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Altered binding of thioflavin t to the peripheral anionic site of acetylcholinesterase after phosphorylation of the active site by chlorpyrifos oxon or dichlorvos

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ABSTRACT

The peripheral anionic site of acetylcholinesterase, when occupied by a ligand, is known to modulate reaction rates at the active site of this important enzyme. The current report utilized the peripheral anionic site specific fluorogenic probe thioflavin t to determine if the organophosphates chlorpyrifos oxon and dichlorvos bind to the peripheral anionic site of human recombinant acetylcholinesterase, since certain organophosphates display concentration-dependent kinetics when inhibiting this enzyme. Incubation of 3 nM acetylcholinesterase active sites with 50 nM or 2000 nM inhibitor altered both the B_{max} and K_d for thioflavin t binding to the peripheral anionic site. However, these changes resulted from phosphorylation of Ser203 since increasing either inhibitor from 50 nM to 2000 nM did not alter further thioflavin t binding kinetics. Moreover, the organophosphate-induced decrease in B_{max} did not represent an actual reduction in binding sites, but instead likely resulted from conformational interactions between the acylation and peripheral anionic sites that led to a decrease in the rigidity of bound thioflavin t. A drop in fluorescence quantum yield, leading to an apparent decrease in B_{max}, would accompany the decreased rigidity of bound thioflavin t molecules. The organophosphate-induced alterations in K_d represented changes in binding affinity of thioflavin t, with diethylphosphorylation of Ser203 increasing K_d, and dimethylphosphorylation of Ser203 decreasing K_d. These results indicate that chlorpyrifos oxon and dichlorvos do not bind directly to the peripheral anionic site of acetylcholinesterase, but can affect binding to that site through phosphorylation of Ser203.

Introduction

Previous studies have reported that certain oxygen analogs of organophosphorus insecticides display concentration-dependent inhibition of the enzyme acetylcholinesterase (EC 3.1.1.7) (Kardos and Sultatos, 2000; Kousba et al., 2004; Rosenfeld and Sultatos, 2006; Kaushik et al., 2007). For example, Kaushik et al. (2007) have shown that the inhibitory capacity of chlorpyrifos oxon (*O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphate) (Fig. 1) decreased as the chlorpyrifos oxon concentration increased up to about 10 nM, but failed to change further at higher oxon levels. While the mechanism of this phenomenon is not known, the reversible binding of these inhibitors to a secondary site on acetylcholinesterase that modulates events at the active site gorge remains a plausible explanation. Such putative binding has been particularly intriguing since a secondary binding site (known as the peripheral anionic site) for acetylcholine and other ligands has been identified and characterized (Bergmann et al., 1950; Changreux, 1966; Aldridge and Reiner, 1969; Taylor and Radić, 1994; Barak et al., 1995; Bourne et al., 2003, 2006; Colletier et al., 2006). Occupation of the peripheral anionic site, which is located on the rim of the active site gorge (Bourne et al., 2006), has been shown to lead to decreased acetylthiocholine hydrolysis through steric blockade of the released thiocholine (Szegletes et al., 1999; Mallender et al., 2000; Johnson et al., 2003; Bourne et al., 2006; Colletier et al., 2006). Although steric blockade of the 3,5,6-trichloro-2-pyridinol released upon phosphorylation of Ser203 by chlorpyrifos oxon could not account for the concentration-dependent inhibition by this organophosphate (Sultatos, 2007), the possibility of the binding of this inhibitor to the peripheral anionic site nevertheless should be considered in view of the role this site can play in modulating events within the active site gorge (Bourne et al., 2006).

Thioflavin t (3,6-dimethyl-2-(4-dimethylaminophenyl)-benzothiazolium) (Fig. 1) is a benzothiazolium dye that has been used to stain amyloid tissues (Vassar and Culling, 1959; LeVine, 1999) as well as DNA (Ilanchelian and Ramaraj, 2004). Interestingly, this fluorophore has been shown to bind selectively to the peripheral anionic site of acetylcholinesterase, and increase its fluorescence by more than 1000-fold over unbound thioflavin t (De Ferrari et al., 2001), thereby identifying it as a valuable tool for studying ligand interactions with the peripheral anionic site (De Ferrari et al., 2001, and Johnson et al., 2003). The fluorescent properties of thioflavin t have been studied by Stsiapura et al. (2007), who have documented that thioflavin t has a nonplanar conformation in its ground state (unexcited), with a torsion angle φ of about 37° between the benzthiazole and the dimethylaminobenzene rings (Fig. 1). When excited by light, thioflavin t has been reported to undergo a twisted internal charge transfer, where the torsion angle φ increases to about 90° (Stsiapura et al., 2007).

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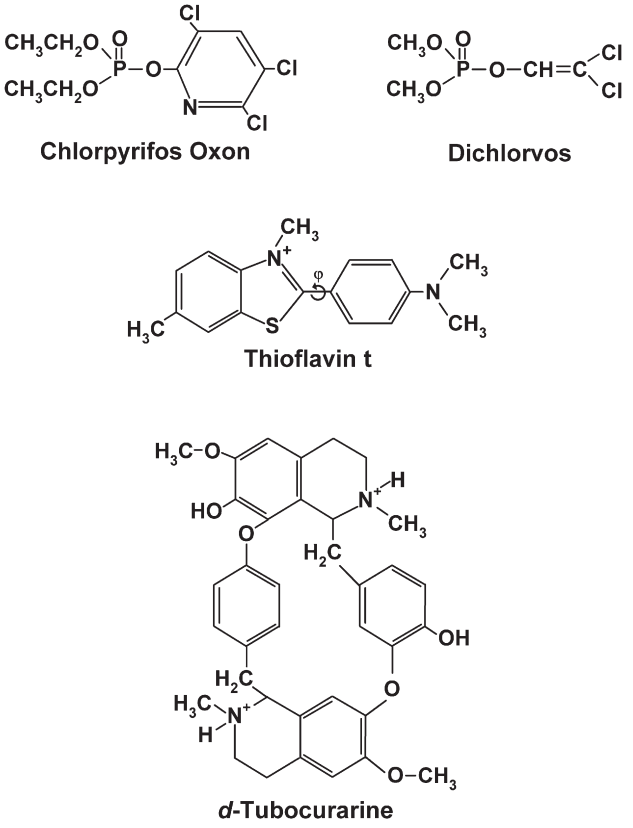

Fig. 1. Chemical structures of chlorpyrifos oxon, dichlorvos, thioflavin t, and *d*-tubocurarine. For thioflavin t, the torsion angle φ represents the site of rotation that can occur upon excitation with light (Stsiapura et al., 2007).

However, if thioflavin t is located in a more rigid microenvironment, such as in a viscous solvent or bound to a protein, the internal rotation of the torsion angle φ has been reported to be blocked upon excitation by light, and the fluorescence quantum yield of this more rigid thioflavin t molecule increases markedly (Stsiapura et al., 2007).

In the current study, thioflavin t has been used as a tool to determine if the organophosphates chlorpyrifos oxon and dichlorvos

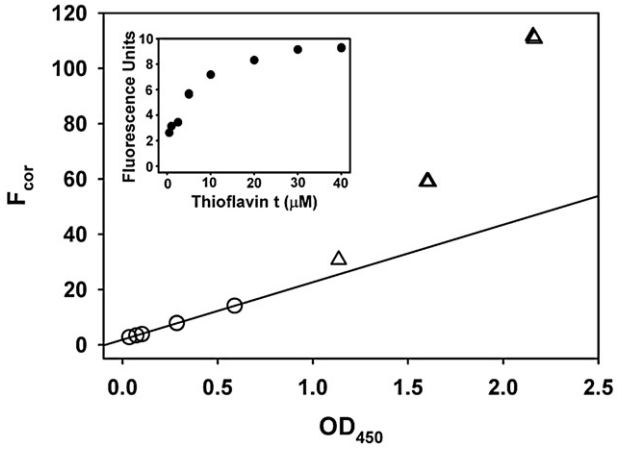

Fig. 2. Relationship between F_{cor} and absorbance of thioflavin t. The open circles represent thioflavin t concentrations of 10 μM and lower, while the open triangles represent thioflavin t concentrations of 20 μM and greater. The solid line signifies the linear regression of thioflavin t concentrations of 10 μM and lower, demonstrating the deviation from linearity of the higher thioflavin t concentrations. The inset shows the achievement of a plateau in F_{ob} at thioflavin t levels of 20 μM and higher. Two observations were performed at each thioflavin t concentration, although some data points are not apparent because of the low variability of the data.

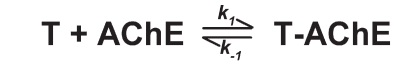

Fig. 3. Kinetic scheme descriptive of the binding of thioflavin t to the peripheral anionic site of acetylcholinesterase. In this scheme T and AChE represent free thioflavin t and acetylcholinesterase, respectively, while T-AChE represents thioflavin t bound reversibly to the peripheral anionic site. The dissociation constant, K_d, is defined as k_{-1}/k_1.

(2,2-dichlorovinyl dimethylphosphate) (Fig. 2) bind to the peripheral anionic site of human recombinant acetylcholinesterase. Chlorpyrifos oxon and dichlorvos were selected, in part, because they are diethyl and dimethyl organophosphates, respectively (Fig. 1), and because they do not significantly absorb or fluoresce at the wavelengths required to assess the binding of thioflavin t to the peripheral anionic site. Additionally, the inhibitory capacity of chlorpyrifos oxon has been shown to change as a function of inhibitor concentration (Kaushik et al., 2007).

Methods

Chemicals. Chlorpyrifos oxon and dichlorvos were purchased from Chem Services (West Chester, PA). Stock solutions were made in ethanol, and stored at −20° until use. Human recombinant acetylcholinesterase and all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

Kinetic studies. All incubations were carried out in 100 nM sodium phosphate buffer (pH 7.4) containing bovine serum albumin at a concentration of 1 mg/ml in order to stabilize recombinant acetylcholinesterase (Shafferman et al., 1992, and Rosenfeld and

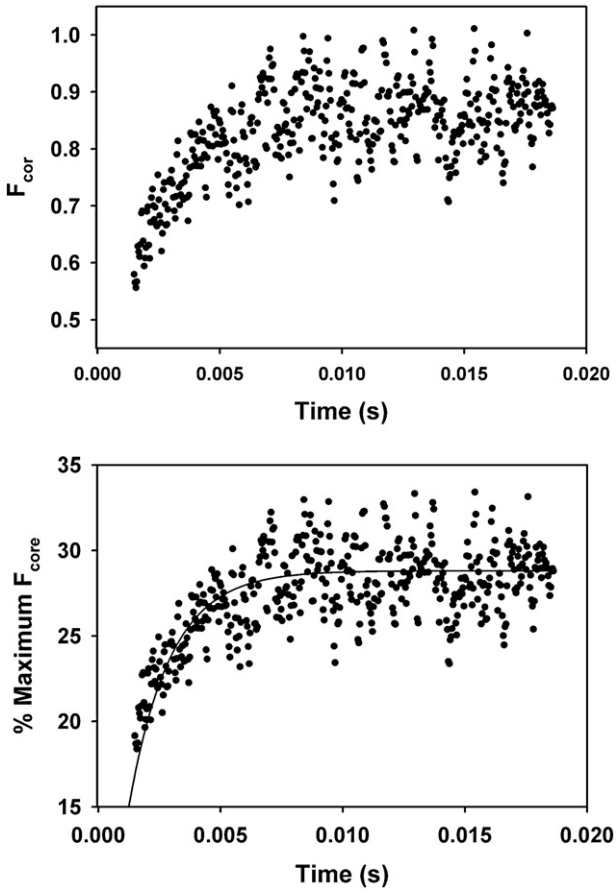

Fig. 4. An example of the enhanced fluorescent signal (F_{cor}) of thioflavin t resulting from its binding to the peripheral anionic site. The upper panel shows F_{cor} with time following the mixing of 100 nM thioflavin t and 3 nM acetylcholinesterase. F_{core} is the mean of all F_{cor} values after 0.01 s (at equilibrium), and was found to be 0.86 in this example. The lower panel shows the same data transformed as a percentage of the maximum fluorescence signal possible (3.01, taken from Fig. 5). The solid line in the lower panel presents the optimized solution of Eqs. (3) (4) (5), which yielded the following values: K_d = 245 nM; k_1 = 1.7 nM^{-1} s^{-1}; and k_{-1} = 416.5 s^{-1}.