Crotonase-Catalyzed β-Elimination Is Concerted: A Double Isotope Effect Study†

Brian J. Bahnson‡ and Vernon E. Anderson*
Department of Chemistry, Box H, Brown University, Providence, Rhode Island 02912
Received December 5, 1990; Revised Manuscript Received March 20, 1991

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 $^{D}(V/K)$ and $^{18}(V/K)$ are 1.60 and 1.053 [Bahnson, 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., Roeske, 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 α-secondary $^{D}(V/K)$ 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 α-secondary effect where the chemical reaction is completely rate determining requires that both bond cleavages be concerted or that the substitution of ^2H at the primary position not significantly alter the partitioning of a hypothetical carbanion. The observation of a solvent discrimination isotope effect determined from the relative incorporation of ^2H from 50% D_2O of 1.60 ± 0.03, identical with the primary $^{D}(V/K)$, 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 $^{D}(V/K)$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 α–β unsaturated CoA thioesters (Willadsen & Eggerer, 1975). The nearly diffusion limited V/K of 5×10^7 M^{-1} s^{-1} and the small $^{D}(V/K)$[1] on the dehydration of 3-hydroxybutyryl-CoA (Person, 1981) showed that large external commitments[2] 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 (Bahnson & Anderson, 1989).

The primary $^{D}(V/K)$, $^{18}(V/K)$, and secondary $^{\alpha\text{-}D}(V/K)$,[3] $^{\beta\text{-}D}(V/K)$, and $^{\beta'\text{-}D}(V/K)$ on the dehydration of (*S*)-3-hydroxybutyrylpantetheine (HBP)[4] in the presence of the activator, 3′,5′-ADP, were measured by the equilibrium perturbation technique (Bahnson & 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 carbocation 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 acetoacetyl-CoA (AcAc-CoA) to crotonase (Waterson & Hill, 1972) suggests that there may be an enolate intermediate. All well-characterized enzyme-catalyzed β-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

†This work was supported by Grant GM 36562 from the National Institute of General Medical Sciences and a Biomedical Research Support Grant. V.E.A. is a Sloan Foundation Fellow.
‡Current address: Department of Chemistry, University of California, Berkeley, CA.

[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 $^{\alpha\text{-}D}(V/K)$ is for ^2H substitution at the carbon that is bonded to the heavy atom leaving group, the secondary $^{\beta\text{-}D}(V/K)$ is for ^2H substitution at the carbon whose C–H bond is broken, and the secondary $^{\beta'\text{-}D}(V/K)$ is for ^2H substitution at the nonreacting β-carbon (Cook, 1976).

[4] Abbreviations: AcAc-CoA, acetoacetyl-CoA; AcAc-P, *S*-acetoacetylpantetheine; 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.

0006-2960/91/0430-5894$02.50/0

anti eliminations that proceed through discrete carbanion 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; Reinsh et al., 1980; Pohl et al., 1986). Nonenzymatic β-elimination reactions with protons activated by adjacent carbonyl functionalities have been shown to react through carbanion intermediates (Fedor, 1967, 1969; Robert et al., 1980), but a change in mechanism from stepwise to concerted was shown for a β-elimination adjacent to a carbonyl when the leaving group was activated through ring strain (Mayer 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 carbanion stepwise to concerted.

In the present study, four experimental approaches are reported that are designed to detect the presence of a carbanion intermediate in the crotonase-catalyzed hydration of *trans*-crotonylpantetheine (CrP). (1) A double isotope effect study is reported in which the secondary $^{\alpha\text{-D}}(V/K)$ on the hydration of CrP was measured in H_2O and D_2O. If deuterium substitution in the solvent alters the partitioning of a carbanion intermediate, which will occur if there is a significant primary $^{D}(V/K)$, the secondary $^{\alpha\text{-D}}(V/K)$ measured in D_2O should be closer to the measured secondary equilibrium isotope effect, $^{\alpha\text{-D}}K_{eq}$. (2) The distribution of [(*R*)-2-^{1}H]- and [(*R*)-2-^{2}H]-HBP following the hydration of CrP in 50:50 H_2O:D_2O was determined. If the proton donated to C-2 of a carbanion intermediate exchanges rapidly with solvent, then a solvent discrimination isotope effect (SDIE) larger than the $^{D}(V/K)$ 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 $D_2{}^{18}O$. 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 carbanion intermediate. (4) The solvent $^{D}(V/K)$ 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 up 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 ϵ_{280} = 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. (*S*)-3-Hydroxyacyl-CoA dehydrogenase was isolated from bovine liver according to the method of 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 solution (pH 6.6, 20% glycerol, 1 mM mercaptoethanol, 1 mM EDTA). Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (type XXIII), hexokinase (EC 2.7.1.1) from baker's yeast (type c-300), and esterase (EC 3.1.1.1) from porcine liver were from Sigma Chemical Co.

Chemicals. Diketene, deuterium oxide (99.96% ^{2}H, 0.5-mL glass vials), *trans*-crotonic anhydride, pentafluorobenzyl bromide, and dihydropyran were from Aldrich Chemical Co. D-Pantethine, 3′,5′-ADP, NADH, D-glucose 6-phosphate, ATP, and NAD^+ were from Sigma Chemical Co. Deuterium oxide (99.9 atom % ^{2}H) was from Cambridge Isotope Laboratories. [1-^{2}H]Glucose (98 atom % ^{2}H) was from Omicron Biochemicals. Unnormalized $D_2{}^{18}O$ (98 atom % ^{18}O) was from Icon Services, Inc. All other chemicals were of reagent grade and were used without further purification.

Thioester Substrates. *S*-Acetoacetyl-pantetheine (AcAc-P), HBP, CrP, [3-^{2}H]HBP, [3-^{2}H]CrP, and AcAc-CoA were synthesized as described by Bahnson and Anderson (1989). *trans*-Crotonyl-CoA (Cr-CoA) was synthesized by the reaction of coenzyme A (10 μmol) with *trans*-crotonic anhydride (50 μmol) in a solution of 2 mL of 0.5 M $NaHCO_3$ (pH 8.5) and 1 mL of EtOH. The solution was acidified to pH 4.0 with 1 M HCl and extracted three times with equal volumes of ethyl acetate. (*S*)-3-Hydroxybutyryl-CoA (HB-CoA) was formed by the crotonase-catalyzed hydration of Cr-CoA. The pantetheine thioesters were purified by HPLC on an 1.0 cm × 25.0 cm octadecylsilyl Econosphere (Alltech) reverse-phase column, and peaks were detected by UV absorbance at 232 nm. HBP was collected at 20 min when the column was eluted isocratically at 3 mL/min with 25% methanol. CrP was separated from an equilibrium with HBP when the column was eluted isocratically at 3 mL/min with 25% methanol for 20 min then eluted isocratically with 40% methanol and the peak was collected at 35 min. HB-CoA was purified by HPLC eluted isocratically at 3 mL/min on the same column as above with an 11% methanol and 10 mM sodium phosphate solution (pH 4.5). The HB-CoA peak was collected at 8–12 min. The Cr-CoA peak was collected at 17–20 min of isocratic elution with a 16% methanol and 10 mM sodium phosphate solution (pH 4.5).

Initial Velocities. 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 kinetic measurements. Initial velocity measurements were performed to compare the V_{max} for the crotonase-catalyzed reaction of HBP in the presence of saturating 3′,5′-ADP activator to the V_{max} of the physiological substrate Cr-CoA. Initial velocities were measured with the substrate HBP varied from 0.76 to 6.08 mM and the activator 3′,5′-ADP varied from 0.5 to 7 mM. Initial velocities of the Cr-CoA hydration were measured with a saturating substrate concentration of 150 μM. The reactions were followed at 280 nm [ϵ_{280} = 3600 M^{-1} cm^{-1}, ϵ_{263} = 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 is decreased. Assays were run in 0.1 M 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 7.00, 0.1 M ionic strength with K^+ salt and 1 mM EDTA). For measurements with HBP and 3′,5′-ADP activator, the enzyme concentration was estimated by UV absorbance to be 0.33 μM crotonase. This enzyme stock solution was accurately diluted 100-fold to 3.3 nM crotonase for kinetics measurements performed with the substrate Cr-CoA. Initial velocities were measured at saturating substrate

concentrations in order to directly compare the V_{max} values of HB-CoA relative to Cr-CoA. The reactions, monitored by UV absorbance at 280 nm, were run with 165 μM HB-CoA, 175 μM 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 $^{\alpha\text{-D}}(V/K)$ was measured for the hydration reaction in H_2O and D_2O with an approximately 50:50 mixture of [3-^{2}H]- and [3-^{1}H]CrP ([3-^{1}H/^{2}H]CrP). The secondary $^{\alpha\text{-D}}(V/K)$ for the hydration reaction run in H_2O is abbreviated as $^{\alpha\text{-D}}(V/K)_{H_2O}$, and the secondary $^{\alpha\text{-D}}(V/K)$ measured in D_2O is abbreviated as $^{\alpha\text{-D}}(V/K)_{D_2O}$. (2) The product distribution of [(*R*)-2-^{1}H/^{2}H]HBP for the hydration of CrP in 50:50 H_2O:D_2O was measured. (3) The isotope exchange of the 2-*R* proton and 3-hydroxy group of HBP with solvent was measured in $D_2{}^{18}O$. 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 μM 3′,5′-ADP as an activator in a total volume of 1.0 mL. Reactions were quenched by the addition of 100 μL 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 hydrolysis of HBP, which was performed under identical conditions, except in D_2O at pL 7.0. Preliminary experiments indicated that base hydrolysis of HBP at pH 10.5 showed a 1–5% exchange of the α-protons when performed in D_2O. The carboxylic acids were derivatized by adding a 10-fold excess of pentafluorobenzyl bromide. Ethanol (1 mL) was added as a cosolvent, and the reaction was stirred at 60 °C for 30 min. The pentafluorobenzyl esters were extracted into CH_2Cl_2 from the reaction mixture with two extractions and brought to dryness under a stream of nitrogen. In some cases the hydroxyl group of pentafluorobenzyl 3-hydroxybutyrate was protected by reaction with dihydropyran. The pentafluorobenzyl 3-hydroxybutyrate was dissolved in 1 mL of CH_2Cl_2 with 50 μL of dihydropyran and a catalytic amount of *p*-toluenesulfonic acid, and the solution was stirred at 25 °C for 1.5 h. The solution was extracted twice with 5% $NaHCO_3$ then brought to dryness under a nitrogen stream.

Mass Spectrometric Analysis of Isotopically Labeled Substrates. The isotope ratio analyses of the pentafluorobenzyl esters were performed with negative chemical ionization (NCI) mass spectrometry with selected ion monitoring (SIM). At a source temperature of 250 °C and a methane pressure of 1 Torr, the pentafluorobenzyl esters fragment to give predominantly a mass/charge (m/z) peak corresponding to the carboxylate fragment. A Hewlett Packard 5988A mass spectrometer system with a Hewlett Packard 5890 gas chromatograph was tuned in the NCI mode with perfluorotributylamine (PFTBA). The mass offset was adjusted to give peak widths of 0.3 atomic mass units. Pentafluorobenzyl 3-hydroxybutyrate was routinely injected into the GC and eluted isothermally at 130 °C for SIM measurements. A broad GC peak eluted between 5 and 6 min, which allowed many cycles of SIM per injection. When an isotope ratio of two m/z peaks was desired, for example the m and m+1 peak, 10 separate m/z settings differing by 0.05 m/z were selected for each ion and monitored for 5 ms to give a cycle time of under 160 ms. Figure 1 shows a scan selected from a SIM acquisition of a 1:1 mixture of pentafluorobenzyl [3-^{1}H]- and [3-^{2}H]-3-hydroxybutyrate. The non-Gaussian shape of the peaks is expected with quadrupole mass filters. When the isotope ratios of four separate isotopic peaks were desired, for example, the m–1, m, m+1, and m+2, then each peak was monitored at five m/z settings with a 0.05 m/z increment and a 5-ms dwell time. Cycle times remained under 160 ms. Isotope ratios were obtained by summing the intensities of the individual m/z settings and subsequently integrating across the entire GC peak after subtracting the baseline. Figure 2 shows an ion chromatogram that has been integrated over the entire GC peak for the m/z ion ranges 103.05–103.50 and 104.05–104.50 for a 1:1 mixture of the derivative of [3-^{1}H] and [3-^{2}H]HBP. In all cases, the m/z settings were centered around the peak intensity following a preliminary injection of the sample.

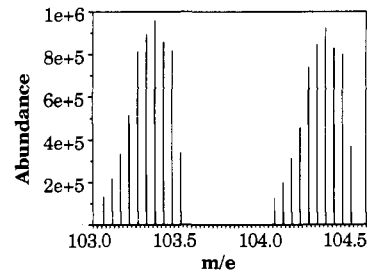


FIGURE 1: SIM scan of a 1:1 mixture of pentafluorobenzyl [3-^{1}H]- and [3-^{2}H]HBP-3-hydroxybutyrate. The m/m+1 ratio of this sample was determined by monitoring 10 separate ion ranges incremented by 0.05 m/z per m/z peak for 5 ms to give a cycle time of under 160 ms. The ion ranges were 103.05–103.50 and 104.05–104.50 for the m and m+1 peaks, respectively.

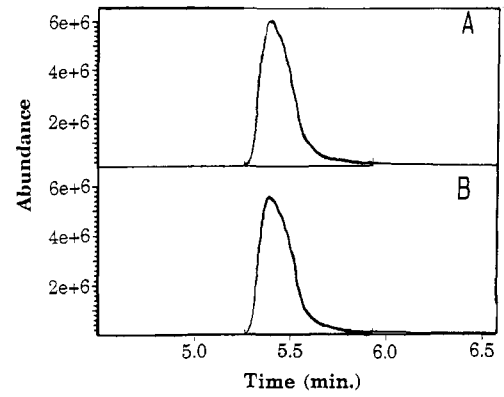


FIGURE 2: Ion chromatogram showing the GC peaks for the SIM acquisition described in the legend to Figure 1 of a 1:1 mixture of pentafluorobenzyl [3-^{1}H]- and [3-^{2}H]-3-hydroxybutyrate. The ion range 103.05–103.50 corresponds to the m peak and is shown in A. The ion range 104.05–104.50 corresponds 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 $^{\alpha\text{-D}}(V/K)$ Measured in H_2O and D_2O. The secondary $^{\alpha\text{-D}}(V/K)$ was measured from the crotonase-catalyzed hydration of [3-^{1}H/^{2}H]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_{eq} of 3.5 (Stern & del Campillo, 1956). To compare the measured secondary $^{\alpha\text{-D}}(V/K)_{H_2O}$, when a proton is transferred to the primary 2*R* position, to the secondary $^{\alpha\text{-D}}(V/K)_{D_2O}$, when a deuteron is transferred to the primary 2*R* position, the hydration reaction was run under identical conditions first in H_2O and then in D_2O. In a given experiment, the same batch of [3-^{1}H/^{2}H]CrP was used to ensure that an identical isotope ratio was present in the initial substrate. The concentration of [3-^{1}H]CrP was

matched to [3-^{2}H]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-^{1}H/^{2}H]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 D_2O were adjusted to a pL of 7.00 in order to compare the reactions run in H_2O at pH 7.00. The buffer was prepared by drying pH 6.37 MOPS (0.1 M, 1 mM EDTA) and resuspending it in D_2O. This solution was dried again to remove residual protons and resuspended in D_2O (glass vials, 99.96 atom % ^{2}H). Due to an isotope effect on the glass electrode, the measured pH_{app} of 6.6 corresponds to a pL of 7.00. The correction is given by eq 1 (Schowen & Schowen, 1982), where X_D is the mole fraction of deuterium in water.

$$pL = pH_{app} + X_D(0.4) \quad (1)$$

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-^{1}H/^{2}H]CrP was derivatized and the [3-^{1}H/^{2}H] ratio was determined by mass spectrometric scanning from 80–90 m/z. Following HPLC separation from the remaining CrP, the product [3-^{1}H/^{2}H]HBP was derivatized to its pentafluorobenzyl ester, and the mass spectrometric isotope ratio was determined. For the secondary $^{\alpha\text{-D}}(V/K)$ measured in H_2O, the m/z ion ranges 103.05–103.50 and 104.05–104.50 were scanned. For the secondary $^{\alpha\text{-D}}(V/K)$ measurement performed in D_2O, 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 D_2O 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*)-^{2}H,3-^{1}H/^{2}H]HBP.

Solvent Discrimination Isotope Effect in 50:50 H_2O:D_2O. 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 H_2O:D_2O at pL 7.00. The reactions were run with 5–10 mM CrP and monitored by UV spectroscopy at 308–312 nm. The pL 7.00 buffer was prepared by twice drying pH 6.69 MOPS (0.1 M, 1 mM EDTA) and resuspending in 50:50 H_2O:D_2O. The pH measured with a glass electrode was 6.80, which corresponds to a pL of 7.00 by eq 1. The 50:50 H_2O:D_2O was made by pipetting equimolar amounts of H_2O and D_2O (99.96 atom % ^{2}H) onto a Mettler digital balance and correcting by mass for the ratio of H_2O to D_2O. Following HPLC separation of the substrate CrP, the isotopic composition of the pentafluorobenzyl 3-hydroxybutyrate obtained from the product [(*R*)-2-^{1}H/^{2}H]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 dihydropyranylated 3-hydroxybutyrate, respectively.

Isotope Exchange of HBP in $D_2{}^{18}O$. The crotonase-catalyzed dehydration of HBP (5 mg) in $D_2{}^{18}O$ (98 atom % ^{18}O, unnormalized) was performed to measure the relative rates of exchange with solvent of the 2*R* proton and the 3-hydroxyl group. $D_2{}^{18}O$ was added to an evaporated buffer that was prepared identically to the buffer used to achieve a pL of 7.00 for the secondary $^{\alpha\text{-D}}(V/K)$ measurement in D_2O (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 ^{2}H and/or ^{18}O 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 ^{2}H, increases of the $m+2$ peak corresponded to exchange of only ^{18}O, and increases of the $m+3$ peak corresponded to exchange of both ^{2}H and ^{18}O 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 ^{1}H and ^{16}O present in the $D_2{}^{18}O$ solvent as described in the appendix.

Solvent $^D(V/K)$ by D_2O Equilibrium Overshoot. An overshoot was measured by UV absorbance at 263 nm for the crotonase-catalyzed dehydration reaction in D_2O. HBP (200 μM) and 3′,5′-ADP (51 μM) were suspended in buffered D_2O (pL 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 D_2O (25 μL, 100 nM final concentration, 25 °C). To describe the magnitude of the overshoot required the measurement of an initial absorbance (A_o), a maximum absorbance A_{max}, and the final equilibrium absorbance (A). The experimental overshoot will be reported as a fractional overshoot as in

$$\text{fractional overshoot} = (A_{max} - A)/(A - A_o) \quad (2)$$

Controls in H_2O were run to demonstrate the lack of an equilibrium overshoot in the absence of D_2O.

$^{\alpha\text{-}2}H$ Equilibrium Isotope Effect. The $^{\alpha\text{-D}}K_{eq}$ was measured from the crotonase-catalyzed reaction of [3-^{1}H/^{2}H]CrP (5 mg), which was fully equilibrated with [3-^{1}H/^{2}H]HBP. The reaction, monitored by UV absorbance at 308 nm, was equilibrated at 25 °C for 150 half-lives as determined from the initial exponential approach to equilibrium. The reaction was quenched with 100 μL of acetic acid, and the HBP and CrP were separated by HPLC. The $m/(m+1)$ isotope ratios of both pentafluorobenzyl esters were determined. Pentafluorobenzyl crotonate derived from [3-^{1}H/^{2}H]CrP was measured over the m/z ion ranges 84.30–84.50, 85.30–85.50, 86.30–86.50, and 87.30–87.50, which correspond to the $m-1$, m, $m+1$, and $m+2$ peaks, respectively. The pentafluorobenzyl ester derived from [3-^{1}H/^{2}H]HBP 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.

Theory

Secondary $^{\alpha\text{-D}}(V/K)$ Measured in H_2O and D_2O. α-Secondary isotope effects were measured by the determination of product isotope ratios at a small fraction of reaction. Enzyme-catalyzed hydration of a mixture of [3-^{1}H]- and [3-^{2}H]CrP is an example of the internal competition method that gives only a V/K isotope effect (Cleland, 1987). When measuring secondary isotope effects by product analysis, the ^{1}H/^{2}H isotope ratio of the product (R_p) relates to the $^D(V/K)$ with a dependence on the fraction of reaction (f) and the

$^1H/^2H$ isotope ratio of the initial substrate (R_o) by the following equation (Melander & Saunders, 1980).

$$^{D}(V/K) = \log (1 - f)/\log [1 - f(R_o/R_p)] \quad (3)$$

When f is very small, eq 3 simplifies to

$$^{D}(V/K) = R_p/R_o \quad (4)$$

Our interest is in the variation of $^{\alpha\text{-}D}(V/K)$ measured in H_2O and D_2O. 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 $^{\alpha\text{-}D}(V/K)$ measurements for the hydration reaction in H_2O and D_2O 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 $^{\alpha\text{-}D}(V/K)_{H_2O}/^{\alpha\text{-}D}(V/K)_{D_2O}$. Additionally, this ratio is independent of R_o since the initial substrate [3-^{1}H/^{2}H]CrP was made experimentally identical by division of the sample to perform the secondary $^{\alpha\text{-}D}(V/K)$ measurements in H_2O and then in D_2O.

The effect of substituting deuterium in a primary position on $^{\alpha\text{-}D}(V/K)$ has been experimentally determined for dehydrogenases (Hermes et al., 1984), on primary ^{13}C isotope effects (Hermes et al., 1982; Rendina et al., 1984), on primary ^{15}N isotope effects (Hermes et al., 1985), and on a primary deuterium isotope effects (Belasco et al., 1986a). To quantitatively interpret the effect of deuteration of 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 enzymic bases involved in the reaction. The measurement of the secondary $^{\alpha\text{-}D}(V/K)$ for the hydration reaction in H_2O and D_2O was designed to minimize the need to have information on the fractionation factor of the enzymic bases. To distinguish whether the secondary $^{\alpha\text{-}D}(V/K)$ and the primary $^{D}(V/K)$ occur in a concerted or stepwise manner, the effect on the $^{\alpha\text{-}D}(V/K)$ when deuterium is substituted in the primary position will be explored for both cases.

Secondary $^{\alpha\text{-}D}(V/K)$ Step Precedes $^{D}(V/K)$ Step. Equations 6–9 were derived from a kinetic model for a stepwise reaction as described in eq 5 (and more chemically depicted in Scheme IV) where A is CrP, P is HBP, and X is the carbanion intermediate.

$$E \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EX \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EP \overset{k_7}{\rightharpoonup} \quad (5)$$

$$^{D}(V/K)_A = \frac{^{D}k_5 + k_5/k_4(1 + k_3/k_2) + {}^{D}K_{eq}k_6/k_7}{1 + k_5/k_4(1 + k_3/k_2) + k_6/k_7} \quad (6)$$

$$^{\alpha\text{-}D}(V/K_A)_{H_2O} = \frac{^{\alpha\text{-}D}k_3 + k_3/k_2 + {}^{\alpha\text{-}D}K_{eq}(k_4/k_5)(1 + k_6/k_7)}{1 + k_3/k_2 + (k_4/k_5)(1 + k_6/k_7)} \quad (7)$$

$$^{\alpha\text{-}D}(V/K_A)_{D_2O} = \frac{^{\alpha\text{-}D}k_3 + k_3/k_2 + {}^{\alpha\text{-}D}K_{eq}(k_4/k_5)(^{D}k_5 + k_6/k_7{}^{D}K_{eq})}{1 + k_3/k_2 + (k_4/k_5)(^{D}k_5 + k_6/k_7{}^{D}K_{eq})} \quad (8)$$

The analysis assumes that k_3 and k_4 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 ^2H substitution at the primary position will only alter k_5 and k_6, where the C–H bond at C-2 is broken or formed. For a stepwise reaction, the substitution of ^2H at the primary position reduces both k_5 and k_6. The reduction in k_5 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., $k_7 > k_6$, the commitment increases by a factor of $^{D}k_5$. This increase in c_r makes the observed value of $^{\alpha\text{-}D}(V/K)_{D_2O}$ closer to $^{\alpha\text{-}D}K_{eq}$ than $^{\alpha\text{-}D}(V/K)_{H_2O}$. 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 $^{\alpha\text{-}D}(V/K)$ should reflect this shift in the rate-determining transition state. The predicted reduction of $^{\alpha\text{-}D}(V/K)_{D_2O}$ relative to $^{\alpha\text{-}D}(V/K)_{H_2O}$ can be calculated with equation 9[5] and from the known values of $^{\alpha\text{-}D}(V/K)_{H_2O}$, $^{\alpha\text{-}D}K_{eq}$, and $^{D}(V/K)$.

$$\frac{^{\alpha\text{-}D}(V/K_A)_{H_2O} - {}^{\alpha\text{-}D}K_{eq}}{^{\alpha\text{-}D}(V/K_A)_{D_2O} - {}^{\alpha\text{-}D}K_{eq}} = {}^{D}(V/K)_A \quad (9)$$

Secondary $^{\alpha\text{-}D}(V/K)$ and $^{D}(V/K)$ Steps Are Concerted. 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.

$$E \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EP \overset{k_5}{\rightharpoonup} \quad (10)$$

$$^{D}(V/K)_A = \frac{^{D}k_3 + c_f + {}^{D}K_{eq}c_r}{1 + c_f + c_r} \quad (11)$$

$$^{\alpha\text{-}D}(V/K_A)_{H_2O} = \frac{^{\alpha\text{-}D}k_3 + c_f + {}^{\alpha\text{-}D}K_{eq}c_r}{1 + c_f + c_r} \quad (12)$$

$$^{\alpha\text{-}D}(V/K_A)_{D_2O} = \frac{^{\alpha\text{-}D}k_3 + c_f/{}^{D}k_3 + {}^{\alpha\text{-}D}K_{eq}c_r{}^{D}K_{eq}/{}^{D}k_3}{1 + c_f/{}^{D}k_3 + c_r{}^{D}K_{eq}/{}^{D}k_3} \quad (13)$$

The forward and reverse commitments, c_f and c_r, correspond to k_3/k_2 and k_4/k_5, respectively. Deuterium substitution for the transferred proton slows down the chemical step, reducing c_f and c_r by ^{D}k and $^{D}k/{}^{D}K_{eq}$, respectively. For a concerted reaction, the value of $^{\alpha\text{-}D}(V/K)_{D_2O}$ will be closer to the intrinsic value, $^{\alpha\text{-}D}k_3$, 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 H_2O:D_2O. A SDIE can be determined by running a reaction to a small fractional conversion in 50:50 H_2O:D_2O. To obtain the measured SDIE the proteo/deutero product isotope ratio (R_p) is divided by the mole fraction ratio of H_2O to D_2O.

$$\text{SDIE} = R_p/(X_{H_2O}/X_{D_2O}) \quad (14)$$

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

$$\text{SDIE} = {}^{D}k_x'(E_{B:H}/E_{B:D}) \quad (15)$$

where $^{D}k_x'$ is the isotope effect on the net rate constant (Cleland, 1975) for converting the enzyme form represented by $E_{B:H}$ and $E_{B:D}$ (which are the fraction of enzyme where the protonated base will donate protium or deuterium to the substrate, respectively) to product. The ratio of $E_{B:D}$ to $E_{B:H}$ 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.,

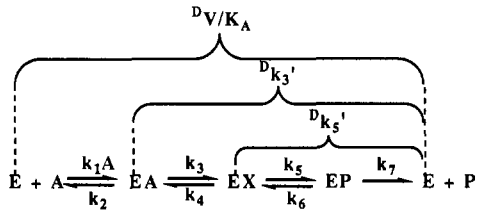
[5] This is most easily proven by substituting eqs 7 and 8 into the left side of eq 9 and simplifying to give eq 6.

Table I: Secondary $^{\alpha\text{-D}}(V/K)_{H_2O}/^{\alpha\text{-D}}(V/K)_{D_2O}$

sample[a]	correction	m/z isotope ratio	first expt[b]	second expt[b]
standard 3-hydroxybutyrate		$m-1/m$ (102/103)	0.0341 ± 0.0021 (3)	0.0110 ± 0.0001 (3)
[(*R*)-2-^{2}H,3-^{1}H/^{2}H]HBP from D_2O hydration		$m/m+1$ (103/104)	0.0615 ± 0.0040 (4)	0.0223 ± 0.0012 (3)
[(*R*)-2-^{2}H,3-^{1}H/^{2}H]HBP from D_2O hydration		$m+1/m+2$ (104/105)	0.8942 ± 0.0027 (8)	1.0660 ± 0.0050 (5)
[(*R*)-2-^{2}H,3-^{1}H/^{2}H]HBP from D_2O hydration	contaminating primary protons[c]	$m+1/m+2$ (104/105)	0.8676 ± 0.0061	1.0548 ± 0.0050
[3-^{1}H/^{2}H]HBP from H_2O hydration		$m/m+1$ (103/104)	0.8691 ± 0.0027 (10)	1.0586 ± 0.0038 (6)
		$^{\alpha\text{-D}}(V/K)_{H_2O}/^{\alpha\text{-D}}(V/K)_{D_2O}$[d] =	1.0017 ± 0.0061	1.0036 ± 0.0050

[a] The sample was derivatized to its pentafluorobenzyl ester. [b] The number of determinations is in parentheses, and the reported error is the standard deviation. [c] The $m+1/m+2$ ratio of the sample from the D_2O hydration was corrected for the contaminating primary proton contribution of the $m+1$ peak as described in the text. [d] The ratio of $^{\alpha\text{-D}}(V/K)_{H_2O}/^{\alpha\text{-D}}(V/K)_{D_2O}$, which is the important ratio for the interpretation of the chemical mechanism, was calculated by dividing the $m/m+1$ ratio of [3-^{1}H/^{2}H]HBP by the $m+1/m+2$ ratio of [(*R*)-2-^{2}H,3-^{1}H/^{2}H]HBP, which has been corrected for protons contaminating the deuterated primary position.

Scheme I



1987). Scheme I represents the SDIE measured, dependent on which enzyme form exchanges rapidly, assuming a fractionation factor of the enzymic base of unity. If k_7 is fast relative to k_6 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 isotope effect on the product ratio will be $^Dk_3'$. Finally, if the proton only exchanges rapidly from free enzyme, then the SDIE will be $^Dk_1'$, which is $^D(V/K)_A$.

Solvent $^D(V/K)$ by 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

$$dA/dT = -(V/K_A)A \quad (16)$$

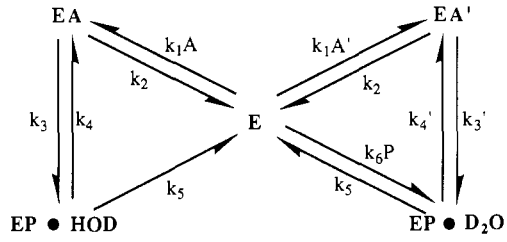
$$dA'/dT = [(V/K_A)'/K_{eq\text{-}D}][A^\circ - A - A'(K_{eq\text{-}D} + 1)] \quad (17)$$

where the pseudo-first-order rate constant for labeled substrate is $(V/K_A)'$, the initial substrate concentration is A°, and $K_{eq\text{-}D}$ is the equilibrium constant between the labeled substrate and product in D_2O. A numerical solution of the differential equations was obtained with an IBM AT computer using ASYSTANT software. By variation of the $^D(V/K)$, 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 $D_2{}^{18}O$.

Results

Initial Velocities. The relative V_{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_{max} value with Cr-CoA as the substrate was 6 ± 1 times larger than with HBP, which had saturating 3',5'-ADP. In a separate experiment, the relative V_{max} values of HB-CoA and Cr-CoA were measured. The V_{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_{max} values for the pantetheine or CoA substrates.

Scheme II



Secondary $^{\alpha\text{-D}}(V/K)$ in H_2O and D_2O. Mass spectrometric isotope ratio measurements were obtained to determine the effect that substituting deuterium for the transferred proton has on the secondary $^{\alpha\text{-D}}(V/K)$ and are reported in Table I. In order to make conclusions from this experiment, the isotope ratios of the product [(*R*)-2-^{2}H,3-^{1}H/^{2}H]HBP from the D_2O hydration and [3-^{1}H/^{2}H]HBP from the H_2O hydration reaction are the most important to compare. The critical ratio for interpreting the chemical mechanism is $^{\alpha\text{-D}}(V/K)_{H_2O}/^{\alpha\text{-D}}(V/K)_{D_2O}$; and, if life were simple, it would be calculated by dividing the $m/m+1$ ratio of [3-^{1}H/^{2}H]HBP formed during the hydration in H_2O by the $m+1/m+2$ ratio of [(*R*)-2-^{2}H,3-^{1}H/^{2}H]HBP formed during the hydration in D_2O. Since D_2O is not 100% ^{2}H, a correction needs to be made. This correction is described in the appendix. The key result shown in the bottom line of Table I is that $^{\alpha\text{-D}}(V/K)$ is not different in H_2O or D_2O, i.e., $^{\alpha\text{-D}}(V/K)_{H_2O}/^{\alpha\text{-D}}(V/K)_{D_2O}$ 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}H content in the D_2O. These two experimental improvements reduced the magnitude of the corrections required.

In order to calculate absolute values of $^{\alpha\text{-D}}(V/K)_{H_2O}$ and $^{\alpha\text{-D}}(V/K)_{D_2O}$ with eq 3, additional corrections need to be made to the $m+1/m+2$ ratio of the D_2O hydration product, the $m/m+1$ ratio of the H_2O hydration product, and the $m/m+1$ ratio of the initial substrate [3-^{1}H/^{2}H]CrP by subtracting the predicted contributions of the natural abundances of ^{13}C, ^{2}H, and ^{17}O to the $m+1$ and $m+2$ peaks. The corrections are made and the values of $^{\alpha\text{-D}}(V/K)_{H_2O}$ and $^{\alpha\text{-D}}(V/K)_{D_2O}$ are reported in Table II for comparison with the value determined previously by equilibrium perturbation.

Table II: Absolute Secondary $^{\alpha\text{-D}}(V/K)_{H_2O}$ and $^{\alpha\text{-D}}(V/K)_{D_2O}$

sample[a]	correction	m/z isotope ratio	first expt[b]	second expt[b]
[3-^{1}H/^{2}H]CrP initial substrate		m/m+1 (85/86)	1.001 ± 0.01 (2)	1.249 ± 0.01 (2)
[3-^{1}H/^{2}H]CrP initial substrate	natural abundance[c]	m/m+1 (85/86)	1.048 ± 0.01	1.323 ± 0.01
[(*R*)-2-^{2}H,3-^{1}H/^{2}H]HBP from D_2O hydration	natural abundance[d]	m+1/m+2 (104/105)	0.9079 ± 0.0027	1.1112 ± 0.0050
[3-^{1}H/^{2}H]HBP from H_2O hydration	natural abundance[c]	m/m+1 (103/104)	0.9073 ± 0.0027	1.1158 ± 0.0038
		$^{\alpha\text{-D}}(V/K)_{D_2O}$ =	0.863 ± 0.01[e]	0.838 ± 0.01[e]
		$^{\alpha\text{-D}}(V/K)_{H_2O}$ =	0.863 ± 0.01[f]	0.841 ± 0.01[f]

[a] The sample was derivatized to its pentafluorobenzyl ester. [b] The number of determinations is in parentheses, and the reported error is the standard deviation. [c] The ^{13}C, ^{2}H, and ^{17}O natural abundance contribution to the m+1 peak was subtracted. [d] The ^{13}C, ^{2}H, and ^{17}O natural abundance contribution to the m+2 peak was subtracted. [e] The $^{\alpha\text{-D}}(V/K)_{D_2O}$ was calculated from the corrected m+1/m+2 ratio of [(*R*)-2-^{2}H,3-^{1}H/^{2}H]HBP and the corrected m/m+1 ratio of [3-^{1}H/^{2}H]CrP by using eq 3. [f] The $^{\alpha\text{-D}}(V/K)_{H_2O}$ was calculated from the corrected m/m+1 ratios of [3-^{1}H/^{2}H]HBP and [3-^{1}H/^{2}H]CrP by using eq 3.

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

sample[a]	m/z	isotope abundance relative to largest peak[b]	isotope abundance corrected for natural abundance[b,c]
standard 3-hydroxybutyrate (2)	m−1 (102)	0.00187 ± 0.00004	
	m (103)	1	
	m+1 (104)	0.04623 ± 0.00025	
	m+2 (105)	0.00752 ± 0.00011	
[(*R*)-2-^{1}H/^{2}H]HBP alcohol unprotected (6)	m−1 (102)	0.00403 ± 0.00010	
	m (103)	1	1
	m+1 (104)	0.6612 ± 0.0018	0.6150 ± 0.0020
	m+2 (105)	0.03737 ± 0.00022	
			^{2}H KIE = 1.618 ± 0.005[d]
[(*R*)-2-^{1}H/^{2}H]HBP alcohol protected by dihydropyran (2)	m−1 (186)	0.00130 ± 0.00018	
	m (187)	1	1
	m+1 (188)	0.7415 ± 0.0138	0.627 ± 0.014
	m+2 (189)	0.07034 ± 0.00279	
			^{2}H KIE = 1.59 ± 0.03[d]

[a] The sample was derivatized to its pentafluorobenzyl ester. The number of determinations is in parentheses. [b] The reported error is the standard deviation. [c] The ^{13}C, ^{2}H, and ^{17}O natural abundance contribution to the m+1 peak was subtracted. [d] 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 dervatives showed a considerable fragmentation peak that corresponds to the elimination of H_2O. There are potentially both an α-secondary and primary isotope effect on elimination in the mass spectrometer. We attempted to detect both of these effects. To detect an α-secondary effect, the [3-^{1}H/^{2}H]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 α-secondary isotope effect in the gas-phase elimination in the mass spectrometer source.

The presence of a large primary ^{2}H 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 ^{2}H 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-^{2}H]HBP did not differ significantly from 1.0 after correction for natural abundance ^{13}C, indicating the gas-phase elimination has no preference for threo 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-^{1}H/^{2}H]HBP catalyzed by crotonase was measured in 50:50 H_2O:D_2O. The product m−1, m, m+1, and m+2 isotope abundances relative to the m peak of [(*R*)-2-^{1}H/^{2}H]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, ^{2}H, and ^{17}O using the m/m+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/z 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-^{1}H/^{2}H]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 $D_2{}^{18}O$

sample[a]	m/z	isotope abundance relative to largest peak[b]	isotope abundance corrected for natural abundance[b,c]	isotope abundance corrected for rehydration of CrP[d]
standard 3-hydroxybutyrate (6)	m−1 (102)	0.00185 ± 0.00010		
	m (103)	1		
	m+1 (104)	0.04544 ± 0.00033		
	m+2 (105)	0.00741 ± 0.00009		
HBP exchanged in $D_2{}^{18}O$ to 20% reaction (6)	m (103)	1	1	
	m+1 (104)	0.05822 ± 0.00023	0.01278 ± 0.00056	<0.004
	m+2 (105)	0.05342 ± 0.00017	0.04543 ± 0.00059	<0.012
	m+3 (106)	0.10469 ± 0.00027	0.10253 ± 0.00083	0.000
HBP exchanged in $D_2{}^{18}O$ to isotopic equilibrium (6)	m (103)	0.06266 ± 0.00051	0.06364 ± 0.00052	
	m+1 (104)	0.08480 ± 0.00064	0.08323 ± 0.00117	
	m+2 (105)	0.32963 ± 0.00030	0.33054 ± 0.00178	
	m+3 (106)	1	1	

[a] The sample was derivatized to its pentafluorobenzyl ester. The number of determinations is in parentheses. [b] The reported error is the standard deviation. [c] The ^{13}C, ^{2}H, and ^{17}O natural abundance contribution to the m+1, m+2, and m+3 peaks was subtracted. [d] The correction applied is described in the appendix and assumes all of the m+3 peak arises from rehydration of CrP.

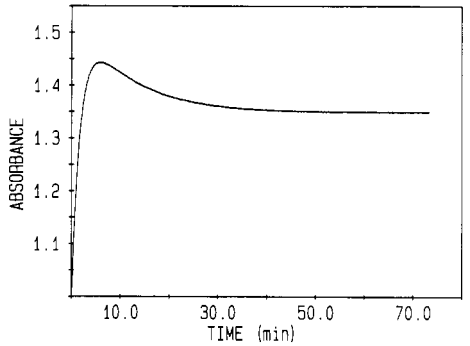


FIGURE 3: Spectrophotometric time course of an equilibrium overshoot for the crotonase-catalyzed dehydration of HBP (200 μM) in D_2O in the presence of 51 μM 3′,5′-ADP. Crotonase (100 nM) was added at time = 0 to a temperature-equilibrated solution, and the absorbance at 263 nm was monitored.

scribed in the appendix had to be made. The object of this isotope exchange experiment was to detect the potential exchange of the abstracted C-2 proton with solvent beyond that explained by simple rehydration of previously formed CrP. By adding the fraction of HBP that had exchanged only the C-2 proton to its standard deviation, the maximum possible exchange of 0.7% was obtained. The numerical integration indicated that 23% of the initial HBP has undergone conversion to CrP. The ratio 0.7/23 = 0.03 reflects the maximum rate of exchange of the C-2 proton relative to reaction. We do not believe that we have unequivocally identified exchange in the absence of reaction. In fact, due to potential errors in the numerical estimation of the extent of reaction, it is possible that the ratio is orders of magnitude smaller than the calculated maximum of 0.03 but not significantly larger.

Solvent $^{D}(V/K)$ by D_2O Equilibrium Overshoot. The solvent $^{D}(V/K)$ was measured by allowing crotonase to catalyze the dehydration of HBP in D_2O and measuring the resulting equilibrium overshoot. A typical equilibrium overshoot is shown in Figure 3. Equilibrium overshoots measured had a fractional overshoot, as defined in eq 2, of 21.2 ± 0.4%. The estimated errors are derived from an estimated error of ±0.002 absorbance unit for each measured absorbance. Duplicate overshoots were obtained within the indicated error limits. To obtain a value of $^{D}(V/K)$ from the fractional overshoot required an estimate of the $K_{eq\text{-}D}$ between [(*R*)-2-^{2}H]HBP and CrP in D_2O. The $K_{eq\text{-}D}$ value for the dehydration reaction was calculated to be 0.33 from a $^{D}K_{eq}$ value of 0.88 for the transfer of ^{2}H from [3-^{2}H]ketoglutarate to D_2O (Cleland, 1980) and a K_{eq} of 0.29 for the crotonase-catalyzed dehydration. The theoretical fractional overshoot was matched to the experimental value of 21.2% with a $K_{eq\text{-}D}$ 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 $K_{eq\text{-}D}$ of 0.33 and a $^{D}(V/K)$ of 1.3 had a fractional overshoot of only 10%.

Table V: α-^{2}H Equilibrium Isotope Effect

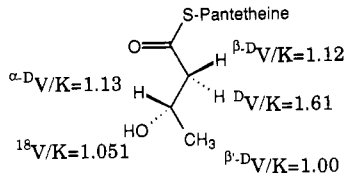
sample[a]	m/z	isotope abundance relative to m peak[b]	isotope abundance corrected for natural abundance[b,c]
standard 3-hydroxybutyrate (3)	m−1 (102)	0.00749 ± 0.00012	
	m (103)	1	
	m+1 (104)	0.04715 ± 0.00057	
	m+2 (105)	0.00739 ± 0.00009	
[3-^{1}H/^{2}H]HBP (6)	m−1 (102)	0.00804 ± 0.00031	
	m (103)	1	1
	m+1 (104)	1.04326 ± 0.00520	0.9962 ± 0.0058
	m+2 (105)	0.0558 ± 0.0004	
[3-^{1}H/^{2}H]CrP (6)	m−1 (84)	0.00575 ± 0.00038	
	m (85)	1	1
	m+1 (86)	0.79156 ± 0.00991	0.7451 ± 0.0105
	m+2 (87)	0.0449 ± 0.0006	
			$^{\alpha\text{-}D}K_{eq}$ = 1.337 ± 0.011[d]

[a] Each sample was derivatized to its pentafluorobenzyl ester. The number of determinations is in parentheses. [b] The reported error is the standard deviation. [c] The ^{13}C, ^{2}H, and ^{17}O natural abundance contribution to the m+1 peak was subtracted. [d] $^{\alpha\text{-}D}K_{eq}$ was calculated from the ratio of the corrected m+1/m of [3-^{1}H/^{2}H]HBP and [3-^{1}H/^{2}H]CrP.

α-^{2}H Equilibrium Isotope Effect. The m/z abundances at m−1, m, m+1, and m+2 isotope abundances relative to the m peak for unlabeled 3-hydroxybutyrate or crotonate are reported in Table V for the [3-^{1}H/^{2}H]HBP and [3-^{1}H/^{2}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 substract ^{13}C, ^{2}H, and ^{17}O natural abundance contributions to the m+1 peak of the [3-^{1}H/^{2}H]HBP sample. The m+1 peak of the [3-^{1}H/^{2}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-hydroxybutyrate. The ratio of the m and m+1 corrected isotope abundances of [3-^{1}H/^{2}H]HBP and [3-^{1}H/^{2}H]CrP gave an $^{\alpha\text{-}D}K_{eq}$ of 1.34 ± 0.01. This value is consistent with the estimated $^{\alpha\text{-}D}K_{eq}$ value of 1.33, which was obtained 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 agreement of the measured primary and secondary kinetic isotope effects with the values previously measured by equilibrium perturbation and the agreement of the secondary

Scheme III



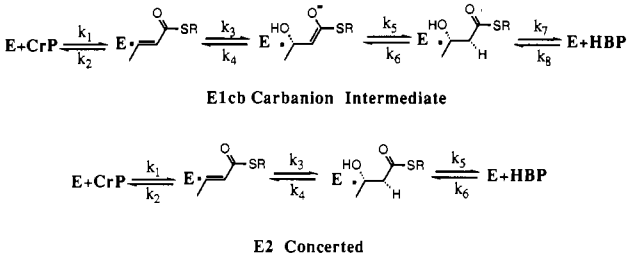
$^{\alpha\text{-D}}K_{eq}$ with the calculated value serve to validate the described whole molecule isotope ratio experimental protocol. The negative ion chemical ionization analysis of pentafluorobenzyl esters has overcome two common drawbacks to isotope ratio measurements. The electron capture, carboxylate expulsion 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 (E1cb) reactions predominate when a carbanion can be stabilized β to the leaving group. Gandler and Jencks (1982) have argued that there is a distinct transition from an E2 to an E1cb 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 E1cb to E2 promoted by the activation of the leaving group by ring strain. The strained lactone ring of 11-oxatricyclo[4.4.3.0^{1,6}]tridecan-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 thioesters is, Does the enzyme predominantly activate the proton for abstraction resulting in the E1cb mechanism, or has the leaving group been activated as well, resulting in a concerted mechanism?

In a previous study we reported the primary $^{D}(V/K)$ and $^{18}(V/K)$ and secondary $^{\alpha\text{-D}}(V/K)$, $^{\beta\text{-D}}(V/K)$, and $^{\beta'\text{-D}}(V/K)$ for the crotonase-catalyzed elimination of HBP (Bahnson & Anderson, 1989). These results are summarized in Scheme III and are consistent with an E1cb 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 $^{\beta'\text{-D}}(V/K)$ significantly greater than unity. The micromolar binding to crotonase of the enol(ate) form of AcAc-CoA (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 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_ms for the pantetheine substrates are over 100-fold greater than the K_ms 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 $^{\alpha\text{-D}}(V/K)_{H_2O}/^{\alpha\text{-D}}(V/K)_{D_2O}$ *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 $^{\alpha\text{-D}}(V/K)$ for the hydration reaction in H_2O and D_2O should provide a distinction between a concerted or stepwise reaction.

The isotope effects for the crotonase-catalyzed dehydration of HBP, shown in Scheme III, are only consistent with a carbanion stepwise or concerted mechanism with carbanion character (Bahnson & Anderson, 1989). The primary $^{18}(V/K)$ and $^{D}(V/K)$ 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.[6] Figure 4 qualitatively illustrates the predicted relationship between the $^{\alpha\text{-D}}(V/K)_{D_2O}$ and the $^{\alpha\text{-D}}(V/K)_{H_2O}$ for the carbanion 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_2O has an α-secondary sensitive transition state that is less rate limiting due to the primary ^2H label, resulting in the $^{\alpha\text{-D}}(V/K)_{D_2O} < {}^{\alpha\text{-D}}(V/K)_{H_2O}$. For the concerted mechanism, in which there is only one isotopically sensitive transition state, deuterium

[6] 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.

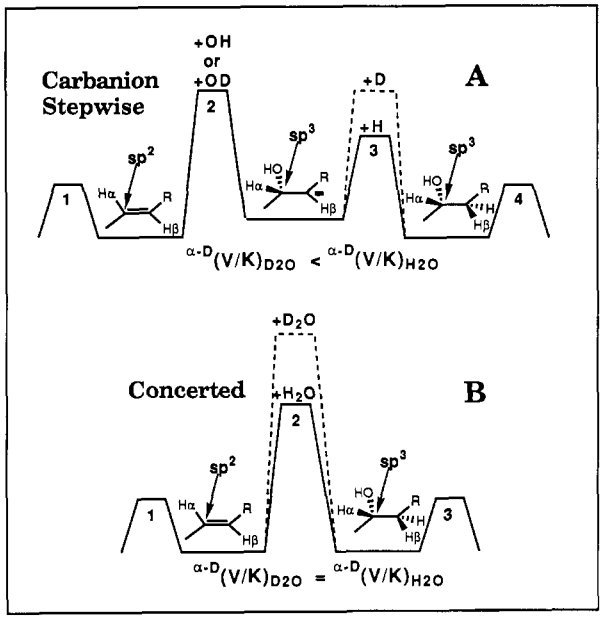


FIGURE 4: Qualitative free energy diagram for a carbanion stepwise (A) and a concerted (B) mechanism for the crotonase-catalyzed hydration of CrP to HBP. The dashed line in each mechanism denotes the effective increased free energy barrier introduced by deuterium substitution. (A) The transition states for C–O (2) and C–H (3) bond formation are rate limiting, and those for substrate association (1) and product dissociation (4) are kinetically insignificant. The α-secondary sensitive transition state (2) is slightly more rate limiting than the C–H bond-forming transition state.[5,7] When the hydration reaction is run in D_2O, the α-secondary sensitive transition state becomes less rate limiting, resulting in ${}^{\alpha\text{-D}}(V/K)_{D_2O} < {}^{\alpha\text{-D}}(V/K)_{H_2O}$. (B) In the concerted mechanism, there is only one isotopically sensitive transition state (2). Deuterium substitution will only make this step more rate limiting, resulting in ${}^{\alpha\text{-D}}(V/K)_{D_2O} = {}^{\alpha\text{-D}}(V/K)_{H_2O}$.

substitution can only make the chemical step more rate limiting, resulting in no change in ${}^{\alpha\text{-D}}(V/K)$.

For the carbanion stepwise mechanism, shown in Scheme IV, the predicted reduction of the ${}^{\alpha\text{-D}}(V/K)_{D_2O}$ can be calculated from eq 9 with the known values of ${}^{\alpha\text{-D}}(V/K)_{H_2O}$, ${}^{\alpha\text{-D}}K_{eq}$, and ${}^{D}(V/K)$ for the hydration of CrP, of 0.85, 0.75 and 1.6, respectively. The calculated value of ${}^{\alpha\text{-D}}(V/K)_{D_2O}$ for a stepwise mechanism is 0.813 compared to a ${}^{\alpha\text{-D}}(V/K)_{H_2O}$ value of 0.850. The ratio, ${}^{\alpha\text{-D}}(V/K)_{H_2O}/{}^{\alpha\text{-D}}(V/K)_{D_2O}$, for the carbanion intermediate mechanism would be 1.046. The experimental ${}^{\alpha\text{-D}}(V/K)_{H_2O}/{}^{\alpha\text{-D}}(V/K)_{D_2O}$ ratios of 1.0017 ± 0.0061 in the first experiment and 1.0036 ± 0.0050 in the second experiment (Table I) are the *strongest evidence against the existence of an intermediate*. As discussed under Theory, the ratio of unity requires that there be a single kinetically significant transition state. A concerted elimination predicts this result and is fully consistent with all of the measured isotope effects.

We have discounted the possibility that the single rate-determining transition state reflects the C–O bond cleavage of a carbanion intermediate, while the C–H bond forming step is much faster (i.e., $k_5 \gg k_4$ in eq 5) for two reasons. First, the primary ${}^{D}(V/K)$ of 1.6 would have to be an equilibrium isotope effect, requiring the fractionation factor of the abstracted proton to be ca. 0.6. In proline racemase where the abstracted proton bound to the base was determined to have such a low fractionation factor, the base was assumed to be a cysteine thiolate (Belasco et al., 1986b). Detailed studies on the inactivation of crotonase with thiol reagents indicate that there is not an essential cysteine residue (Waterson et al., 1972). Second, no precedent exists for the ${}^{\beta\text{-D}}(V/K)$ exceeding the equilibrium isotope effect in a reversible deprotonation stepwise elimination ($E1cb_{rev}$), while it has been observed and theoretically predicted for a concerted elimination (Amin et al., 1988).

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 ${}^{\alpha\text{-D}}(V/K)$ 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 ${}^{\alpha\text{-D}}(V/K)_{NAD}$ 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 carbanion stepwise 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 ${}^{\alpha\text{-D}}(V/K)_{D_2O}$ 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 ${}^{\alpha\text{-D}}(V/K)_{H_2O}$ and ${}^{\alpha\text{-D}}(V/K)_{D_2O}$ 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 carbanion mechanism would still result in equivalent values of ${}^{\alpha\text{-D}}(V/K)_{H_2O}$ and ${}^{\alpha\text{-D}}(V/K)_{D_2O}$. Qualitatively, if a primary intrinsic solvent KIE on the C–O bond forming step existed and was comparable to the ${}^{D}k$ for the C–H bond forming step, then the partitioning of a carbanion intermediate would be the same in H_2O as in D_2O. 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 carbanion intermediate unaffected by the substitution of D_2O. Quantitatively this can be shown in eq 18 by first simplifying eq 8 for the case where there are no external commitments, i.e., $k_2 \gg k_3$ and $k_7 \gg k_6$.

$$ {}^{\alpha\text{-D}}(V/K_A)_{D_2O} = \frac{{}^{\alpha\text{-D}}k_3 + {}^{\alpha\text{-D}}K_{eq}\,{}^{D}k_5(k_4/k_5)}{1 + {}^{D}k_5(k_4/k_5)} \qquad (18) $$

The c_r for the C–O bond-forming step is increased in D_2O by a factor of ${}^{D}k_5$. An intrinsic solvent KIE on C–O bond

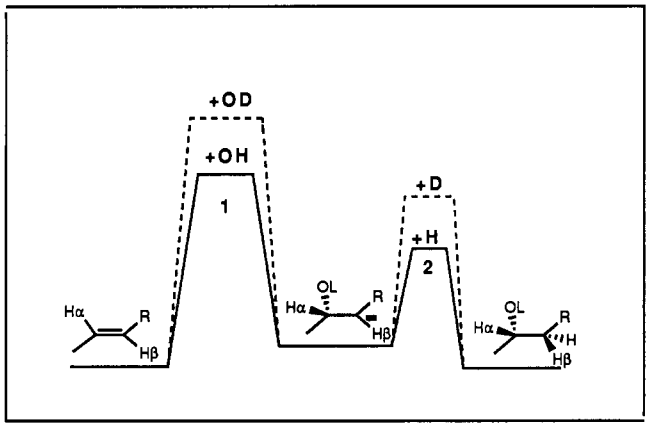


FIGURE 5: Free energy diagram illustrating a hypothetical situation for a carbanion stepwise mechanism where a primary intrinsic solvent KIE on the C–O bond-forming step (transition state 1) existed and was comparable to the intrinsic ^{D}k for the C–H bond-forming step (transition state 2). The presence of an intrinsic solvent KIE in transition state 1 makes this step more rate limiting compared to the C–H bond-forming step, thus reducing the measured primary $^{D}(V/K)$.

cleavage would reduce the c_r by the factor $^{D(c\text{-}o)}k_4$ as shown by

$$^{\alpha\text{-}D}(V/K_A)_{D_2O} = \frac{^{\alpha\text{-}D}k_3 + {}^{\alpha\text{-}D}K_{eq}\,{}^{D}k_5(k_4/k_5)/{}^{D(c\text{-}o)}k_4}{1 + {}^{D}k_5(k_4/k_5)/{}^{D(c\text{-}o)}k_4} \quad (19)$$

where $^{D(c\text{-}o)}k_4$ is the intrinsic ^{2}H solvent KIE on the C–O bond cleavage step. When $^{D}k_5$ is equal to $^{D(c\text{-}o)}k_4$, $^{\alpha\text{-}D}(V/K)_{D_2O}$ is equal to $^{\alpha\text{-}D}(V/K)_{H_2O}$ 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 $k_7 \gg k_6$.

$$^{D}(V/K)_A = \frac{^{D}k_5 + k_5/k_4}{1 + k_5/k_4} \quad (20)$$

The presence of a large $^{D(c\text{-}o)}k_4$ would significantly reduce the measured $^{D}(V/K)$ according to

$$^{D}(V/K)_A = \frac{^{D}k_5 + {}^{D(c\text{-}o)}k_4(k_5/k_4)}{1 + {}^{D(c\text{-}o)}k_4(k_5/k_4)} \quad (21)$$

An estimation can be made of the $^{D}(V/K)$ for a stepwise mechanism, when $^{D}k_5$ is equivalent to $^{D(c\text{-}o)}k_4$ and minimum values of $^{D}k_5 = 3.1$ 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 $^{\alpha\text{-}D}(V/K)_{H_2O}$ to equal the $^{\alpha\text{-}D}(V/K)_{D_2O}$ for the carbanion stepwise mechanism.

[7] Minimum values of ^{D}k and k_5/k_4 were obtained for the stepwise mechanism by assigning ^{18}k to 1.072, which is the largest observed ^{18}O effect (Blanchard & Cleland, 1980), and estimating the internal commitments that would reduce the ^{18}k to the measured $^{18}(V/K)$ of 1.051 (Bahnson & Anderson, 1989).

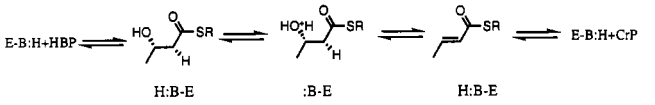
Solvent Discrimination Isotope Effect. Proton exchange of an enzyme–substrate or an enzyme–intermediate complex can be probed by measuring solvent KIEs in mixed H_2O:D_2O solutions. Yamada and O'Leary (1977) measured a hydrogen isotope discrimination in 50:50 H_2O:D_2O of the proton transfer that follows 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., $^{D}k_5$ 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 α–β unsaturated nitriles, and by microscopic reversibility the elimination from β-cyano thioethers, 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 $D_2{}^{18}O$. The isotope exchange of HBP in $D_2{}^{18}O$ 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 β-eliminations are not unprecedented. Dohner and Saunders (1986) identified syn eliminations from standard 2,2-diarylethyl 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_β-H bond being cleaved to a greater extent than the C_α leaving group bond. Extensive studies at the boundary between E1cb 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^+H_2 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

Scheme V



would be shorter than a single vibration of the C–O^+H_2 bond. Fishbein and Jencks (1988) estimated expulsion rates for substituted thiophenolate ions α to nitrile-stabilized carbanions and concluded that they range from $10^{9.7}$ to $10^{12.4}$ s^{-1}, just slower than the bond vibrational frequency of ca. 3×10^{13} s^{-1}. We expect that the rate constant for elimination of H_2O whose pK_a is from 4 to 10 pH units lower than the thiophenolate 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_a is over 8 pH units greater than the poorest thiophenolate 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 enol(ate) intermediate would be negligible.

The microscopic reverse of an elimination proceeding after a preequilibrium protonation is the addition of neutral H_2O to the α–β unsaturated thioester. The attack of the poor nucleophile is possible in the crotonase reaction because of the enhanced electrophilicity of the β-carbon in α–β unsaturated thioesters relative to α–β unsaturated carboxylates. This electrophilic property of α–β unsaturated CoA esters may be more important to the crotonase mechanism than the better recognized enhanced acidity of the α-protons (Bruice & Benkovic, 1966).

Appendix

Corrections for Isotope Ratios Used To Determine $^{\alpha\text{-D}}(V/K)$. The primary difficulty is correcting for the presence of [3-^{2}H,2-^{1}H]HBP because of the presence of a significant amount of protium in the D_2O. This is shown in eqs A1 and A2, which indicate the contributing factors to the lighter isotopic species for hydration in H_2O and D_2O, respectively,

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

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

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_2O hydration product and the $m{-}1/m$ 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/z peaks by the mass spectrometer (line 1, Table I). If there were no ^{1}H in the D_2O, the $m/m{+}1$ ratio for the sample hydrated in D_2O (line 2, Table I) 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-^{2}H,2-^{1}H]HBP. Note that the corrected value (line 4, Table I) 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-^{2}H,2-^{1}H]hydroxybutyrate, the correction to the $m{+}1$ peak of the D_2O hydration product was solved iteratively according to

$$1 = X_i(A - B)C = X_{i+1} \quad \text{(A3)}$$

where X_i is the $m{+}1$ peak of the D_2O hydration product that was initially assigned a value of 1.0, A is the $m/m{+}1$ isotope ratio of the D_2O 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_2O hydration product. The value X_{i+1} was substituted into the X_i term of eq A3 during each iterative cycle until X_i equaled X_{i+1} to four significant figures. The corrected $m{+}1/m{+}2$ isotope ratio of the D_2O hydration product was calculated by multiplying the X_i term by the uncorrected $m{+}1/m{+}2$ isotope ratio of the D_2O hydration product and is shown in line 4, Table I. We did not add the correction to the $m{+}2$ peak, because there is an analogous contribution to the m peak from [3-^{1}H,2-^{1}H]HBP that would then have to be added to the $m{+}1$ peak.

Corrections for ^1H *and* ^{16}O *in* $D_2{}^{18}O$. 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 $D_2{}^{18}O$, the reaction is made irreversible by loss and dilution of the initial ^{16}O at C-3. Unfortunately, the $D_2{}^{18}O$ 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_2O. The ^{13}C contribution was determined by the $m{+}1/m$ ratio of standard 3-hydroxybutyrate and directly subtracted from the $m{+}1/m$ ratio for the 23% reaction sample. The correction for hydration of CrP with D_2O 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 $D_2{}^{18}O$. A calculated amount of CrP hydrated by D_2O 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 $m{+}1/m$ 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_2O.

References

Amin, M., Price, R. C., & Saunders, W. H., Jr. (1988) *J. Am. Chem. Soc. 110*, 4085.

Anderson, V. E. (1981) Ph.D. dissertation, University of Wisconsin, Madison.

Anderson, V. E. (1991) in *Enzyme mechanism from isotope effects* (Cook, P. F., Ed.) CRC Press, Cleveland, OH.

Anderson, V. E., Bahnson, B. J., Wlassics, I. D., & Walsh, C. T. (1990) *J. Biol. Chem. 265*, 6255–6261.

Bach, R. D., Badger, R. C., & Lang, T. J. (1979) *J. Am. Chem. Soc. 101*, 2845.

Bahnson, B. J., & Anderson, V. E. (1989) *Biochemistry 28*, 4173–4181.

Belasco, J. G., Albery, W. J., & Knowles, J. R. (1983) *J. Am. Chem. Soc. 105*, 2475–2477.
Belasco, J. G., Albery, W. J., & Knowles, J. R. (1986a) *Biochemistry 25*, 2552–2558.
Belasco, J. G., Bruice, T. W., Albery, W. J., & Knowles, J. R. (1986b) *Biochemistry 25*, 2558–2564.
Blanchard, J. S., & Cleland, W. W. (1980) *Biochemistry 19*, 4506.
Bruice, T. C., & Benkovic, S. (1966) *Bioorganic Mechanisms*, Vol. 1, p 294, Benjamin, New York.
Cardinale, G. J., & Abeles, R. H. (1968) *Biochemistry 7*, 3970.
Clark, J. D., O'Keefe, S. J., & Knowles, J. R. (1988) *Biochemistry 27*, 5961.
Cleland, W. W. (1975) *Biochemistry 14*, 3220.
Cleland, W. W. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) p 252, University Park Press, Baltimore, MD.
Cleland, W. W. (1980) *Methods Enzymol. 64*, 104.
Cleland, W. W. (1982) *CRC Crit. Rev. Biochem. 13*, 385–428.
Cleland, W. W. (1987) *Biorg. Chem. 15*, 303–327.
Cook, D. (1976) *J. Org. Chem. 41*, 2173–2179.
Cook, P. F., Oppenheimer, N. J., & Cleland, W. W. (1981) *Biochemistry 20*, 1817.
Dewar, M. J. S., & Dieter, K. M. (1988) *Biochemistry 27*, 3302.
Dohner, B. R., & Saunders, W. H., Jr. (1986) *J. Am. Chem. Soc. 108*, 245.
Fedor, L. R. (1967) *J. Am. Chem. Soc. 89*, 4479.
Fedor, L. R. (1969) *J. Am. Chem. Soc. 91*, 913.
Fishbein, J. C., & Jencks, W. P. (1988) *J. Am. Chem. Soc. 110*, 5075–5086.
Gandler, J. R., & Jencks, W. P. (1982) *J. Am. Chem. Soc. 104*, 1937–1951.
Hass, G. M., & Hill, R. L. (1969) *J. Biol. Chem. 244*, 6080.
Hermes, J. D., Roeske, C. A., O'Leary, M. H., & Cleland, W. W. (1982) *Biochemistry 21*, 5106–5114.
Hermes, J. D., Morrical, S. W., O'Leary, M. H., & Cleland, W. W. (1984) *Biochemistry 23*, 5479–5488.
Hermes, J. D., Weiss, P. M., & Cleland, W. W. (1985) *Biochemistry 24*, 2959–2967.
Huskey, W. P., & Schowen, R. L. (1983) *J. Am. Chem. Soc. 105*, 5704.
Kim, S. C., & Raushel, F. M. (1986) *Biochemistry 25*, 4744.
Kresge, A. J. (1964) *Pure Appl. Chem. 8*, 243–258.
Kurz, L. C., & Frieden, C. (1980) *J. Am. Chem. Soc. 102*, 4198–4203.
Lynen, F., & Ochoa, S. (1953) *Biochim. Biophys. Acta 12*, 299.
Marshall, D. R., Thomas, P. J., & Stirling, C. J. M. (1977) *J. Chem. Soc., Perkin Trans. 2*, 1914–1919.
Mayer, B. J., Spencer, T. A., & Onan, K. D. (1984) *J. Am. Chem. Soc. 106*, 6343–6348.
Melander, L., & Saunders, W. H., Jr. (1980) in *Reaction Rates of Isotopic Molecules*, Wiley, New York.
More-O'Ferral, R. A., & Warren, P. J. (1975) *J. Chem. Soc., Chem. Commun.*, 483–484.
Murfin, W. W. (1974) Ph.D. Thesis, Washington University, St. Louis, MO.
Northrop, D. B. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) p 122, University Park Press, Baltimore, MD.
Nuiry, I. I., Hermes, J. D., Weiss, P. M., Chen, C.-Y., & Cook, P. F. (1984) *Biochemistry 23*, 5168.
Person, N. B. (1981) Ph.D. Dissertation, State University of New York at Buffalo.
Pohl, B., Raichle, T., & Ghisla, S. (1986) *Eur. J. Biochem. 160*, 109–115.
Reinsh, J., Katz, A., Wean, J., Aprahamian, G., & McFarland, T. J. (1980) *J. Biol. Chem. 19*, 9093–9097.
Rendina, A. R., Hermes, J. D., & Cleland, W. W. (1984) *Biochemistry 23*, 6257.
Robert, R. D., Ferran, H. E., Jr., Gula, M. J., & Spencer, T. A. (1980) *J. Am. Chem. Soc. 102*, 7054.
Saunders, W. H., Jr. (1985) *J. Am. Chem. Soc. 107*, 164.
Schowen, K. B., & Schowen, R. L. (1982) *Methods Enzymol. 87*, 551–606.
Staack, H., Binstock, J. F., & Schulz, H. (1978) *J. Biol. Chem. 253*, 1827.
Steinman, H. M., & Hill, R. L. (1973) *J. Biol. Chem. 248*, 892–900.
Steinman, H. M., & Hill, R. L. (1975) *Methods Enzymol. 35*, 136–151.
Stern, J. R., & del Campillo, A. (1956) *J. Biol. Chem. 218*, 985.
Stubbe, J., & Abeles, R. H. (1980) *Biochemistry 19*, 5505.
Waterson, R. M., & Hill, R. L. (1972) *J. Biol. Chem. 247*, 5258–5265.
Waterson, R. M., Hass, G. M., & Hill, R. L. (1972) *J. Biol. Chem. 247*, 5252–5257.
Weiss, P. M., Boerner, R. J., & Cleland, W. W. (1987) *J. Am. Chem. Soc. 109*, 7201–7202.
Willadsen, P., & Eggerer, H. (1975) *Eur. J. Biochem. 54*, 247.
Wlassics, I. D., & Anderson, V. E. (1989) *Biochemistry 28*, 1627–1633.
Yamada, H., & O'Leary, M. H. (1977) *J. Am. Chem. Soc. 99*, 1660.