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An investigation into the butyrylcholinesterase-catalyzed hydrolysis of formylthiocholine using heavy atom kinetic isotope effects

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ABSTRACT

Heavy atom kinetic isotope effects (KIEs) were determined for the butyrylcholinesterase-catalyzed hydrolysis of formylthiocholine (FTC). The leaving-S, carbonyl-C, and carbonyl-O KIEs are $^{34}k = 0.994 \pm 0.004$, $^{13}k = 1.0148 \pm 0.0007$, and $^{18}k = 0.999 \pm 0.002$, respectively. The observed KIEs support a mechanism for both acylation and deacylation where the steps up to and including the formation of the tetrahedral intermediate are at least partially rate determining. These results, in contrast to previous studies with acetylthiocholine, suggest that the decomposition of a tetrahedral intermediate is not rate-determining for FTC hydrolysis. Structural differences between the two substrates are likely responsible for the observed mechanism change with FTC.

1. Introduction

Thioesters are energy-rich functional groups that often serve as enzyme substrates for a variety of reactions in metabolism. Many families of proteins utilize thioesters as substrates. For example, acetyl transferases, thiolases, and thioesterases catalyze thioester exchange, Claisen condensations, and hydrolysis of thioesters, respectively [1,2]. Cholinesterases are one family of enzymes that can be used to study the mechanism of thioester hydrolysis. Although thioesters are not the natural substrate, these enzymes provide insight into the mechanism of thioester hydrolysis and can provide valuable information for the design of transition state inhibitors.

Cholinesterases, such as butyrylcholinesterase (BChE) and acetylcholinesterase (AChE), are serine hydrolases with the α/β hydrolase fold. While the exact role played by BChE is still unknown, it is thought to be primarily responsible for detoxification reactions in the serum, liver, lungs, and intestinal mucosa [3]. It is also found in the brain and is believed to support acetylcholinesterase in hydrolyzing acetylcholine at the synapse [4]. Enzymes in this family have active sites that consist of a catalytic triad. They follow a mechanism similar to that of a serine protease in which the activated serine attacks the acyl-substrate to form an acyl-enzyme intermediate. The acyl-enzyme intermediate then reacts directly with a water molecule at the active site. Structural and kinetic data provide strong evidence that both the acylation and deacylation reactions occur through a tetrahedral intermediate [5,6]. In the current paper we report the results of a continuing heavy atom kinetic isotope effect (KIE) investigation of BChE-catalyzed thioester hydrolysis. The substrate for these investigations was formylthiocholine (FTC) and the measured KIEs include the leaving-S, the carbonyl-C, and the carbonyl-O.

2. Theory and calculations

KIEs are reported as a ratio of rate constants for molecules containing different isotopes at a particular position. For example, a carbon KIE is the ratio of $^{12}k/^{13}k$ and is written as $^{13}k_{obs}$ [7]. Rather than a single rate constant, the observed KIE is the empirical KIE and can be influenced by a variety of partially rate-determining steps. In this study the KIEs were measured using natural abundance carbon, oxygen, and sulfur. After a partial reaction, the final isotopic analysis was completed by an isotope ratio mass spectrometer (IRMS) and was reported as a per mil difference (δ) of the sample relative to that for a standard material.

All KIEs in this paper are measured using the competitive method [8]. To calculate the KIE from the δ values three data points are necessary: (a) the δ for the unreacted substrate or completely reacted product (δ_o); (b) the δ for the isolated starting material or product from the partial reaction (δ_s or δ_p); and (c) the fraction of reaction. The KIE can be calculated using substrate or product analysis with Eqs. (1) and (2), respectively.

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$$\mathrm{KIE} = \log(1-f)/[(1-f)(\delta_s/1000+1)/(\delta_o/1000+1)] \quad (1)$$

$$\mathrm{KIE} = \log(1-f)/[1-(f(\delta_p/1000+1)/(\delta_o/1000+1))] \quad (2)$$

3. Results and discussion

The family of cholinesterases includes acetylcholinesterase and butyrylcholinesterase. This family of enzymes has an acylation reaction where the acyl substrate is covalently bound to the enzyme and a deacylation reaction where water acts as a nucleophile to hydrolyze the acyl-enzyme intermediate (see Fig. 1). BChE was chosen for this study because substrate inhibition with FTC does not occur as observed in AChE, simplifying analysis.

The mechanism of BChE-catalyzed hydrolysis of acetylthiocholine (ATC) was studied previously using KIE experiments. Quinn et al. used the direct method to independently measure the rates of the natural abundance and isotopically enriched substrates [9]. This method allows determination of both the KIE on k_{cat}/K_m and k_{cat}. Using acetyl-$^2H_3$-thiocholine, a secondary KIE of $^{D3}(k_{cat}/K_m)$ = 0.98 was found. KIEs on k_{cat}/K_m include only steps up to the first irreversible step (i.e. product release for acylation) [10]. This small inverse secondary KIE led to the conclusion that some step (or steps) other than chemical formation of the tetrahedral intermediate were partially rate determining. The observed KIE on k_{cat} was $^{D3}k_{cat}$ = 1.10 [10]. KIEs on k_{cat} measure the effects on the steps after formation of the ES complex through product release. Quinn attributed this normal KIE as arising from an sp^3-hybridized carbon in a tetrahedral intermediate going through an sp^2-like transition state. This lead to the unusual conclusion that breakdown of a tetrahedral intermediate to an acyl product is rate determining and further implies that a tetrahedral intermediate accumulates during the reaction under saturating conditions. Although unusual, the conclusion is consistent with X-ray crystal structures that find a stable tetrahedral intermediate with the human enzyme, stabilized by a three-pronged oxyanion hole in the BChE active site [6]. Quinn's et al., work with equine serum BChE could not determine which of the two tetrahedral intermediates accumulates, although subsequent work with *Drosophila melanogaster* AChE was consistent with accumulation of the tetrahedral intermediate in second, deacylation reaction [5].

Several KIEs for BChE-catalyzed hydrolysis of FTC have been measured previously in our labs using the competitive method, which measures only k_{cat}/K_m isotope effects (Table 1) [11]. In these studies FTC was used as the substrate instead of ATC. The reason for choosing FTC was the existence of analytical procedures for formate which allowed measurement of multiple KIEs. These k_{cat}/K_m KIEs fall into two categories. The carbonyl-C, carbonyl-O, formyl-H and leaving-S KIEs provide mechanistic information up to release of thiocholine (the acylation reaction), whereas the nucleophile-O KIE provides mechanistic evidence only for the deacylation reaction.

The magnitude of observed formyl-H KIEs on FTC hydrolyses range from 0.89 to 0.75 (Table 1). In addition, calculated values for a model reaction indicate that very early transition states (little hybridization change at the carbonyl-C) should yield a formyl-H KIE near 0.98, whereas very late transition states will yield values near 0.75 [12]. As a result the observed small inverse formyl-H KIE of $^{D}(k_{cat}/K_m)$ = 0.89 for BChE-catalyzed hydrolysis of FTC is consistent with a somewhat early transition state that occurs during rate determining formation of the tetrahedral intermediate. A second possibility is that formation of the tetrahedral intermediate is only partially rate determining and is masked by other prior steps (e.g. binding).

The observed nucleophile-O KIE was small and inverse (^{18}k = 0.9925) [11]. If it is assumed that the oxygen equilibrium isotope effect for partitioning water between bulk solvent and the active site of the enzyme is negligible, the observed nucleophile-O KIE will then only report on the deacylation step. It is during deacylation that water is a substrate for the hydrolysis of the acyl enzyme, resulting in all the significant bonding changes to the nucleophile-O. If breakdown of the tetrahedral intermediate in the deacylation step is rate determining, a large inverse KIE (due mostly to the $^{18}K_{eq}$ for formation of the tetrahedral intermediate) would be expected [13]. Since the observed KIE is only slightly inverse, steps up to and including formation of the second, deacylation tetrahedral intermediate are at least partially rate determining.

The leaving-S KIE measured in this study was determined with natural abundance FTC using the competitive method and gives the isotope effect on k_{cat}/K_m. The observed inverse leaving-S KIE of 0.994 for BChE-catalyzed hydrolysis is statistically different than unity (>98% C.I.), but not statistically different than the leaving-S KIEs for the acid-catalyzed and neutral hydrolyses of FTC (also >98% C.I.) determined previously (Table 1) [14,15]. These inverse S-KIEs are somewhat surprising since leaving group heteroatom KIEs for oxoesters and amides are most often normal [8]. The inverse sulfur KIEs for the hydrolysis of FTC in neutral and acidic conditions were attributed to new bending modes for the sulfur during formation of the tetrahedral intermediate which leads to stiffer bonding to the sulfur atom and an inverse KIE. This trend toward a more inverse sulfur KIE in later transition states was also observed with phosphothioate ester hydrolysis [15]. In the present case of BChE-catalyzed hydrolysis, the inverse leaving-S KIE leads to the conclusion that breakdown of the tetrahedral intermediate during acylation is not rate determining, since rate-determining breakdown of the tetrahedral intermediate should give rise to a normal KIE. Because accumulation of the tetrahedral intermediate requires its breakdown to be rate determining, this also means that the tetrahedral intermediate for acylation does not accumulate in the hydrolysis of FTC. However, the reason for the observed inverse effect must be different than the non-enzymatic cases where late sp^3-like transition states are proposed on the basis of larger inverse

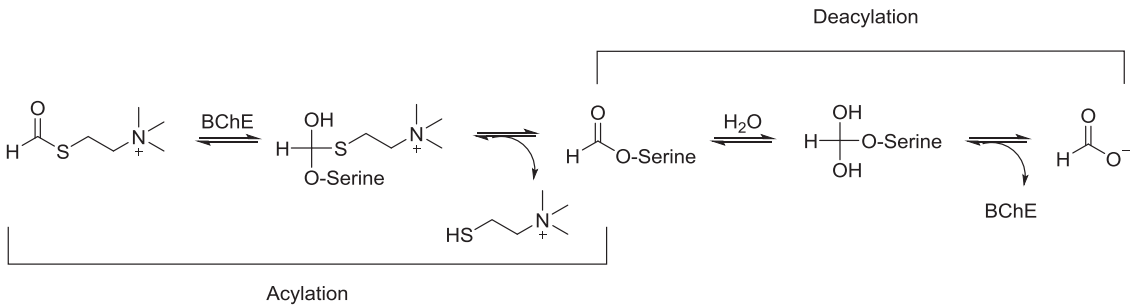

Fig. 1. Mechanism of BChE-catalyzed hydrolysis of FTC.