step. When \( Dk_3 \) is equal to \( \alpha-D(c\rightarrow 0)k_4 \), \( \alpha-D(V/K)_{D_2O} \) is equal to \( \alpha-D(V/K)_{H_2O} \) for a carbanion stepwise mechanism.

In the present study, this possibility was eliminated by measuring the primary \( D(V/K) \) by the equilibrium overshoot method in \( D_2O \). Figure 5 illustrates a second consequence of a primary solvent KIE. The increase in the free energy barrier of the \( C-O \) bond-forming step in \( D_2O \) relative to the \( C-H \) bond-forming step should result in a large decrease of the \( D(V/K) \) measured in \( D_2O \). We have looked for this decrease in \( D(V/K) \) by using the equilibrium overshoot method in \( D_2O \). Equation 6, which describes the reduction of the measured \( D(V/K) \) by commitments for a carbanion stepwise mechanism, can be simplified to eq 20 for the case of \( k_2 \gg k_3 \) and \( k_1 \gg k_6 \).

\[
D(V/K)_{A} = \frac{Dk_3 + k_5/k_4}{1 + k_3/k_4}
\]

(20)

The presence of a large \( \alpha-D(c\rightarrow 0)k_4 \) would significantly reduce the measured \( D(V/K) \) according to

\[
D(V/K)_{A} = \frac{Dk_3 + \alpha-D(c\rightarrow 0)k_4(k_5/k_4)}{1 + D(c\rightarrow 0)k_4(k_5/k_4)}
\]

(21)

An estimation can be made of the \( D(V/K) \) for a stepwise mechanism, when \( Dk_3 \) is equivalent to \( \alpha-D(c\rightarrow 0)k_4 \) and minimum values of \( Dk_3 = 3.1 \) and \( k_5/k_4 = 2.5 \) are used.\(^7\) The upper limit of \( D(V/K) \) obtained from eq 21 is 1.24, which corresponds to a fractional overshoot of less than 10%. The experimentally measured fractional overshoot of 21.1%, which corresponds to a \( D(V/K) \) of 1.6, rules out the hypothetical situation described above, which could have permitted the \( \alpha-D(V/K)_{H_2O} \) to equal the \( \alpha-D(V/K)_{D_2O} \) for the carbanion stepwise mechanism.

\(^7\) Minimum values of \( Dk_3 \) and \( k_5/k_4 \) were obtained for the stepwise mechanism by assigning \( ^1H \) to 1.072, which is the largest observed \( ^1H \) effect (Blanchard & Cleland, 1980), and estimating the internal commitments that would reduce the \( ^1H \) to the measured \( ^1H(V/K) \) of 1.051 (Bahnsen & Anderson, 1989).

**Solvent Discrimination Isotope Effect.** Proton exchange of an enzyme-substrate or an enzyme-intermediate complex can be probed by measuring solvent KIEs in mixed \( H_2O:D_2O \) solutions. Yamada and O'Leary (1977) measured a hydrogen isotope discrimination in 50:50 \( H_2O:D_2O \) of the proton transfer that follows the decarboxylation step in the reaction catalyzed by glutamate decarboxylase. Since the proton transfer followed an irreversible step, the lack of proton exchange during the lifetime of the quinoid intermediate resulted in a SDIE that reflected the fractionation factor of the enzyme base. The interpretation changes when all the steps of the enzyme mechanism are reversible as in the crotonase-catalyzed reaction. A rapid exchange of the transferred proton with solvent during the lifetime of a carbanion intermediate would result in the measurement of an intrinsic proton transfer isotope effect, i.e., \( \alpha-Dk_3 \) in Scheme I. Fishbein and Jencks (1988) used the difference between the small observed solvent kinetic isotope effect and the larger SDIE as one of the primary pieces of evidence that addition of thiols to \( \alpha-\beta \) unsaturated nitriles, and by microscopic reversibility the elimination from \( \beta-\gamma \) cyanothioethers, proceeds through a carbanion intermediate.

The measured SDIE of 1.6 for the crotonase reaction corroborates the concerted mechanism by providing evidence against a carbanion intermediate where the abstracted proton exchanges freely with solvent, which would give rise to a SDIE of at least 3.1 (see above). This result then puts an additional limitation on a potential carbanion intermediate mechanism. In an Elcb mechanism, the proton donor would have to be secluded from solvent and monoprotic, since a polyprotic base (e.g., lysine) would show significant isotopic discrimination through rotation of the \( C-N \) bond even with slow solvent exchange. The SDIE of 1.6 is expected for a concerted mechanism and is not dependent on which enzyme form exchanges protons with solvent.

**Isotope Exchange in \( D_{18}O \).** The isotope exchange of HBP in \( D_{18}O \) is consistent with the SDIE described above and further corroborates the conclusion of a concerted mechanism for the crotonase reaction. Because the large background correction resulted in subtracting two comparably sized numbers, the solvent exchange experiment is only able to give an upper estimate on how fast the abstracted proton, from a potential carbanion intermediate, exchanges compared to the overall reaction rate. Our results indicate that loss of hydroxide from a hypothetical carbanion intermediate would have to be at least 30 times faster than exchange of the abstracted proton with solvent.

**Implications of a Concerted Mechanism.** Concerted syn-eliminations are not unprecedented. Döhner and Saunders (1986) identified syn eliminations from standard 2,2-diaryl-ethyl tosylates. Ab initio calculations by Bach et al. (1979) suggested that concerted syn eliminations are possible but will have a carbanion character arising from the \( C_2-H \) bond being cleaved to a greater extent than the \( C_n \) leaving group bond. Extensive studies at the boundary between Elcb and concerted E2 eliminations where the abstracted proton is acidic have shown that activation of the leaving group can result in a concerted reaction (More-O’Ferral and Warren, 1975; Marshall et al., 1977; Mayer et al., 1984). While none of our experiments require the activation of the leaving group to occur by a preequilibrium protonation of the C-3 hydroxy, it is consistent with all of the isotope effects measured. The cleavage of a C-O+H2 bond constrained to be parallel to the p orbital containing the lone electron pair of an incipient carbanion may be fast enough that the concerted mechanism is enforced, i.e., the lifetime of the carbanion intermediate...
Crotonase $\beta$-Elimination Is Concerted

Fishbein and Jencks (1988) estimated expulsion rates for substituted thioenolate ions $\alpha$ to nitrile-stabilized carbanions and concluded that they range from $10^9$ to $10^{12.4}$ s$^{-1}$, just slower than the bond vibrational frequency of ca. $3 \times 10^{13}$ s$^{-1}$. We expect that the rate constant for elimination of H$_2$O whose pK$\alpha$ is from 4 to 10 pH units lower than the thioenolate and is presumably in the proper geometric orientation for elimination would face a smaller barrier for elimination. By the same argument, it is difficult to conceive of how hydroxide could be eliminated in a concerted fashion since its pK$\alpha$ is over 8 pH units greater than the poorest thioenolate examined. Although there is currently no other evidence, a preequilibrium protonation of the leaving group coupled with the syn stereochemistry of the elimination would permit a single active site base to mediate both proton transfers as shown in Scheme V.

Alternatively, it is possible that the syn stereochemistry is enforced by a carboxylate functioning as both proton donor and acceptor in a cyclic transition state. The important feature of either alternative is that the leaving group has to be activated sufficiently so that the lifetime of the enolate intermediate would be negligible.

The microscopic reverse of an elimination proceeding after a preequilibrium protonation is the addition of neutral H$_2$O to the $\alpha$-$\beta$ unsaturated thioester. The attack of the poor nucleophile is possible in the crotonase reaction because of the enhanced electrophilicity of the $\beta$-carbon in $\alpha$-$\beta$ unsaturated thioesters relative to $\alpha$-$\beta$ unsaturated carboxylates. This electrophilic property of $\alpha$-$\beta$ unsaturated CoA esters may be more important to the crotonase mechanism than the better recognized enhanced acidity of the $\alpha$-protons (Bruce & Benkovic, 1966).

**APPENDIX**

**Corrections for Isotope Ratios Used To Determine $\delta$D($V/K$).** The primary difficulty is correcting for the presence of $[3$-$^{13}$$H,2$-$^2$$H]$HBP because of the presence of a significant amount of protium in the D$_2$O. This is shown in eqs A1 and A2, which indicate the contributing factors to the lighter isotopic species for hydration in H$_2$O and D$_2$O, respectively,

\[ m = [3$-$^{13}$$H,2$-$^2$$H]$HBP + bleed over from $m+1$ \] (A1)

\[ m+1 = [3$-$^{13}$$H,2$-$^2$$H]$HBP + [3$-$^{13}$$H,2$-$^2$$H]$HBP + bleed over from $m+2$ \] (A2)

where $m$ is the mass of unlabeled 3-hydroxybutyrate.

In order to correct for the contaminating protons in the C-2 primary position of the product, the measurement of the $m$/$m+1$ isotope ratio of the D$_2$O hydration product and the $m$/$m+1$ isotope ratio of a standard sample of pentafluorobenzyl 3-hydroxybutyrate were required. The $m+1$/$m$ ratio of the standard was measured to determine the fraction of the m peak that bleeds over to the m$-$1 peak due to the incomplete resolution of m$+2$ peaks by the mass spectrometer (line 1, Table 1). If there were no $^1$H in the D$_2$O, the $m$/$m+1$ ratio for the sample hydrated in D$_2$O (line 2, Table 1) should be the same.

The difference ($A$ - $B$ in eq A3) is taken as an initial estimate of the correction that needs to be subtracted from the raw $m+1$/$m+2$ ratio to eliminate the contribution from $[3$-$^{13}$$H,2$-$^2$$H]$HBP. Note that the corrected value (line 4, Table 1) is within experimental error of line 3 - (line 2 - line 1). Because these are ratios, the simple subtraction only works exactly if the $m+1$/$m+2$ ratio is unity. To account for the deviation from unity and to eliminate a contribution of bleed over from the $[3$-$^{13}$$H,2$-$^2$$H]$hydroxybutyrate, the correction to the m$+1$ peak of the D$_2$O hydration product was solved iteratively according to

\[ 1 = X(A - B)C = X_{t+1} \] (A3)

where $X_t$ is the m$+1$ peak of the D$_2$O hydration product that was initially assigned a value of 1.0, A is the m$+1$/m$+2$ isotope ratio of the D$_2$O hydration product, B is the m$+1$/$m$ isotope ratio of the standard 3-hydroxybutyrate, and C is the m$+2$/$m+1$ isotope ratio of the D$_2$O hydration product. The value $X_{t+1}$ was substituted into the $X_t$ term of eq A3 during each iterative cycle until $X_t$ equaled $X_{t+1}$ to four significant figures.

The corrected m$+1$/m$+2$ isotope ratio of the D$_2$O hydration product was calculated by multiplying the $X_t$ term by the uncorrected m$+1$/m$+2$ isotope ratio of the D$_2$O hydration product and is shown in line 4, Table 1. We did not add the correction to the m$+2$ peak, because there is an analogous contribution to the m peak from $[3$-$^{13}$$H,2$-$^2$$H]$HBP that would then have to be added to the m$+1$ peak.

**Corrections for $^1$H and $^{15}$O in D$_2$O**. We hoped to identify the exchange of the abstracted C-2 proton with solvent faster than reaction. This would result in an increase in the m$+1$ peak relative to the m peak for the unreacted HBP. Ideally this is done when the reaction is irreversible, which is not technically possible with crotonase. However by using D$_2^1$O, the reaction is made irreversible by loss and dilution of the initial $^{15}$O at C-3. Unfortunately, the D$_2^1$O we were supplied contained significant amounts of both lighter isotopes. Two corrections have to be made to the m$+1$ peak: (1) for the $^{13}$C contribution of the m peak, and (2) for the rehydration of CrP with D$_2$O. The $^{13}$C contribution was determined by the m$+3$/$m+2$ isotope ratio of the standard 3-hydroxybutyrate and directly subtracted from the m$+1$/m$+2$ ratio for the 23% reaction sample. The correction for hydration of CrP with D$_2$O was made by assuming the m$+3$/m$+1$ ratio would be identical with that obtained from the fully equilibrated sample. It was assumed that all of the m$+3$ peak was derived from hydration of CrP with D$_2^1$O. A calculated amount of CrP hydrated by D$_2$O was obtained by multiplying the m$+3$ peak for the 23% reaction sample by the m$+1$/m$+3$ ratio for the equilibrium sample and subtracting this contribution from the m$+1$ peak. This correction yields an m$+1$/m$+2$ ratio of 0.004. This analysis ignores the solvent discrimination isotope effect of 1.6 we measured, which would decrease the size of the correction but not in a simple fashion since the same solvent discrimination isotope effect increases the m$+2$ peak at the expense of the m$+3$ peak. Because of these difficulties, the conclusion we feel justified in drawing is that at most 0.4% of the HBP that was remaining contained $^2$H that was introduced by solvent exchange and not hydration of CrP with D$_2$O.

**REFERENCES**


