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An evaluation of the inhibition of human butyrylcholinesterase and acetylcholinesterase by the organophosphate chlorpyrifos oxon

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ABSTRACT

Acetylcholinesterase (EC 3.1.1.7) and butyrylcholinesterase (EC 3.1.1.8) are enzymes that belong to the superfamily of α/β-hydrolase fold proteins. While they share many characteristics, they also possess many important differences. For example, whereas they have about 54% amino acid sequence identity, the active site gorge of acetylcholinesterase is considerably smaller than that of butyrylcholinesterase. Moreover, both have been shown to display simple and complex kinetic mechanisms, depending on the particular substrate examined, the substrate concentration, and incubation conditions. In the current study, incubation of butyrylthiocholine in a concentration range of 0.005–3.0 mM, with 317 pM human butyrylcholinesterase in vitro, resulted in rates of production of thiocholine that were accurately described by simple Michaelis–Menten kinetics, with a K_m of 0.10 mM. Similarly, the inhibition of butyrylcholinesterase in vitro by the organophosphate chlorpyrifos oxon was described by simple Michaelis–Menten kinetics, with a k_i of 3048 nM^{-1} h^{-1}, and a K_D of 2.02 nM. In contrast to inhibition of butyrylcholinesterase, inhibition of human acetylcholinesterase by chlorpyrifos oxon in vitro followed concentration-dependent inhibition kinetics, with the k_i increasing as the inhibitor concentration decreased. Chlorpyrifos oxon concentrations of 10 and 0.3 nM gave k_is of 1.2 and 19.3 nM^{-1} h^{-1}, respectively. Although the mechanism of concentration-dependent inhibition kinetics is not known, the much smaller, more restrictive active site gorge of acetylcholinesterase almost certainly plays a role. Similarly, the much larger active site gorge of butyrylcholinesterase likely contributes to its much greater reactivity towards chlorpyrifos oxon, compared to acetylcholinesterase.

Introduction

Acetylcholinesterase (EC 3.1.1.7) and butyrylcholinesterase (EC 3.1.1.8) are enzymes that belong to the superfamily of α/β-hydrolase fold proteins (Valle et al., 2008). These two enzymes share about 54% amino acid sequence identity (Lockridge et al., 1987), but differ in their specificity towards various substrates and inhibitors (Valle et al., 2008). Their crystal structures have revealed similar architecture, with one catalytic triad located at the bottom of a deep gorge (Nicolet et al., 2003; Masson et al., 2008). The hydrolysis of substrate by both enzymes proceeds through a transacylation step that involves nucleophilic and general acid-base elements (Quinn, 1987). However, the butyrylcholinesterase active site gorge is lined with 6 aromatic amino acid residues rather than the 14 found in acetylcholinesterase (Darvesh et al., 2003). Moreover, the phenylalanine residues of the acyl pocket in acetylcholinesterase (Phe-295 and Phe-297) are replaced with Lys-286 and Val-228 in butyrylcholinesterase, leading to a larger acyl pocket that can accommodate larger substrates (Vellom et al., 1993; Darvesh et al., 2003; Nicolet et al., 2003). The minimal reaction mechanism for both enzymes can be represented kinetically as shown in the upper panel of Fig. 1.

Located in the vicinity of the rim of the acetylcholinesterase gorge is a region referred to as the peripheral anionic site (which includes Tyr-72, Tyr-124, Trp-286, Tyr-341, and Asp-74) that when occupied by certain ligands, including acetylcholine or acetylthiocholine, modifies activity through steric blockade and/or induction of confirmation changes in residues within the active site gorge (Barak et al., 1995; Bourne et al., 2003). Although butyrylcholinesterase was initially thought to lack a peripheral anionic site, later studies established that Asp-70 and Tyr-332 constitute the peripheral anionic site in this enzyme (Masson et al., 1996, 2001). A more complex reaction mechanism for both enzymes, which includes binding of substrate to the peripheral anionic site, is shown in the lower panel Fig. 1.

The classic report of Main (1964) developed the inhibitory rate constant k_i to describe the inhibitory capacity of organophosphorus inhibitors towards acetylcholinesterase (Fig. 1). While the k_i scheme has served for many years as the basis for our understanding of how organophosphorus inhibitors interact with acetylcholinesterase kinetically, more recent studies have indicated that it is incomplete in certain instances. Studies from this laboratory have documented that

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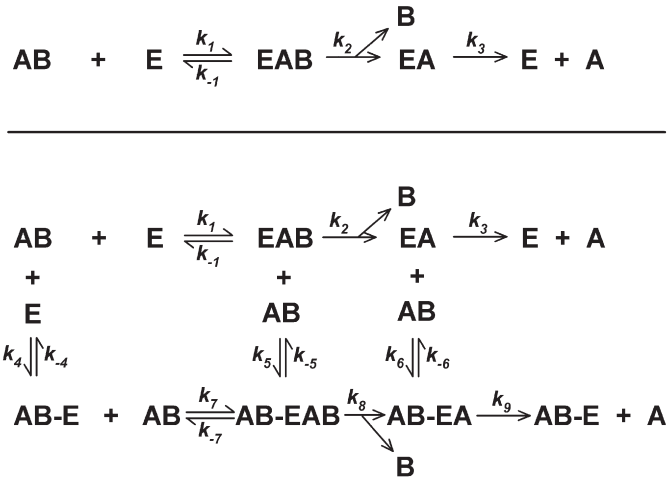

Fig. 1. Proposed kinetic schemes descriptive of the interactions of substrates or organophosphorus inhibitors with human acetylcholinesterase or butyrylcholinesterase. The upper panel contains the minimal reaction scheme based on simple Michaelis–Menten kinetics where in the case of an organophosphorus inhibitor, $k_i = k_2/K_D$ (K_D = [E]*[AB]/([E] − [AB] and $K_D = k_{-1}/k_1$) (developed by Main, 1964). E designates free enzyme; AB represents substrate or inhibitor; EAB represents the substrate or inhibitor reversibly bound to enzyme (Michaelis complex); EA signifies the acylated or phosphorylated intermediate; B represents the leaving group; and A designates acetate (for the substrates acetylcholine or acetylthiocholine), butyrate (for the substrates butyrylcholine or butyrylthiocholine), or the di-alkoxy phosphate moiety (for an organophosphate). The lower panel contains a more complex kinetic scheme where the substrate or inhibitor also binds reversibly to a secondary site, thereby altering events at the active site. In this lower panel, AB–E represents substrate or inhibitor bound reversibly to a secondary site; AB–EAB designates enzyme with reversibly bound substrate or inhibitor at both the active site and a secondary site; and AB–EB represents enzyme acylated or phosphorylated at the active site, with substrate or inhibitor bound reversibly to a secondary site. All other symbols have the same meaning as in the upper panel.

certain oxons of organophosphorus insecticides, such as chlorpyrifos oxon or methyl paraoxon, have k_is towards acetylcholinesterase in vitro that change as a function of inhibitor concentration, thereby displaying concentration-dependent inhibition kinetics (Rosenfeld and Sultatos, 2006; Kaushik et al., 2007; Sultatos, 2007; Sultatos and Kaushik, 2008). Stated differently, these studies have demonstrated that the capacity of individual inhibitor molecules to inhibit acetylcholinesterase (as evidenced by the k_i) decreases as the inhibitor concentration increases. Interestingly, this concentration-dependent inhibition kinetics is likely not mediated through the peripheral anionic site since chlorpyrifos oxon did not displace the peripheral anionic site ligand thioflavin t (Sultatos and Kaushik, 2008).

The current report was undertaken to determine if inhibition of human butyrylcholinesterase by chlorpyrifos oxon displays concentration-dependent inhibition kinetics, as is the case with acetylcholinesterase. Given the documented similarities and differences between acetylcholinesterase and butyrylcholinesterase, comparisons of patterns of their inhibition by chlorpyrifos oxon could yield important insights into the mechanism of concentration-dependent inhibition kinetics.

Materials and methods

Chemicals. Chlorpyrifos oxon (*O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphate) was purchased from Chem Services (West Chester, PA). Human recombinant acetylcholinesterase, human butyrylcholinesterase, and all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

Measurement of enzyme activity. Acetylcholinesterase activity was monitored with the substrate acetylthiocholine in a plate reader (BIOTEK Instruments, Winoski, VT), as previously described (Kaushik et al., 2007). Butyrylcholinesterase activity was measured by conventional and stopped-flow spectroscopy. For conventional spectroscopy, a Shimadzu UV-2550 UV-Vis spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) was used at 24° with the wavelength set at 412 nm. The incubation volumes were 1 ml, containing 317 pM butyrylcholinesterase, 0.1 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), and various concentrations of the substrate butyrylthiocholine ranging from 0.005 to 3 mM. The concentration of butyrylcholinesterase was determined by titration with chlorpyrifos oxon (Amitai et al., 1998). Incubations were typically 10–15 min and were linear throughout. The change in optical density was converted to amount of thiocholine produced by construction of a standard curve with glutathione serving as the sulfhydryl source for DTNB (this is possible since the increased optical density at 412 nm results from the liberation of 5-thio-2-nitro-benzoate upon reaction of DTNB with a sulfhydryl group (Ellman et al., 1961)).

For stopped-flow spectroscopy, a SX20 stopped-flow spectrophotometer (Applied Photophysics Limited, Leatherhead, UK) was used. The instrument had a 20 μl flow cell at 24°, with the light path set at 10 mm. The wavelength was set to 412 nm, with monochromator slit openings of 5 mm. All solutions were prepared in 100 mM sodium phosphate buffer (pH 7.4), and the flow cell contained 0.5 mM butyrylthiocholine, various concentrations of butyrylcholinesterase ranging from 100 to 400 pM, and 1 mM DTNB. The concentration of DTNB was increased, compared to that used in conventional spec-

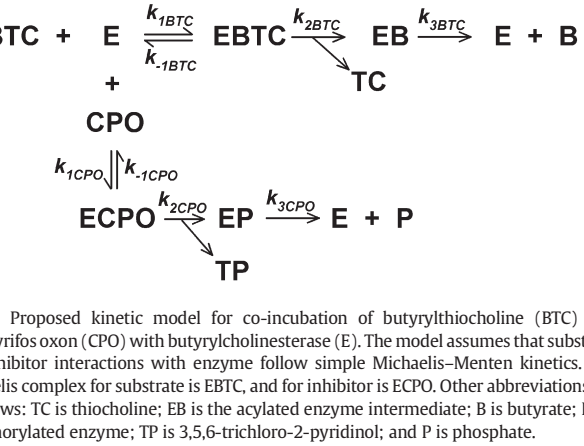

Fig. 2. Proposed kinetic model for co-incubation of butyrylthiocholine (BTC) and chlorpyrifos oxon (CPO) with butyrylcholinesterase (E). The model assumes that substrate and inhibitor interactions with enzyme follow simple Michaelis–Menten kinetics. The Michaelis complex for substrate is EBTC, and for inhibitor is ECPO. Other abbreviations are as follows: TC is thiocholine; EB is the acylated enzyme intermediate; B is butyrate; EP is phosphorylated enzyme; TP is 3,5,6-trichloro-2-pyridinol; and P is phosphate.

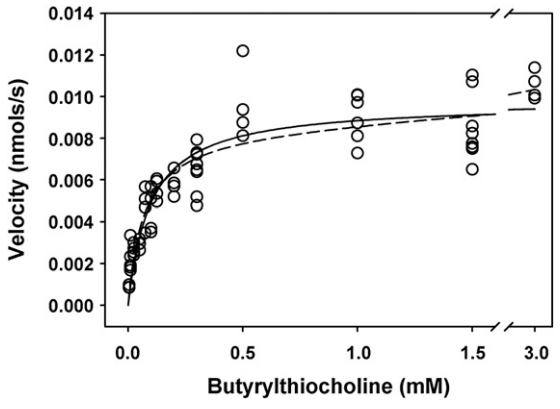

Fig. 3. Determination of kinetic parameters descriptive of the hydrolysis of butyrylthiocholine by human butyrylcholinesterase. The open circles represent empirical data, where each circle is indicative of a single determination of the slope of linear production of thiocholine over 10 min at 24°. The enzyme active site concentration was 317 pM. Fitting the data to the Michaelis–Menten equation (Eq. (1)) or the Haldane equation (Eq. (2)) yielded overlapping, indistinguishable fitted curves that are represented by the solid line. The dashed line designates the curve fit to the Webb equation (Eq. (3)). Kinetic parameters are shown in Table 1.