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A Kinetic Isotope Effect and Isotope Exchange Study of the Nonenzymatic and the Equine Serum Butyrylcholinesterase-Catalyzed Thioester Hydrolysis

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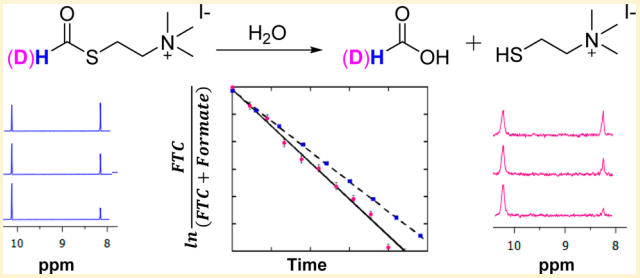
Supporting Information

ABSTRACT: Formylthiocholine (FTC) was synthesized and found to be a substrate for nonenzymatic and butyrylcholinesterase (BChE)-catalyzed hydrolysis. Solvent (D_2O) and secondary formyl-H kinetic isotope effects (KIEs) were measured by an NMR spectroscopic method. The solvent (D_2O) KIEs are ^{D_2O}k = 0.20 in 200 mM HCl, ^{D_2O}k = 0.81 in 50 mM HCl, and ^{D_2O}k = 4.2 in pure water. The formyl-H KIEs are ^{D}k = 0.80 in 200 mM HCl, ^{D}k = 0.77 in 50 mM HCl, ^{D}k = 0.75 in pure water, ^{D}k = 0.88 in 50 mM NaOH, and $^{D}(V/K)$ = 0.89 in the BChE-catalyzed hydrolysis in MES buffer at pH 6.8. Positional isotope exchange experiments showed no detectable exchange of ^{18}O into the carbonyl oxygen of FTC or the product, formate, under any of the above conditions. Solvent nucleophile-O KIEs were determined to be ^{18}k = 0.9917 under neutral conditions, ^{18}k = 1.0290 (water nucleophile) or ^{18}k = 0.989 (hydroxide nucleophile) under alkaline conditions, and $^{18}(V/K)$ = 0.9925 for BChE catalysis. The acidic, neutral, and BChE-catalyzed reactions are explained in terms of a stepwise mechanism with tetrahedral intermediates. Evidence for a change to a direct displacement mechanism under alkaline conditions is presented.

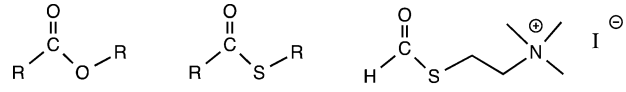


INTRODUCTION

Thioesters are energy-rich acyl groups that are of considerable importance in both organic chemistry and biochemistry. In organic chemistry, the reactive C−S bond serves as a gateway to other acyl groups. In biochemistry, the combination of thioesters with nucleophiles encompasses reactions as diverse as thioester exchange (acetyl transferases), Claisen condensations (thiolases), and hydrolysis (thioesterases).[1,2] These enzyme-catalyzed reactions are vital to fatty acid biosynthesis,[3] polyketide and nonribosomal peptide synthesis,[4,5] control of levels of acyl CoAs,[6,7] and S-palmitoylation.[8−10]

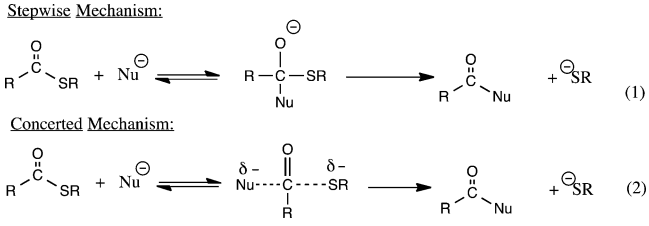
The reactivity of thioesters has been compared to the more thoroughly investigated oxoesters. Certain thioesters have been shown to be 100 times more reactive than oxoesters toward amine- and thiol-based nucleophiles but up to 2000 times more reactive than oxoesters toward carbanion nucleophiles.[11−15] The difference in reactivity between oxo and thioesters is thought to be a result of the lower resonance stabilization of thioesters, which stems from suboptimal overlap of the larger 3p orbital of sulfur with the smaller 2p orbital of the carbonyl-C.[11] On the other hand, the alkaline hydrolysis rates of certain oxoesters and thioesters have been found to be surprisingly similar. This difference in reactivity has yet to be fully explained mechanistically.

Three general mechanisms have been proposed for the reaction of thioesters with nucleophiles, two of which are



Oxoester **Thioester** **Formylthiocholine (FTC)**

shown in eqs 1 and 2. On the basis of the results of classic physical organic experiments, the most likely mechanism for



(1)

(2)

this reaction is believed to be that of eq 1.[16−19] Permutations of this mechanism take into account the differing charges on the tetrahedral intermediates as a function of pH. The concerted (or direct displacement) mechanism in eq 2 has been proposed for a leaving group that is exceptionally reactive, but evidence for this type of mechanism is relatively rare.[20,21] The third

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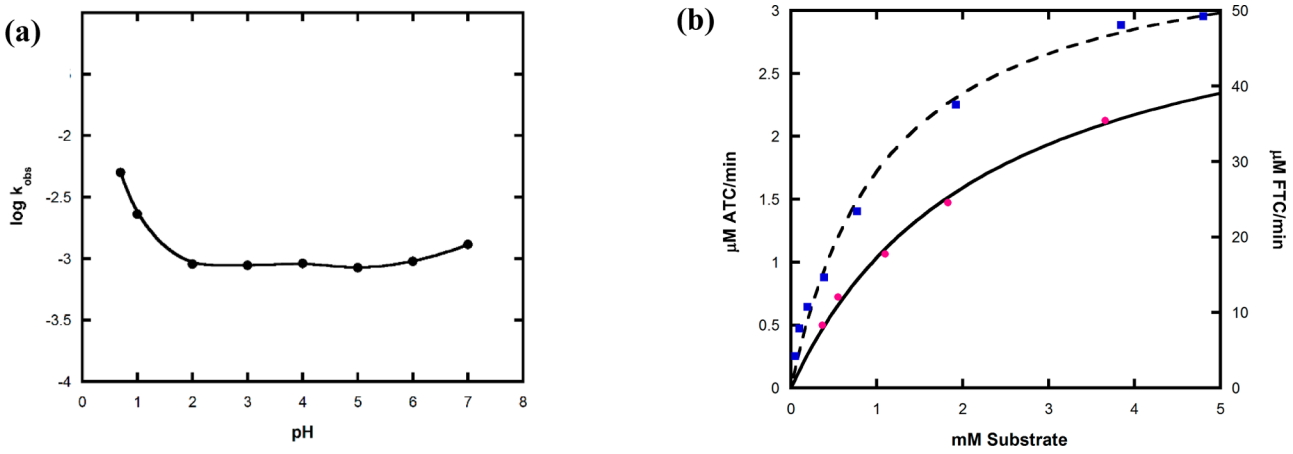

Figure 1. (a) Plot of log k_{obs} vs pH for the nonenzymatic hydrolysis of FTC at 25 °C. (b) Michaelis–Menten curves for BChE-catalyzed hydrolysis of ATC (blue squares) and FTC (red circles). The lines represent fits to the Michaelis–Menten equation.

mechanism (not shown) is an elimination–addition mechanism. This mechanism cannot operate in the present case because formyl thioesters lack the requisite hydrogen atom on the α-carbon.[22]

The pH rate profile for the hydrolysis of alkyl thioesters (where the leaving group is an alkylthiol) shows several distinct regions. Typically the rate is dependent on the hydroxide concentration above pH 7 and shows a plateau in neutral to mildly acidic regions. Under more highly acidic conditions, however, differing behaviors have been reported. Bruice and others have studied acyl-activated thioesters, such as ethyl trifluorothioacetate, and found that hydrolysis was inhibited by high concentrations of acid (pH < 2).[19] On the other hand, thioesters without acyl-activated groups (such as thioesters of acetic and formic acid) display acid-catalyzed hydrolysis below pH 2.[17] Schmir has studied a range of acyl groups and determined that hydrolysis switches relatively smoothly from acid inhibition for the most highly electron-withdrawing acyl groups to acid catalysis for the least electron-withdrawing acyl groups.[18] This was attributed to a change in the ratio of rate constants for breakdown of the neutral tetrahedral intermediate to give products and reactants.

Positional isotope exchange (PIX) and kinetic isotope effect (KIE) experiments have played an important role in the elucidation of the mechanism of thioester hydrolysis. Bruice measured the single-solvent (D_2O) KIE on the hydrolysis of ethyl trifluorothioacetate for the uncatalyzed "water reaction" (^{D_2O}k = 3.0). This experiment aided in establishing the commonly held mechanism for the water reaction, in which one water molecule catalyzes the attack of a second water molecule on ethyl trifluorothioacetate. A later proton inventory study on this reaction by Hogg was consistent with Bruice's proposed mechanism for the water reaction.[23] In addition, carbonyl-^{18}O PIX experiments by Bender demonstrated that ethyl trifluorothioacetate exchanged ^{18}O from the carbonyl-O into solvent during hydrolysis below pH 5.[24] These results added to the growing evidence that thioester hydrolysis follows a stepwise mechanism. Bruice also determined the single-solvent KIE for the hydroxide-promoted reaction (^{D_2O}k = 0.78).[19] This result supported the idea of direct attack by hydroxide under alkaline conditions.

The present paper reports a mechanistic investigation of the hydrolysis of formylthiocholine (FTC) using isotope effects and isotope exchange as probes. The measurement of KIEs is one of the best methods to obtain a detailed bonding picture of the transition state. This is true because isotopic substitution has a very modest effect on the rate of reaction, and this in turn rarely results in changing the natural mechanism. Other physical organic methods, such as Hammett σ–ρ correlations, often result in very large rate changes that can induce a change in the mechanism one is attempting to investigate. Our substrate, FTC, also has structural/physical advantages over the acetyl thioesters discussed in the preceding paragraphs. First, FTC is water-soluble and does not require cosolvents. Second, there are established analytical procedures for measuring KIEs for all of the atoms at the chemically reactive site of this thioester. Finally, FTC is a substrate for enzyme-catalyzed hydrolysis by butyrylcholinesterase (BChE), which allows for comparison of the organic reaction mechanism for hydrolysis to one of biological origin.

RESULTS

Synthesis. The synthesis of FTC was carried out in two steps (see the Supporting Information). The first step involved the formylation of 2-(*N*,*N*-dimethylamino)ethanethiol hydrochloride with acetic formic anhydride to give formyl 2-(*N*,*N*-dimethylamino)ethanethiol hydrochloride (FDC) in 95% crude yield. Since the formyl group of FDC proved too labile in aqueous potassium carbonate, FDC was deprotonated using Hünig's base immobilized on polystyrene resin followed by methylation with iodomethane to give a 27% overall crude yield of FTC. The crude FTC was crystallized from ethanol (70% recovery). This synthetic scheme was also adapted to make 1-*d*-FTC and 1-^{13}C-FTC using 1-*d*-formic acid and ^{13}C-formic acid, respectively.

Nonenzymatic pH–Rate Profile. A ^{1}H NMR spectroscopic method was used to measure the rate constant for FTC hydrolysis as a function of pH (from pH 0.5 to pH 7.0). The formyl-H resonances of both FTC and formate (or formic acid) were integrated during the course of the reaction. The value of k_{obs} was determined from a plot of the logarithm of the fraction of FTC remaining versus time. The pH–log k_{obs} profile (Figure 1a) shows two distinct regions: a water-catalyzed reaction (pH ~2 to pH 7) and an acid-catalyzed reaction (below pH ~2). The reaction above pH 7 is extremely fast, and the rate constant could not be measured with equipment available in this lab. However, on the basis of the results for other thioesters (as well as oxygen esters), the reaction is most likely first-order in

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hydroxide. All of the rate constant values are given in units of min^{-1}. One can roughly compare the magnitudes of k_{obs} for FTC and methyl thioformate during the plateau regions of the plots of log k_{obs} versus pH. Between pH 3.6 and 4.6, the magnitude of k_{obs} for FTC is approximately 0.00090 min^{-1}. In that same region, the k_{obs} for methyl thioformate[17] is approximately 0.00025 min^{-1}. However, it must be noted that methyl thioformate required nonaqueous cosolvents, whereas FTC was freely soluble in water.

BChE Kinetics. The kinetic parameters of the BChE-catalyzed hydrolysis of acetylthiocholine (ATC) and FTC were determined at pH 6.8 in MES buffer. The rate of hydrolysis of ATC was measured by means of a continuous assay at 324 nm that uses 2,2′-dithiodipyridine (DTDP) to monitor the production of thiocholine. The rate of FTC hydrolysis was monitored with a point assay using 5-(3-carboxy-4-nitrophenyl)disulfanyl-2-nitrobenzoic acid (DTNB). Because the extinction coefficient of TNB^- varies with buffer composition and pH, the extinction coefficient was determined to be 12.6 mM^{-1} cm^{-1} at pH 6.8 in 50 mM MES buffer. The value of K_m for ATC was determined to be 1.1 ± 0.2 mM, and that for FTC was 2.3 ± 0.3 mM. The k_{cat} for ATC was (8.1 ± 0.4) × 10^2 min^{-1}, and that for FTC was (1.1 ± 0.1) × 10^4 min^{-1}. The k_{cat} was calculated from the activity data provided by the manufacturer. The Michaelis–Menten plots of the kinetic data are given in Figure 1b.

Solvent KIEs. The solvent KIEs for hydrolysis under neutral and acidic conditions were determined by direct measurement of the rate constants using an ^1H NMR spectroscopic assay in which the relative fractions of FTC and formate were determined by integration of their respective formyl-H resonances. The pL (pL = pH or pD) was adjusted to a common value at the start of the reaction. The rate constants were determined as described in the Nonenzymatic pH–Rate Profile section above. The relative fractions of FTC and formate were determined by integration of their respective formyl-H resonances. An example of this NMR assay is given in the Supporting Information, and an example of the kinetic plot to determine the solvent KIE is shown in Figure 2. All of the KIEs measured in this work are summarized in Table 1. The alkaline hydrolysis of FTC was too rapid to be followed by instrumentation available in this lab. Because the measurement of all solvent KIEs requires the direct method, the solvent KIE for alkaline hydrolysis could not be determined.

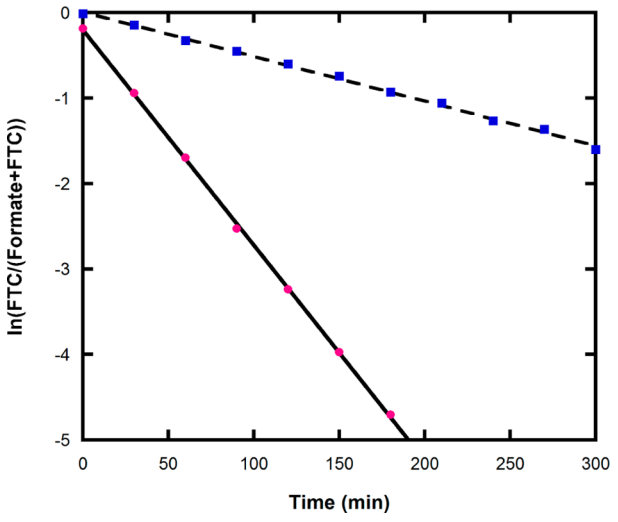

Figure 2. Solvent isotope effect for FTC hydrolysis in 200 mM HCl (blue squares) and 200 mM DCl (red circles).

Formyl-H KIEs. The formyl-H KIEs under neutral and acidic conditions were determined by direct measurement of the rate constants for H-FTC and D-FTC. Measurement of the rate constants utilized the NMR spectroscopic assay described above. The only difference was the use of ^2H NMR spectroscopy to measure the rate of D-FTC hydrolysis. An example of this NMR assay is given in the Supporting Information. An example of the kinetic plot to determine the formyl-H KIE is given in Figure 3, and the KIEs are summarized in Table 1.

The formyl-H KIEs for the alkaline hydrolysis and BChE-catalyzed hydrolysis required the competitive method (not direct measurement of the individual rate constants). In the alkaline case this was due to the high rate of the reaction; in the enzymatic case this was due to the uncertainty in measuring the exact amount of enzyme required for preparation of the separate solutions of H-FTC and D-FTC. In the competitive method, H-FTC and D-FTC were present in a single reaction mixture. The extents of the reactions of H-FTC and D-FTC were measured by ^1H NMR and ^2H NMR spectroscopy, respectively. These rate ratios were converted into the observed KIEs using published equations.[25] The KIEs are summarized in Table 1.

Nucleophile-O KIEs. Determination of the nucleophile-O KIE requires three experimentally determined isotope ratios: (a) the $^{18}\delta(O)$ of the original carbonyl-O of FTC, (b) the $^{18}\delta(O)$ of the water used in the experiment, and (c) the average $^{18}\delta(O)$ of the oxygen atoms of formate, which is the product of hydrolysis. The definition of $^{18}\delta(O)$ is given by eq 3,

$$^{18}\delta(O) = [(R_S - R_{std})/R_{std}] \times 1000 \quad (3)$$

in which R_s is the isotope ratio of the sample (i.e., $^{18}O/^{16}O$) and R_{std} is the same isotope ratio for standard CO_2. The value of $^{18}\delta(O)$ represents the per mil difference between the sample and a tank standard. A positive (+) value of $^{18}\delta(O)$ means the sample is enriched in ^{18}O compared with the standard; a negative (−) value means it is depleted in the heavier isotope. The determination of $^{18}\delta(O)$ for part (a) was accomplished using a modified published procedure involving the conversion of FTC (instead of methyl formate) to CO, followed by conversion of CO to CO_2 and measurement of the $^{18}\delta(O)$ of the resulting CO_2 by isotope-ratio mass spectrometry (IRMS).[26] Because the conversion of FTC to CO_2 is quantitative, this method preserves the isotopic composition of the carbonyl-O of FTC (Scheme 1a). The determination of $^{18}\delta(O)$ for part (b) utilized the published procedure involving exchange of oxygen from water into a limiting amount of CO_2 followed by measurement of the $^{18}\delta(O)$ by IRMS.[27] Using known fractionation factors, the $^{18}\delta(O)$ for water (and hydroxide for alkaline reactions) can be calculated.[28] The determination of $^{18}\delta(O)$ for part (c) was accomplished by complete hydrolysis of FTC to formate under the specified reaction conditions. Because this hydrolysis is quantitative and there is no exchange of ^{18}O from the solvent into the carbonyl-O, the measured $^{18}\delta(O)$ for the CO_2 derived from formate represents an average for the two oxygen atoms in the original formate. This is called $^{18}\delta(O)_{obs}$. One of these oxygen atoms in formate is derived from the original carbonyl-O of FTC, and its $^{18}\delta(O)$ is called $^{18}\delta(O)_{C=O}$; the other oxygen is from the

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Table 1. Positional Isotope Exchange (PIX), Solvent (D and ^{18}O) KIEs, and Formyl-H KIEs on the Hydrolysis of FTC in Aqueous Solutions at 25 °C and an Ionic Strength of 200 mM

conditions[a]	solvent KIE (^{D_2O}k)	formyl-H KIE (^{D}k)	nucleophile-O KIE (^{18}k)[b]	PIX (k_h/k_e)[c]
200 mM HCl	0.20 ± 0.02	0.80 ± 0.02	n.d.	>25
50 mM HCl	0.81 ± 0.02	0.77 ± 0.02	n.d.	>25
neutral H_2O	4.2 ± 0.02	0.75 ± 0.02	0.9917 ± 0.0009	>25
alkaline	n.d.	0.88 ± 0.02	1.029 ± 0.001 (H_2O) 0.989 ± 0.001 (HO^-)	>25
BChE-catalyzed	n.d.	0.89 ± 0.02	0.9925 ± 0.0008	>25

[a]The solvent KIE (^{D_2O}k) and formyl-H KIE (^{D}k) were obtained from triplicate experiments. [b]The nucleophile-O KIE (^{18}k) was obtained from at least 4 determinations. [c]PIX results were obtained from a single determination.

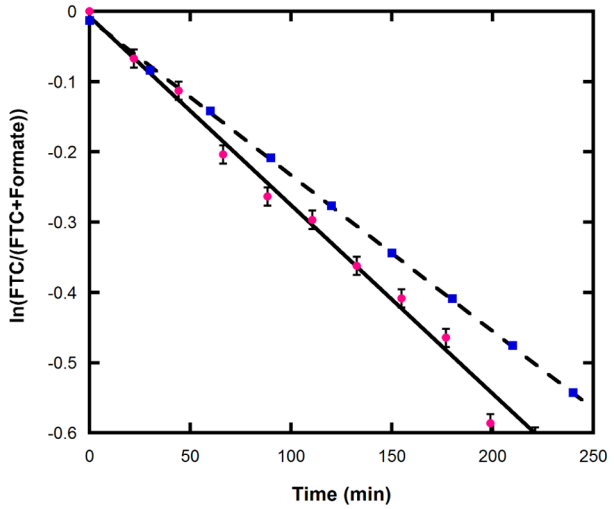
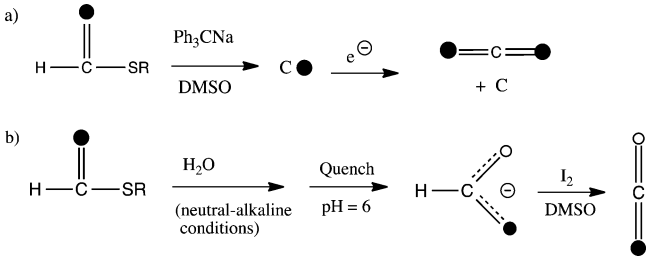


Figure 3. Formyl-H kinetic isotope effect in 50 mM HCl (ionic strength = 200 mM). The rates of hydrolysis of H-FTC (blue squares) and D-FTC (red circles) are shown.

Scheme 1. (a) Measurement of $\delta(^{18}O)$ of the Carbonyl-O of FTC; (b) Measurement of $^{18}\delta(O)$ for Formate, Where One Oxygen Atom Is Derived from the Solvent (○) and the Other from the Carbonyl-O of FTC (●)



nucleophile (either water or hydroxide), and its $^{18}\delta(O)$ is called $^{18}\delta(O)_{Nu}$ (see Scheme 1b). The relationship between the measured $^{18}\delta(O)$ values is given by eq 4:

$$^{18}\delta(O)_{obs} = (0.5)[^{18}\delta(O)_{C=O}] + (0.5)[^{18}\delta(O)_{Nu}] \quad (4)$$

Since $^{18}\delta(O)_{obs}$ and $^{18}\delta(O)_{C=O}$ are known, $^{18}\delta(O)_{Nu}$ can be calculated.

Once $^{18}\delta(O)_{Nu}$ has been calculated, the KIE for the nucleophile can be determined from the difference between $^{18}\delta(O)_{Nu}$ and either $^{18}\delta(O)_{H_2O}$ (water nucleophile) or $^{18}\delta(O)_{OH}$ (hydroxide nucleophile). This relationship holds because water, as the solvent, can be considered as an infinite pool of nucleophilic oxygen atoms with an unchanging isotope ratio. The measured $^{18}\delta(O)$ values for these calculations are given in Table 2. As an example, the calculation of the KIE for alkaline hydrolysis of FTC proceeds as follows: $^{18}\delta(O)_{obs}$ is −48.7, and $^{18}\delta(O)_{C=O}$ is −28.5. From eq 4, $^{18}\delta(O)_{Nu}$ is −68.9. If hydroxide is the nucleophile [$^{18}\delta(O)_{OH}$ = −79.9], the KIE would be (−68.9) − (−79.9) = +11.0 per mil. Since the oxygen atom that enters formate from the nucleophile becomes heavier by 11.0 per mil, the KIE is an inverse one (i.e., ^{18}k = 0.989). If water is the nucleophile [$^{18}\delta(O)_{H_2O}$ = −39.9], the KIE would be ^{18}k = 1.0290. It is important to note that the nucleophile-O KIE could not be measured for reactions under acidic conditions (either 50 or 200 mM HCl) because the product is predominantly formic acid, which exchanges oxygen with the solvent under these conditions. Under neutral and BChE-catalyzed conditions, water is the only possible nucleophile because of the low concentration of hydroxide.

Carbonyl-^{18}O Exchange (PIX). A ^{13}C NMR spectroscopic method based on the effect of the ^{18}O isotope shift on the ^{13}C NMR resonance was used to detect exchange of ^{18}O from water into the carbonyl-O of formate (Scheme 2a).[26] The ^{13}C NMR method was chosen over a more traditional mass spectrometry approach such as GCMS or LCMS because FTC can undergo hydrolysis under the conditions utilized for these methods. By

Table 2. Measured Isotopic Compositions [$\delta^{18}(O)$] for Calculation of the Nucleophile-O KIEs

$^{18}\delta(O)$ of FTC carbonyl-O	$^{18}\delta(O)$ of formate			$^{18}\delta(O)$ of CO_2 exchanged with water	$^{18}\delta(O)$ H_2O (calcd)[a]	$^{18}\delta(O)$ OH^- (calcd)[b]
	neutral hydrolysis	alkaline hydrolysis	BCHE hydrolysis			
−28.4	−29.4	−48.7	−30.8	+1.2	**−39.9**	**−79.9**
−28.1	−30.0	−48.5	−30.3	+1.0		
−29.1	−30.5	−49.4	−30.0	**+1.1**[d]		
−28.3	−30.2	−48.0	−30.4	**(range = 0.1)**		
−28.8	**−30.0 ± 0.5**[c]	**−48.7 ± 0.6**[c]	**−30.4 ± 0.3**[c]			
−28.5 ± 0.4[c]						

[a]From ref 27. [b]From ref 28. [c]Average and standard deviation of all trials. [d]Average of two determinations.

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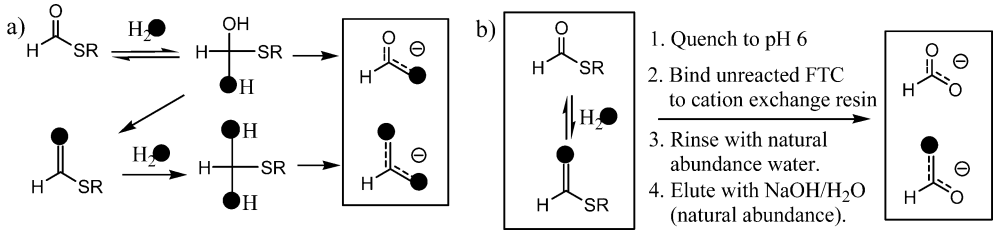
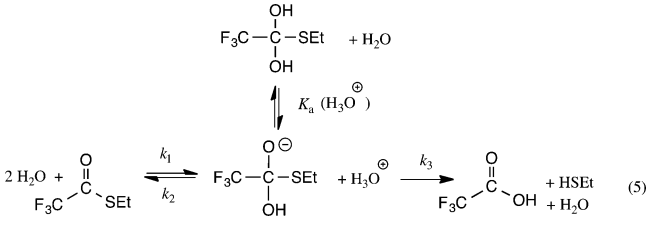
Scheme 2. (a) Analysis of the Extent of ^{18}O Exchange into the Product (Formate) under Conditions Where Formate Does Not Exchange ^{18}O with Water; (b) Analysis of the Extent of ^{18}O Exchange into FTC under Conditions Where the Product, Formic Acid, Exchanges ^{18}O with Water



necessity, the ^{13}C NMR method required measurement of isotope exchange into the product (formate) rather than into the starting thioester because the ^{13}C resonance of FTC was too broad to give baseline separation of the isotope-shifted peaks. The value of T_1 for the carbonyl-C of formate was found to be 10 s. Consequently, a delay time of 60 s was used to ensure quantitative integrations of the isotope-shifted peaks. The fact that formic acid is known to exchange ^{18}O with water under acidic conditions introduced another complication. This problem was overcome by binding the FTC (after partial hydrolysis in ^{18}O-water) to a strong cation-exchange resin and washing off the residual $H_2{}^{18}O$ with a large volume of natural-abundance water. FTC was eluted (and hydrolyzed) with NaOH (where no exchange occurs either during or after hydrolysis). The resulting formate was analyzed by ^{13}C NMR spectroscopy. This procedure is shown in Scheme 2b. In the final analysis, there was no detectable exchange of ^{18}O from water into the carbonyl of FTC under any of the conditions studied. It is estimated that the ^{13}C NMR spectroscopic method would have been able to detect a k_h/k_e (the ratio of the rate of hydrolysis to exchange) of 25 or less. Therefore, in this work the ratio is reported to be $k_h/k_e > 25$.

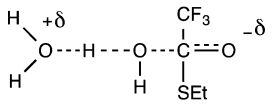
DISCUSSION

Pioneering studies by Fedor and Bruice showed that the hydrolysis of thioesters with electron-withdrawing acyl groups (such as trifluorothioacetate) has three distinct regions between pH 0 and 14: (a) above pH 7, the reaction is first-order in hydroxide; (b) in the region between pH ∼2 to pH 7, the reaction is likely water-catalyzed utilizing two water molecules; and (c) in the more strongly acidic region from pH 0 to 2, the hydrolysis is subject to acid inhibition. Single-solvent KIEs were found to be $^{D_2O}k = 2.97$ for the water reaction and $^{D_2O}k = 0.80$ for the reaction with hydroxide.[19] This and additional kinetic evidence argued strongly for a stepwise mechanism involving tetrahedral intermediates.[16] The mechanism proposed by Bruice in acidic and neutral media is essentially that given in eq 5.

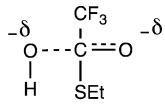


(5)

Bender investigated the exchange of ^{18}O from the carbonyl-O of ethyl trifluorothioacetate into the solvent (water) and found that the ratio of the rate constant for hydrolysis (k_h) to that for exchange (k_e) ranges from $k_h/k_e = 2.5$ at pH 1.1 to no detectable exchange above pH 5.[24] Hogg and Venkatasubban investigated the hydrolysis of ethyl trifluorothioacetate under mildly acidic conditions (0.001 M acid) using the proton inventory technique and concluded that under those conditions the likely mechanism involves the water-catalyzed attack of water on the thioester, leading to the transition state shown as structure **I**.[23] Although a proton inventory under basic conditions was not reported, the inverse solvent effect would be consistent with no protons in flight and a transition state that would resemble structure **II**.

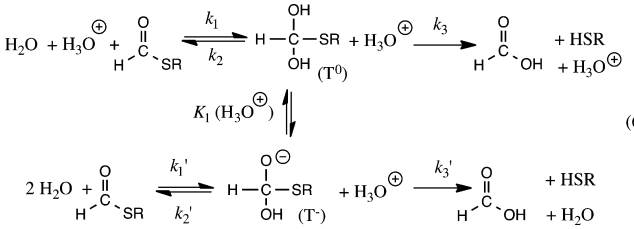


Structure I



Structure II

Schmir and Hershfield studied the rate of hydrolysis of methyl thioformate, a thioester without an electron-withdrawing acyl group.[17] Methyl thioformate was found to be approximately 2000 times less reactive than ethyl trifluorothioacetate under most conditions and displayed acid catalysis (instead of acid inhibition) below pH 2. Schmir also studied the acidic hydrolysis of a series ethyl thioacetates that varied from those with strong electron-withdrawing acyl groups to those with weak electron-withdrawing acyl groups.[18] As expected, there was a relatively smooth transition from acid inhibition to acid catalysis. For methyl thioformate, an expanded mechanism was proposed to account for the occurrence of acid-catalysis. This mechanism is shown in eq 6. At this point, the available



(6)

data for alkyl thioesters like trifluorothioacetate and methyl thioformate support a stepwise mechanism with tetrahedral intermediates. For this reason, the PIX and KIE experiments for FTC will be rationalized within the framework of that mechanism.

Positional Isotope Exchange (PIX) for FTC. Exchange of ^{18}O between the carbonyl-O and water during hydrolysis has been observed for a variety of acyl groups, including O-esters, amides, and ethyl trifluorothioacetate (below pH 5).[24,29] However, to the best of our knowledge, there are no reported

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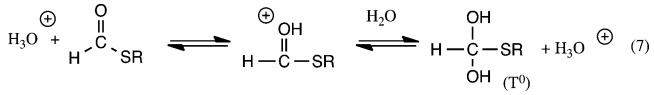
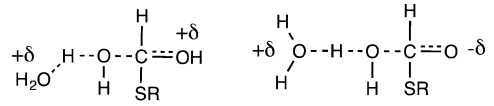
PIX experiments for thioesters exhibiting acid catalysis (i.e., no electron-withdrawing acyl groups). Exchange of ^{18}O from water into the carbonyl-O of FTC was investigated utilizing a published ^{13}C NMR spectroscopic method.[26] The method is sensitive enough to have detected a k_h/k_e ratio of 25 or lower. Under all of the conditions studied (from acidic to alkaline), no exchange was detected within the limits of our method. This leads to one of two conclusions: (a) if symmetrical tetrahedral intermediates are present, the return of the intermediate to reactants (k_2) is much slower than its breakdown to products (k_3); or (b) proton transfers between tetrahedral intermediates are not fast compared with other steps in the mechanism. The first possibility would not be surprising considering that an alkylthiol is a much better leaving group than the alkoxy group. Since we have no evidence for slow proton transfers in the case of FTC hydrolysis, we will utilize the classical assumption of rapid exchange of protons between tetrahedral intermediates. The PIX experiment on the BChE-catalyzed hydrolysis of FTC is a special case and will be discussed later.

Hydrolysis in Acidic Media. The discussion below pertains to the most acidic conditions studied, namely, 200 mM HCl. The relevant data to be interpreted are (a) the lack of ^{18}O exchange between the carbonyl-O and the solvent (k_h/k_e > 25), (b) the formyl-H KIE (^{D}k = 0.80), and (c) the single-solvent (D_2O) KIE (^{D_2O}k = 0.20). Since there is no observable ^{18}O exchange and rapid proton transfer is assumed, the rate-determining step(s) will occur during formation of a tetrahedral intermediate. Formyl-H KIEs are sensitive to changes in hybridization at the carbonyl-C in going from the sp^2 hybridization in the starting thioester to an sp^3-like geometry in the transition state. In 200 mM HCl solution, FTC shows an observed formyl-H KIE of ^{D}k = 0.80 (Table 1). This is similar to that for the acid-catalyzed hydrolysis of methyl formate (^{D}k = 0.81).[30] There are no calculations for thioester hydrolysis that relate the extent of the hybridization change in this type of acyl-transfer reaction to the magnitude of the formyl-H KIE. The best available theoretical model is that reported by Schowen for the addition of hydroxide to acetaldehyde.[31] This model, like the hydrolysis of FTC, involves the addition of a nucleophilic oxygen atom to a carbonyl-C. In addition, the theoretical model is limited to the formation of a tetrahedral intermediate, similar to the rate-determining formation of a tetrahedral intermediate proposed for FTC hydrolysis. The calculations in this model give an estimated numerical relationship between the magnitudes of the β-deuterium, formyl-H, carbonyl-C, carbonyl-O, nucleophile-O KIEs and the Pauling bond order in the transition state. Using this model to approximate the FTC case an observed formyl-H KIE (^{D}k = 0.80) predicts the sp^2 to sp^3 hybridization change of the carbonyl-C to be at least 60% complete in the transition state. This roughly corresponds to the extent of formation of the C−O bond to the nucleophile in the transition state. In all the discussions that follow, this theoretical model will be used to estimate relationships between the various KIEs and transition-state bond orders.

FTC (like methyl thioformate and formate O-esters) exhibits catalysis by hydronium ion at pH < 2, requiring a term for hydronium ion in the rate law. For both FTC and formate O-esters, the single-solvent KIE is significantly inverse (^{D_2O}k = 0.20 for FTC at 200 mM HCl; ^{D_2O}k = 0.64 for ethyl formate at 0.190 M HCl).[32] For both types of esters, this result argues for the prominence of the secondary solvent KIEs and diminishes the magnitude of primary solvent KIEs (protons in flight) during formation of the transition state. Classically, for O-esters the mechanism in eq 6 is expanded to include a pre-equilibrium protonation of the carbonyl-O followed by rate-determining attack of water to form T^0. The analogous expansion of the mechanism for a thioester is given in eq 7. This would be



consistent with the observed lack of carbonyl-^{18}O exchange if expulsion of the thiol is much faster than exchange or if the proton-transfer steps are not rapid. This general mechanism is similar to that proposed for O-esters such as methyl formate.[33] A possible transition state for this mechanism where the second step is rate-limiting is shown as structure **III**. A cyclic "water wire"-type transition state is also possible.



Structure III **Structure IV**

Hydrolysis Under Neutral Conditions. The discussion below pertains to conditions in pure water. The pH changes slightly during the reaction studied because of production of formic acid. However, the rate followed first-order kinetics throughout each run. The relevant data to be interpreted are (a) the lack of ^{18}O exchange between the carbonyl-O and solvent (k_h/k_e > 25), (b) the formyl-H KIE (^{D}k = 0.75), (c) the single-solvent (D_2O) KIE (^{D_2O}k = 4.2), and (d) the nucleophile KIE (^{18}k = 0.9917). The hydrolysis of thioesters has been studied under neutral to weakly acidic conditions (pH 2−7). In this region, the pH−rate profiles for thioesters with and without electron-withdrawing groups are flat and are presumed to follow a common mechanism. This reaction is commonly called the water reaction. As mentioned earlier, ethyl trifluorothioacetate undergoes much less carbonyl-^{18}O exchange under these neutral conditions than under more acidic conditions.[24] In fact, Bender could not detect exchange above pH 5. As mentioned earlier, we could not detect carbonyl-^{18}O exchange between FTC and water under any conditions tested, mirroring the behavior of ethyl trifluorothioacetate at moderate pH. Once more, this result is consistent with rate-limiting formation of a tetrahedral intermediate. The formyl-H KIE for FTC is more inverse under neutral conditions (^{D}k = 0.75) than under acidic conditions (^{D}k = 0.80 in 200 mM HCl). On the basis of the Schowen model[31] described above, the change in hybridization for the carbonyl-C from sp^2 to sp^3 would be at least 80% complete in this very late transition state.

The single-solvent KIE changes dramatically from an inverse effect in 200 mM HCl (^{D_2O}k = 0.20) to a large normal one under neutral conditions (^{D_2O}k = 4.2). In the case of neutral hydrolysis, the primary solvent KIEs (due to protons in flight) are now dominant, and the transition state likely contains one or more protons in flight. This agrees with the results from a solvent KIE study by Hogg and co-workers for ethyl trifluorothioacetate under similar experimental conditions.[23] Their single-solvent KIE was ^{D_2O}k = 3.7, and their proton inventory was consistent with a transition state like that shown in structure **IV** and the mechanism shown as the bottom pathway in eq 6. It is logical that FTC and ethyl trifluorothioacetate share this common mechanism. It is also

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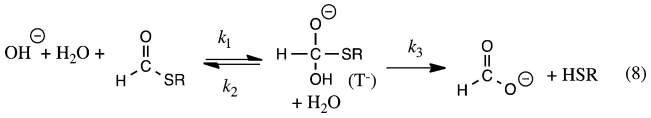
noteworthy that our observed KIEs in 50 mM HCl (^{D}k = 0.77, ^{D_2O}k = 0.81) show transitional values between those at 200 mM HCl and those under neutral conditions (Table 1), indicating a smooth transition between these two mechanisms.

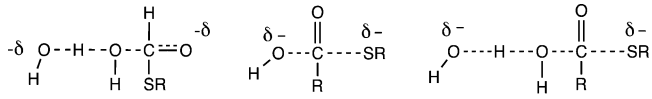
The nucleophile-O KIE was measured for the water reaction and found to be ^{18}k = 0.9917. (It is important to note that this KIE could not be measured under acidic conditions because the product, formic acid, readily exchanges ^{18}O with the solvent.) Because they are difficult to measure, very few nucleophile-O KIEs in aqueous solution have been reported. What perspectives do such nucleophile KIEs provide and how are they interpreted? The answer to this question requires a discussion of the origin of KIEs. Theoretically, all KIEs depend on a temperature-independent factor (TIF) and temperature-dependent factor (TDF).[34] The TIF is due to reaction coordinate motion; this contribution to the overall KIE is normal. The TDF results from the creation of new vibrational modes in the transition state. Stiffer transition-state bonding to the isotope leads to inverse effects; looser bonding leads to normal ones. Usually the TIF is expected to dominate for nucleophile KIEs because there should be considerable reaction coordinate motion on the part of the nucleophile. What can lead to an inverse nucleophile KIE like that observed in the present study? The first possibility is a late transition state containing a high degree of bond order between the nucleophile and the carbonyl-C during formation of the tetrahedral intermediate. This increases the effect of the newly created bonding modes, thereby increasing the inverse contribution of the TDF. The second possibility occurs when formation of the bond between the carbonyl-C and nucleophile occurs during an equilibrium step prior to rate-determining breakdown of the tetrahedral intermediate. Clearly, the second possibility is unlikely under neutral conditions, where there is a lack of carbonyl-^{18}O exchange and where sulfur is expected to be a much better leaving group than oxygen. In summary, all of the KIEs under acidic and neutral conditions in the current study are consistent with a mechanism involving an sp^3-like transition state that resembles the tetrahedral intermediate. The transition state for this mechanism is consistent with that in structure **IV** in which the bond to the nucleophile is highly formed.

Hydrolysis under Alkaline Conditions. The alkaline conditions studied were without the complications of a buffered system. The relevant data to be interpreted are (a) the lack of ^{18}O exchange between the carbonyl-O and solvent (k_h/k_e > 25), (b) the formyl-H KIE (^{D}k = 0.88), and (c) the nucleophile-O KIE (^{18}k = 1.029 for water or ^{18}k = 0.989 for hydroxide). Alkaline hydrolysis of FTC was too rapid to determine the actual reaction order with respect to hydroxide given the equipment available in this lab. However, it is likely that the reaction is first-order in hydroxide, as has been found for O-esters and other thioesters.[19,30] The currently accepted mechanism has only two steps with the formation of a single anionic tetrahedral intermediate (eq 8). There are two



(8)

permutations of this mechanism. The first is the direct attack of hydroxide on the carbonyl-C; the second involves action of hydroxide as a general base to abstract a proton from a water molecule in the proximity of the carbonyl-C. The transition state for the first possibility was given previously as structure **II**; that for the second possibility is given as structure **V**. It should be noted that structure **V** contains one water molecule but more are possible.



Even though the reaction in alkali is very rapid, the formyl-H KIE could still be measured using the competitive method and a limiting amount of hydroxide. Under these alkaline conditions, FTC exhibits a small inverse formyl-H KIE of ^{D}k = 0.88. Together with the observed lack of ^{18}O exchange, this indicates a somewhat early, sp^2-like transition state during formation of the tetrahedral intermediate. The Schowen model predicts that the change in hybridization for the carbonyl-C from sp^2 to sp^3 would be about 40% complete in this early transition state.

The nucleophile-O KIE has been used to differentiate between the two possible nucleophiles (hydroxide and water).[35] For FTC, if hydroxide is the actual nucleophile, the nucleophile-O KIE would be calculated to be ^{18}k = 0.989; if water is the actual nucleophile, the nucleophile-O KIE would be ^{18}k = 1.0290 (see the Results). The water nucleophile scenario has been supported in the case of the alkaline hydrolysis of methyl formate (an oxygen ester) on the basis of KIE studies[35] and on the basis of a proton inventory study of ethyl acetate.[36] Is it possible to choose between these possibilities for FTC? Unfortunately, the answer is not as clear-cut as in the methyl formate case. On the basis of the theoretical model of Schowen, a water nucleophile with a KIE of ^{18}k = 1.0290 would predict a formyl-H KIE of approximately ^{D}k = 0.98, whereas a hydroxide nucleophile with a KIE of ^{18}k = 0.989 would predict a formyl-H KIE of approximately ^{D}k = 0.82. Neither choice is a good fit for the observed formyl-H KIE for alkaline hydrolysis (^{D}k = 0.88).

A third possibility that might overcome this problem is a change in mechanism from stepwise (eq 1) to direct displacement (eq 2). Williams has proposed a change to direct displacement for acyl transfers involving very good nucleophiles and very good leaving groups.[37] Cleland and Hengge have found isotope effect evidence for the direct displacement mechanism for the reaction of a variety of nucleophiles (including hydroxide) with *p*-nitrophenyl acetate (PNPA).[20,21] Both FTC and PNPA have excellent leaving groups with low pK_a values. Thiocholine has a pK_a of about 8, whereas the pK_a of *p*-nitrophenol is 7.15. Both reactions were performed under strongly nucleophilic conditions. Thus, it would not be surprising to find the direct displacement mechanism to be operating under these conditions. The transition-state structure for the direct displacement mechanism is shown as structure **VI** or **VII**, depending on the actual nucleophile (hydroxide or water, respectively).

BChE-Catalyzed Hydrolysis. The cholinesterase family of enzymes is a highly efficient catalytic group of proteins. The best known is acetylcholinesterase (AChE), for which the k_{cat}/K_m value for hydrolysis of its natural substrate, acetylcholine, approaches diffusion control (i.e., a "perfect" catalyst).[38,39] BChE is somewhat less efficient but was chosen for the present study because it does not exhibit the complicating substrate

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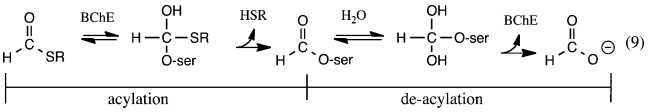
inhibition displayed by AChE. Both AChE and BChE utilize thioesters as substrates. In this study, FTC was shown to be a substrate for BChE with K_m = 2.3 mM and V_{max} = 57 μM/min (extrapolated values from the data in Figure 1). The chemical mechanism for BChE-catalyzed hydrolysis is thought to involve the typical catalytic triad present in all serine proteases: a carboxylate, a histidine, and a serine. A truncated mechanism is shown in eq 9.



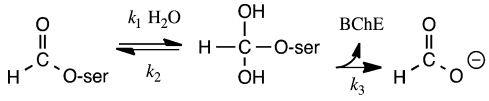
(9)

Quinn recently used β-hydrogen KIEs to investigate the BChE-catalyzed hydrolysis of ATC.[39] Using direct measurement of the rates, he found $^{D3}k_{cat}/K_m$ = 0.98 and $^{D3}k_{cat}$ = 1.10. The $^{D3}k_{cat}/K_m$ KIE encompasses bonding changes in going from reactants to the rate-limiting transition state. This can include any and all steps up to and including the first irreversible step. For BChE, the first irreversible step is release of the thiol, meaning that the observed KIE must be for the acylation only. The small magnitude of $^{D3}k_{cat}/K_m$ implies that hydrolysis is limited by more than one step. On the other hand, the normal $^{D3}k_{cat}$ KIE implies that under saturating conditions a tetrahedral intermediate accumulates (either during the acylation or deacylation steps) and that its decomposition is partially rate-determining.

In the present study of the BChE-catalyzed hydrolysis of FTC, PIX experiments failed to detect any ^{18}O exchange between water and the carbonyl-O of FTC. Carbonyl-^{18}O exchange could only have occurred during formation of the tetrahedral intermediate for the deacylation step because water is a nucleophile only during this step (see eq 9). However, the lack of exchange is not surprising in view of the stereospecificity of enzymes. Enzymes are capable of distinguishing between two atoms that would be identical in solution, such as the two oxygen atoms of a symmetrical tetrahedral intermediate. As a result, the lack of exchange may be due to the fact that hydrolysis is much faster than exchange or it may simply be an outgrowth of the natural stereospecificity of the enzyme.

The transition-state structure for FTC hydrolysis was then investigated via measurement of the formyl-H KIE. This KIE was measured by the competitive method (instead of by direct measurements of rate constants) and is a (V/K) isotope effect, which, like $^{D3}k_{cat}/K_m$, reflects only bonding changes in the acylation steps (see above). The observed formyl-H KIE was $^{D}(V/K)$ = 0.89. The small inverse KIE indicates either an early sp^2-like transition state for rate-determining formation of the tetrahedral intermediate or that more than one step is rate-determining (as postulated by Quinn). Can the magnitude of the formyl-H KIE for FTC be compared to the above-mentioned β-hydrogen KIE for ATC? Both the α-hydrogen KIE and the β-hydrogen KIE are secondary KIEs, where the connection to the hydrogen atom(s) is not severed during the reaction. In addition, they both reflect changes in hybridization at the carbonyl-C in going to the transition state. The difference is largely in the magnitude of the two KIEs; the α-hydrogen KIE is intrinsically larger. The Schowen model provides a basis for a qualitative comparison.[31] In the acetaldehyde system, when the calculated β-hydrogen KIE is between 0.97 and 0.98, the corresponding calculated value of the α-hydrogen KIE is in the range of 0.92−0.95. Therefore, Quinn's secondary KIE and the one measured in this work are in reasonable agreement within the limits of the model.

The observed nucleophile-O KIE is a small, inverse effect, where $^{18}(V/K)$ = 0.9925. Our methodology (see Scheme 1b) follows only the change in $^{18}\delta$(O) between the oxygen atoms of the bulk solvent and those that are incorporated into the product. Thus, only the role of water as the nucleophile is observed. In the enzymatic reaction, this nucleophile (water) does not enter the catalyzed chemical reaction until the deacylation step of BChE catalysis (eq 9). Whereas the formyl-H KIE was interpreted in terms of the acylation step, the nucleophile-O KIE must be interpreted in terms of the deacylation step, as shown in eq 10.



(10)

What can lead to a small inverse nucleophile-O KIE on the deacylation step? First, consider the possibility that breakdown of the tetrahedral intermediate (eq 10) is rate-determining. In this case, the observed KIE on the deacylation step would essentially become the product of the equilibrium isotope effect (EIE) for formation of the tetrahedral intermediate ($^{18}k_1/^{18}k_2$ or $^{18}K_{eq}$) multiplied by that on its breakdown ($^{18}k_3$). This requires the reasonable assumption that isotope effects on the binding of water to BChE are negligible. Because bonding changes to the nucleophilic-O are much greater during formation of the tetrahedral intermediate than during its decomposition, $^{18}k_3$ is quite small and the magnitude of the observed KIE largely reflects $^{18}K_{eq}$. In addition, if k_3 is rate-determining, the tetrahedral intermediate is in equilibrium with the acyl enzyme and water. The expected magnitude of this EIE can be approximated from the results of a KIE study of the urease-catalyzed hydrolysis of formamide.[40] Here the leaving-N KIE was $^{15}(V/K)$ = 1.0321, making the breakdown of the tetrahedral intermediate rate-determining. The observed large inverse nucleophile-O KIE of $^{18}(V/K)$ = 0.9778 for formamide must then mainly reflect the magnitude of $^{18}K_{eq}$ for formation of the tetrahedral intermediate. Moreover, model equilibria agree that this EIE should be approximately 2% inverse.[40] In the present case, the observed nucleophile-O KIE, $^{18}(V/K)$ = 0.9925, is much smaller than the $^{18}K_{eq}$ in the formamide case, arguing against rate-determining breakdown of the tetrahedral intermediate during deacylation as a viable possibility.

The observed small inverse KIE is consistent with a kinetic mechanism where some step (or steps) prior to breakdown of the tetrahedral intermediate is rate-determining. If the rate-determining step is formation of the C−O bond, a late, sp^3-like transition state is most likely. This late transition state would increase the effect of the newly created bonding modes, causing the normal contribution from the TIF to be diminished by the inverse contribution of the TDF. Any possibility involving rate-determining steps prior to breakdown of the tetrahedral intermediate can fit the observed small inverse nucleophile-O KIE, but this implies that the tetrahedral intermediate is not in equilibrium with the acyl enzyme and water.

In summary, two general possibilities for the BChE-catalyzed hydrolysis of FTC have been presented. The first allows for the accumulation of the tetrahedral intermediate during deacylation but does not fit the observed nucleophile-O KIE. The second fits the observed nucleophile-O KIE but does not allow for accumulation of the tetrahedral intermediate during deacyla-

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tion. As a result, if a tetrahedral intermediate accumulates during the BChE-catalyzed hydrolysis of FTC, as was observed in the case of ATC hydrolysis,[38,39] the most likely candidate would be the tetrahedral intermediate formed in the acylation step.

CONCLUSIONS

The hydrolysis of FTC was investigated under acidic, neutral, basic, and BChE-catalyzed conditions. PIX experiments failed to detect any exchange between the carbonyl-O of FTC and water under any of the above conditions. For a stepwise mechanism, this indicates that breakdown of any symmetrical tetrahedral intermediate is much faster than its formation (assuming rapid proton transfers). Under acidic and neutral conditions, the formyl-H and nucleophile-O KIEs fit the long-established stepwise mechanism involving tetrahedral intermediates. The relatively large formyl-H KIEs and the small inverse nucleophile-O KIE (neutral conditions) indicate a late, sp^3-like transition state that occurs during formation of a tetrahedral intermediate.

For alkaline hydrolysis, the observed formyl-H and nucleophile-O KIEs do not easily fit the classic stepwise mechanism. The nucleophile-O KIE experiment gives two possible results, one where water is the nucleophile (^{18}k = 1.029) and the other where hydroxide is the nucleophile (^{18}k = 0.989). A published theoretical model for the stepwise mechanism predicts that when the nucleophile-O KIE is large and normal (1.029), the formyl-H KIE should be less than ^{D}k = 0.95. On the other hand, when the nucleophile-O KIE is large and inverse (0.989), the formyl-H KIE should be less than ^{D}k = 0.82.[31] The observed KIEs do not fit either scenario. Therefore, a possible change to a relatively rare direct displacement mechanism is proposed. Alkaline hydrolysis has the requisite conditions of a good nucleophile and a good leaving group that make this mechanism a distinct possibility, as proposed by Williams.[37] Sulfur leaving group isotope effects, which are currently under development in this lab, are designed to shed light on this issue.

For BChE-catalyzed hydrolysis of FTC, the observed small inverse formyl-H KIE indicates that the formation of the tetrahedral intermediate during the acylation of BChE is only partially rate-determining. On the other hand, the observed small inverse nucleophile-O KIE on the deacylation step is consistent with rate-determining formation of the C−O bond between the nucleophile-O and the carbonyl-C of FTC. If FTC and ATC have similar kinetic mechanisms, this result implies that the tetrahedral species that is proposed to accumulate during hydrolysis is likely to be one formed in the acylation step.[38]

EXPERIMENTAL SECTION

Synthesis of Formylthiocholine. A solution containing formic acid (2.2 equiv, 15.6 mmol, 589 μL) and acetic anhydride (2 equiv, 14.2 mmol, 1342 μL) was stirred at 60 °C for 2 h. The clear solution was cooled to room temperature, and 2-(dimethylamino)ethanethiol hydrochloride (1 equiv, 7.1 mmol, 1.0 g) was added to the resulting mixed anhydride.[41] The reaction mixture was stirred overnight under a nitrogen atmosphere at room temperature. Excess acetic anhydride, acetic acid, and formic acid were removed under reduced pressure using coevaporations with heptane, and FDC was isolated as a white solid (1.13 g) in a crude yield of 95%. The crude FDC (1 equiv, 6.5 mmol, 1.1 g) was then partially dissolved in 25 mL of acetone with continuous stirring for 5 min in a 100 mL round-bottom flask. To the flask was added *N*,*N*-diisopropylamine resin (5 equiv, 3.68 mmol/g, 8.8 g) and the mixture was allowed to stir for approximately 15 min. A glass rod was used to break apart white insoluble material, and both the insoluble material and the resin were filtered using a 10 mL disposable column. The filtrate was collected and again filtered into a round-bottom flask through a 0.45 μm plastic filter fitted to a 20 mL plastic syringe in order to remove all insoluble material. To this round-bottom flask was added methyl iodide (3 equiv, 20.4 mmol, 1.21 mL), and the reaction mixture was allowed to stir until a white solid precipitated out of solution. Once the white precipitate formed, the solution was stirred for an additional 10 min at room temperature. The white solid was isolated by vacuum filtration, washed with cold diethyl ether (15−20 mL), and placed under high vacuum for further drying. The solid (487 mg, 1.77 mmol) was isolated in a crude yield of 27%. The crude formylthiocholine iodide was recrystallized from absolute ethanol, and traces of ethanol were removed by high vacuum with a percent recovery from recrystallization of 70% and an overall yield of 19% (342 mg, 1.24 mmol). Mp = 171−172 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 10.4 (s, 1H), 3.53−3.43 (m, 2H), 3.43−3.34 (m, 2H), 3.19 (s, 9H). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 190.30, 64.03, 52.71, 19.70. IR (neat, cm^{-1}): 3040.82−3007.86, 2846, 1659. HRMS (ESI-TOF) *m*/*z*: [M]$^+$ calcd for $C_6H_{14}NOS$ 148.0791, found 148.0789. 1-*d*-FTC (or D-FTC) and 1-^{13}*C*-FTC were synthesized via the above procedure using *d*-formic acid and ^{13}*C*-formic acid, respectively, as the starting reagents.

Enzymatic Assays. Equine serum BChE activity with ATC as the substrate was determined by means of a continuous assay at 324 nm that uses DTDP to monitor the production of thiocholine.[42] Reaction mixtures contained 1.0 mM DTDP, 50 mM MES (pH 6.8), 4.0 nM BChE, and 0.048−4.8 mM ATC. All of the reaction components were combined in a 1 mL UV quartz cuvette, and the reaction was started upon addition of the enzyme. BChE activity with FTC as a substrate was determined by a point assay in MES buffer (pH 6.8) using DTNB to monitor the formation of thiocholine.[43] The FTC concentration was varied from 0.4 to 4 mM. All of the reaction components except for BChE were combined to make a 4 mL solution. BChE was added, and aliquots were withdrawn at various times, diluted, and placed in a 1 mL quartz UV cuvette. Immediately, a 50 μL aliquot of 5 mM DTNB was added, and the absorbance at 412 nm was recorded. There was negligible residual reaction in the time between dilution and recording of the absorbance. All of the reactions were performed at room temperature.

Deuterium Solvent KIE Procedure. Solutions with volumes of 400 μL were prepared under the following experimental conditions: (a) 200 mM HCl (or DCl), (b) 50 mM HCl (or DCl), or (c) pure H_2O (or D_2O). The solutions in D_2O were at least 97 atom % deuterium. The pH meter readings were adjusted to correct for the differing activities of aqueous HCl and DCl (pD = pH meter reading + 0.40).[44] The ionic strength of each solution was maintained at 200 mM by addition of solid KCl. The reaction was initiated by addition of the appropriate solution to solid FTC, and the mixture was immediately transferred to an NMR tube. The initial concentration of FTC was between 25 and 35 mM. An ^{1}H NMR spectrum was obtained every 30 min over 3−5 h at a probe temperature of 25 °C. The relative concentrations of the formyl proton of FTC and the formyl proton of formate (or formic acid) were determined by integration. The slope of a plot of the natural logarithm of the fraction of reaction versus time was used to determine the first-order rate constant. This direct measurement of the rate constants in H_2O and D_2O allowed for calculation of the KIE.

Formyl-H KIE Procedure. Measurement of the formyl-H KIEs for hydrolysis of H-FTC and D-FTC in 200 mM HCl, 50 mM HCl, and pure H_2O utilized a procedure similar to that given in the previous paragraph to directly measure the rate constants. In each case, the reaction was initiated by adding the appropriate solution to either solid H-FTC or D-FTC. The initial concentration of FTC was 25 mM; that for D-FTC was 50 mM. An ^{1}H NMR spectrum (for H-FTC) or an ^{2}H NMR spectrum (for D-FTC) was obtained every 30 min over 3−5 h. The integration data were plotted as described in the previous paragraph. The formyl-H KIEs for alkaline hydrolysis and BChE-catalyzed hydrolysis were determined via the competitive method. For

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alkaline hydrolysis, 400 μL of a 50 mM KOH solution was added to a mixture of solid H-FTC and D-FTC so that the final solution was 25 mM in H-FTC and 75 mM in D-FTC. Alternating ^{1}H NMR and ^{2}H NMR spectra were taken, and the ratio of rate constants was obtained at a common time point to determine the KIE. The $^{D}(V/K)$ formyl-H KIE determination for the BChE-catalyzed hydrolysis of FTC also employed this competitive method. A 400 μL solution containing 200 mM MES buffer (pH 6.8) and 5 nM BChE was added to a mixture of solid H-FTC and D-FTC so that the final solution was 25 mM in H-FTC and 75 mM in D-FTC. As above, alternating ^{1}H NMR and ^{2}H NMR spectra were taken to determine the ratio of rate constants. In separate control experiments it was shown that the MES-buffer-catalyzed rate of hydrolysis was negligible compared with the enzyme-catalyzed rate of hydrolysis.

Nucleophile-O KIE Procedure. This experiment contained three parts. (a) To produce Ph_3CNa, 0.41 g (6.8 mmol) of NaH (60% oil dispersion) was added to 24 mL of anhydrous DMSO, and this solution was heated to 70 °C for 1.5 h under dry, CO_2-free nitrogen. A 2.5 g (10 mmol) sample of Ph_3CH was added to the above solution, and the solution immediately turned red as a result of the formation of Ph_3C^- anion. For the decarbonylation, 10−15 mg of solid FTC was placed in one side of a Y-tube along with 1.5 mL of anhydrous DMSO. A 3.0 mL aliquot of the Ph_3C anion solution was placed in the other side of the Y-tube. The Y-tube was evacuated, and the contents of the two sides were mixed. The resulting CO was distilled under vacuum through two liquid N_2 traps into a collection tube containing molecular sieves at liquid N_2 temperature. The collection tube was moved to a CO converter, and the sieves were warmed to 180 °C. The CO was electrolytically disproportionated to CO_2 and C in an apparatus described by Crowe.[45] The resulting CO_2 was transferred under vacuum to a sample tube, which was subsequently attached to the IRMS instrument for analysis to determine $^{18}\delta$(O) for the oxygen atoms of CO_2. A definition of $^{18}\delta$(O) is given in the Results. Because the chemical conversion was quantitative, this measured $^{18}\delta$(O) reflected the $^{18}\delta$(O) for the carbonyl-O of FTC.[26,35] (b) Measurement of $^{18}\delta$(O) for water was accomplished by the addition of a small sample of CO_2 (<100 μmol) to an evacuated round-bottom flask containing 20 mL of degassed water and 1 mL of concentrated H_2SO_4. The mixture was stirred overnight, and the CO_2 was isolated under vacuum and analyzed by IRMS. (c) Analysis of the oxygen atoms of formate was accomplished as follows. FTC was hydrolyzed completely under the acidic, neutral, alkaline, or BChE-catalyzed conditions noted above. The solution was passed through an anion exchange resin (acetate form), which bound formate. The formate was eluted with 0.1 M NaCl. The isolated formate and a stir bar were placed in a round-bottom flask that was equipped with two stopcocks. One stopcock was attached to a side arm that was capped with a septum. The second stopcock contained a ground glass joint for attachment to the high-vacuum line. Water from the solution containing formate was evaporated with stirring under high vacuum at room temperature. The solid residue was then further dried under high vacuum at 70 °C overnight. While the system was still under vacuum, 2 mL of anhydrous DMSO containing 200 mg of I_2 was added to the dried formate through the septum-capped side arm. The resulting CO_2 was passed through two pentane/liquid N_2 cold traps and collected in a liquid N_2 trap. The CO_2 was then distilled under vacuum into a collection tube and transferred to the IRMS instrument. Analysis by IRMS gave the $^{18}\delta$(O), which was a weighted average for the two oxygen atoms of CO_2. Analysis by IRMS simultaneously gave the $^{13}\delta$(C) for the carbon atom of CO_2. Again, because conversion of formate to CO_2 was quantitative, the measured $^{18}\delta$(O) of CO_2 was the same as for the two oxygen atoms of formate. The procedure for calculating the KIE from these data is given in the Results.

Positional Isotope Exchange Procedure. The extent of isotope exchange between the carbonyl-O of FTC and water was determined by a previously published ^{13}C NMR method.[26] The ^{13}C resonance of the product, formate, gave baseline separation of the three oxygen-isotope-shifted ^{13}C peaks (^{16}O,^{16}O; ^{18}O,^{16}O; and ^{18}O,^{18}O), and these were integrated. The T_1 value for the ^{13}C resonance of formate was determined to be 10 s, and consequently, a delay time of 60 s was employed to allow for quantitative integration of the isotope-shifted ^{13}C peaks. In theory, one could integrate the oxygen-isotope-shifted peaks of FTC or formate. However, the ^{13}C resonance for the carbonyl of FTC is too broad for accurate integration, leaving analysis of formate as the only possibility. Under neutral conditions, the reaction was initiated by addition of 0.50 mL of 97 atom % ^{18}O-water to solid 1-^{13}C-FTC. The initial [FTC] was 50 mM. The reaction was allowed to proceed beyond 50% total reaction (as determined by ^{1}H NMR spectroscopy), and the ^{13}C spectrum was recorded. Under alkaline conditions, a solution that contained 25 mM KOH and 93 atom % ^{18}O-water was prepared. This was added to enough solid 1-^{13}C-FTC so that the initial concentration of FTC was initially 50 mM. The reaction essentially stopped when the limiting base was consumed, and the ^{13}C spectrum was immediately recorded. For the BChE-catalyzed hydrolysis, the solution contained 100 mM MES buffer (pH 6.8) in 90 atom % ^{18}O-water. The reaction was initiated by addition of the above solution to solid 1-^{13}C-FTC (final [FTC] = 25 mM). This reaction was allowed to reach completion, and then the ^{13}C NMR spectrum was recorded. All of the above solutions had an ionic strength of 200 mM.

Oxygen exchange for hydrolysis of FTC in 50 and 200 mM HCl required a modification of the procedure in the previous paragraph because the product, formic acid, is known to exchange with ^{18}O-water under these acidic conditions.[33] The reactions were separately initiated by addition of 200 or 50 mM HCl in 93 atom % ^{18}O-water to solid 1-^{13}C-FTC. After 50% total reaction as determined by ^{1}H NMR spectroscopy, the reactions were quenched in MES buffer to pH ∼6 and immediately applied to a cation exchange resin (Li^+ form). The bound FTC was washed with 30 mL of natural-abundance water and eluted from the column (and simultaneously hydrolyzed) with 100 mM NaOH (natural abundance). The fractions containing formate were collected, reduced to a volume of 500 μL, and placed in an NMR tube. The results of the PIX experiments under alkaline conditions (previous paragraph) showed no exchange of ^{18}O water into the oxygen carbonyl atoms of formate. Consequently, if exchange into the carbonyl of FTC occurred under these acidic hydrolysis, any exchanged ^{18}O remained in the isolated formate as an ^{18}O,^{16}O isotope-shifted peak.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic scheme for synthesis of FTC, NMR spectra for FTC, sample proton and deuterium NMR spectra for KIE experiments, carbon NMR spectra for PIX experiments, and observed first-order rate constants. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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