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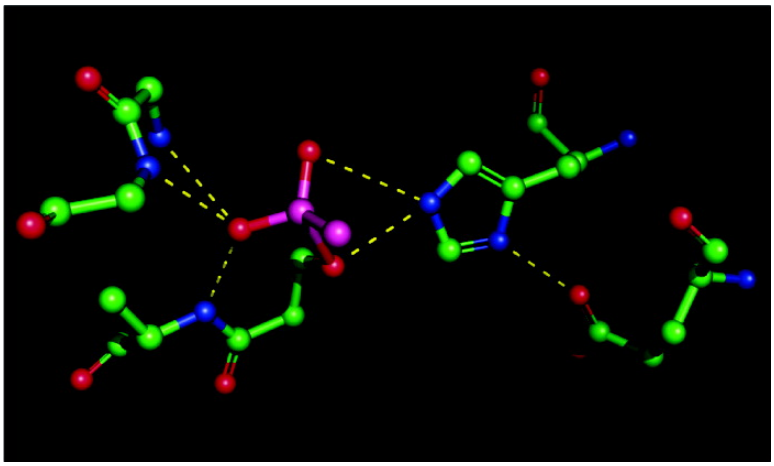

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The Reactant State for Substrate-Activated Turnover of Acetylthiocholine by Butyrylcholinesterase is a Tetrahedral Intermediate

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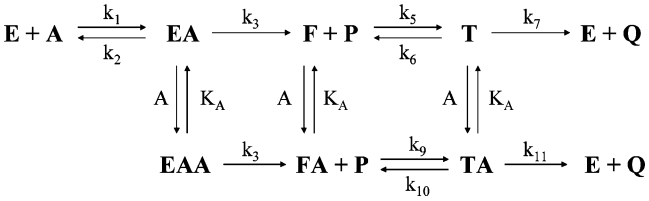
Acetylcholinesterase (AChE[1]) and butyrylcholinesterase (BuChE[1]) catalyze the hydrolysis of choline esters with very high catalytic efficiency. At neutral pH, cholinesterase reactions are $\sim 10^{13}$-fold faster than spontaneous substrate hydrolysis, a factor that corresponds to 74 kJ mol^{-1} of transition state stabilization.[2] Though many structural features that contribute to the catalytic mechanism have been illuminated[3,4]—the Ser-His-Glu catalytic triad, the oxyanion hole, the acyl binding site, the quaternary ammonium binding site—a decisive understanding of cholinesterase catalytic power is yet to be had.

Recently, Nicolet et al.[5] reported crystal structures of human BuChE and complexes of the enzyme. Surprisingly, in the supposed unliganded enzyme structure, determined at a resolution of 2.0 Å, electron density that was proximal to the nucleophilic oxygen of the active site Ser198 was interpreted as a bound butyrate. This ligand could be displaced with 3-bromopropionate, which placed the structural assignment on a firm footing. However, it was not possible to differentiate cleanly between a complex in which the ligand is bound as a tetrahedral intermediate and one which is a BuChE−carboxylate complex. Though angular distortions toward tetrahedrality were noted about the carbonyl carbon of the bound butyrate, the resolution of the structure and the rather long Ser198 γO to butyrate carbonyl carbon distance (2.16 Å) suggest that the complex may be a mixture of tetrahedral intermediate and EP complexes, and perhaps also a covalent acylenzyme.

These unusual observations beg for an experimental probe of the catalytic behavior of BuChE that can address the nature of substrate hybridization changes that accompany catalytic turnover in the steady state. Herein we report on the measurements of secondary β-deuterium kinetic isotope effects with isotopomers of acetylthiocholine (i.e., acetyl-L$_3$-thiocholine, L = ^{1}H or ^{2}H) that provide such a probe.[6] Secondary deuterium isotope effects are inverse (<1.00) when a sp^2 hybridized reactant is converted to a quasi-tetrahedral transition state and normal (>1.00) when an sp^3 reactant state is converted to a transition state that has greater trigonal planar character.[7]

BuChE-catalyzed reactions often show substrate activation at high substrate concentrations.[3] An interaction mechanism that accords with substrate activation is outlined in Scheme 1. E and A are the enzyme and substrate, respectively, EA is the Michaelis complex, F is the acylenzyme intermediate, and EAA is the ternary complex of enzyme with substrate monomers bound at both the active and substrate activation sites. The active site of BuChE is at the bottom of a 20 Å deep gorge,[5] and the substrate activation site is thought to coincide with the peripheral site at the mouth of the gorge.[9] Substrate activation is observed because turnover of EAA via βk_{cat} ($= \beta V_{max}/[E]_T$) is faster than turnover of EA by a factor β. As Figure 1 shows, the dependence of human BuChE-catalyzed hydrolysis of acetyl-^{1}H$_3$-thiocholine on substrate concentration shows deviations from Michaelis−Menten kinetics that are consistent with substrate activation. The data in Figure 1 were fit to eq 1, derived for the kinetic mechanism of Scheme 1 (see Supporting Information for derivation of eq 1). The fit yielded the following parameters: $V_{max} = 20 \pm 2$ mA min^{-1}, $K_m = 80 \pm 20$ μM, $K_a = 3.5 \pm 0.5$ mM, and $\beta = 2.6 \pm 0.2$. Hence, the ternary EAA complex of Scheme 1 turns over to product 2.6 times faster than does the EA complex. Though substrate activation has long been observed for BuChE-catalyzed reactions, an understanding of its functional origins remains elusive. Fortunately, the β-secondary deuterium kinetic isotope effects detailed below shed important new light on this phenomenon.

Scheme 1. Kinetic Mechanism for Substrate Activation of BuChE-Catalyzed Reactions



$$v_i = \frac{V_{max}[A](1 + \beta[A]/K_A)}{K_m(1 + \beta[A]/K_A) + [A](1 + [A]/K_A)} \qquad (1)$$

The least-squares uncertainties that result from the fit in Figure 1 preclude determination of secondary isotope effects on the kinetic parameters from such experiments. An alternate approach is to determine the secondary isotope effect on the initial rate (i.e., $^{D3}v_i = v_i^{H3}/v_i^{D3}$) as a function of substrate concentration. As Figure 2 shows, the isotope effect on the initial rate increases hyperbolically as the substrate concentration increases. These data were fit to eq 2, which provides a quantitative analysis and extrapolation of the isotope effects:

$$^{D3}v_i = {}^{D3}V_{max}\frac{1 + \frac{\beta[A]}{K_A}}{1 + \frac{\beta[A]}{K_A{}^{D3}\beta}} \times \frac{[A]\left(1 + \frac{[A]}{K_A}\right) + K_m\left(1 + \frac{\beta[A]}{K_A{}^{D3}\beta}\right)}{[A]\left(1 + \frac{[A]}{K_A}\right) + K_m\left(1 + \frac{\beta[A]}{K_A}\right)} \qquad (2)$$

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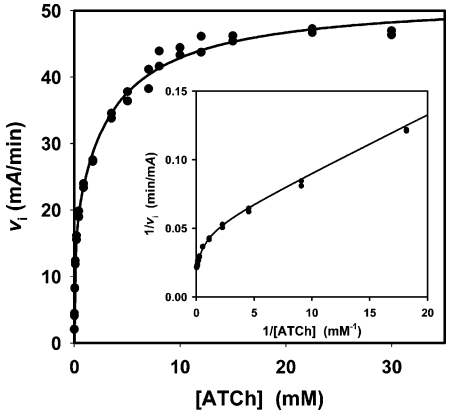

Figure 1. Dependence of initial rate on concentration of acetyl-$^1H_3$-thiocholine. Reactions were monitored by the coupled assay of Ellman et al.[8] at 27.0 ± 0.1 °C and pH = 7.24 in 0.1 M sodium phosphate buffer that contained 0.5 mM DTNB, 0.03 mg/mL of BSA, and 93 pM recombinant human BuChE. Reactions were followed at λ = 412 nm on a Molecular Devices SPECTRA$_{MAX}$ PLUS^{384} UV−visible microplate spectrophotometer. The solid line is least-squares fit to eq 1 of the text. The double-reciprocal plot in the inset clearly shows substrate activation.

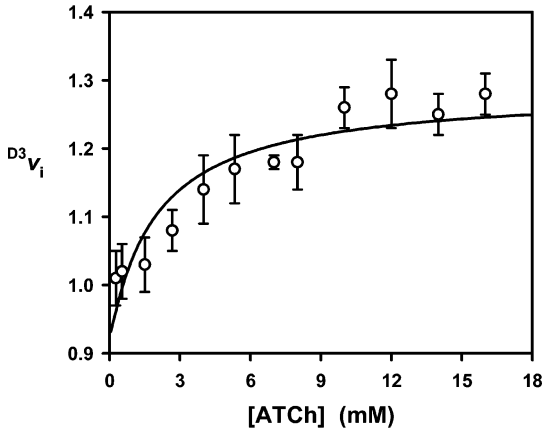

Figure 2. β-Secondary deuterium kinetic isotope effects versus substrate concentration for the recombinant human BuChE-catalyzed hydrolysis of acetyl-L_3-thiocholine (L = H or D). The solid line is a least-squares fit to eq 2 of the text.

The isotope effect extrapolated to zero concentration is $^{D3}V/K$ (the isotope effect on V_{max}/K_m), and $^{D3}\beta$ is the asymptotic value of the isotope effect on substrate activation of V_{max} at infinite concentration. Figure 2 shows a least-squares fit of the isotope effects to eq 2. In determining this fit, the parameters β, K_A, and K_m were constrained to the values determined in the substrate activation plot in Figure 1. The isotope effects that result from the fit in Figure 2 are $^{D3}V/K = 0.93 \pm 0.03$ and $^{D3}\beta = 1.38 \pm 0.06$. Hence, the isotope effect on βV_{max} is 1.29 ± 0.06.

The experiment in Figure 2 does not provide the value of the isotope effect on V_{max}, the parameter that monitors nonsubstrate-activated turnover via the EA complex of Scheme 1. To determine $^{D3}V_{max}$ and to provide a check on the value of $^{D3}V/K$ determined in Figure 2, time course data for the isotopic acetyl-L_3-thiocholines were fit to the integrated form of the Michaelis−Menten equation.[10] The experiments utilized an initial substrate concentration $[A]_0$ = 0.2 mM that was well below K_A, and therefore, the kinetics were not complicated by substrate activation. These experiments gave the following values for the isotope effects on the Michaelis−Menten parameters: $^{D3}V_{max} = 1.02 \pm 0.05$ and $^{D3}V/K = 0.97 \pm 0.06$. Both values are within experimental error of an isotope effect of 1.00, and the value for $^{D3}V/K$ indeed agrees with that determined in Figure 2. Importantly, neither isotope effect approaches the normal isotope effect on substrate-activated V_{max}. Only upon substrate activation is the marked normal isotope effect expressed.

The observation of a covalent tetrahedral adduct in the crystal structure of human BuChE[5] suggests that turnover of the acylenzyme intermediate is rate limiting for V_{max}. Therefore, the sizable normal isotope effect on substrate-activated V_{max} suggests that in the FA complex allosteric modulation of the active site is accompanied by stabilization of the tetrahedral adduct, which then becomes the predominant accumulating species in the steady state. The turnover of the tetrahedral adduct would be marked by rehybridization from sp^3 toward sp^2 in the developing acetate product, consistent with the observed normal isotope effect. Hence, observations from X-ray crystallography and from isotope effects conspire to suggest that an important element in the catalytic power of cholinesterases is their ability to stabilize tetrahedral intermediates that are, in corresponding nonenzymatic reactions, high-energy and metastable species.

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Supporting Information Available: Kinetic parameters (V_{max}, V/K) from fits of time courses to the integrated Michaelis−Menten equation for BuChE-catalyzed hydrolyses of acetyl-L_3-thiocholines. Derivations of eqs 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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(1) Abbreviations: AChE, acetylcholinesterase; ATCh, acetylthiocholine; BuChE, butyrylcholinesterase; BSA, bovine serum albumin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); mA, milli-absorbance unit.
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