Differentiation between Acetylcholinesterase and the Organophosphate-inhibited Form Using Antibodies and the Correlation of Antibody Recognition with Reactivation Mechanism and Rate*

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Organophosphate esters (OPs)1 are a diverse class of compounds (Fig. 1 and Table I) with the most well known applications as insecticides (e.g. malathion and parathion), as protease inhibitors (e.g. diisopropyl fluorophosphate (DFP)), in glaucoma treatment (e.g. echothiophate), and as chemical warfare agents (e.g. sarin and VX). The acute toxic effects of OP compounds correlate well with their ability to inhibit acetylcholinesterase (AChE) by reaction with an essential serine hydroxyl to form a relatively stable phosphoserine ester bond or an OP-AChE conjugate (Fig. 1 and Table I, 2) (1, 2). AChE inactivation results in an accumulation of acetylcholine in cholinergic synapses and, depending on the OP structure and persistence of the OP-AChE conjugate, excessive stimulation of acetylcholine receptors. Excess acetylcholine leads to hyper-stimulation of skeletal muscle endplates, smooth muscle, and secretory glands as well as altered central nervous system and cardiac activity, which can result in cardiovascular and respiratory compromise and, in extreme cases, death (3).

The biochemical mechanism of OP inactivation of AChE is a widely accepted process and initiated by precursory phosphorylation at the catalytic serine residue (Fig. 1) (4). The phosphorylation of AChE by an OP is synchronous with the ejection of a leaving group (Z) to yield a stable, covalent phosphoserine ester bond (Fig. 1 and Table I, 2). The phosphorylated AChE can usually undergo two possible postinhibition fates: (a) reactivation, which is cleavage of the phosphoester-serine bond either spontaneously (water) or mediated by oxime antidotes or (b) “aging,” in which a phosphate bond is cleaved to produce a phosphate oxynion (Fig. 1 and Table I, 3). An “aged” OP-AChE conjugate is considered irreversible inhibition and typically unable to regain enzymatic activity. The inhibition and postinhibition rates and mechanisms are dependent on the structure and reactivity of the phosphorus ester ligands (4, 5). For example, OP-AChE conjugates that contain branched dimethyl esters generally reactivate readily, whereas OP-AChE conjugates that contain larger or branched alkyl groups (e.g. isopropyl, isobutyl, etc.) are more prone to undergo an aging mechanism. Overall OP compounds react with AChE to afford structurally precise phosphoserine esters suggesting that these products can serve as selective indicators of mechanism and possibly a direct correlation with the original OP structure.

Human exposure to OPs is assessed clinically by measuring the depression in serum or erythrocyte cholinesterase activity utilizing a colorimetric assay (6). The colorimetric assay is considered rapid and capable of high throughput analysis, however, several limitations to this method exist: (a) control levels of cholinesterase activity must be obtained prior to analysis of OP exposure, (b) cholinesterase activity and exposure level are not directly correlated, and most importantly (c) this assay does not identify the structure of the OP-AChE conjugates. As indicated, different OP structures can form distinct OP-AChE conjugates that can yield different toxic outcomes, and therefore the need to determine the structure of the OP-AChE conjugate...
is essential to understand the reactivation and/or aging profile for a given OP (3, 7–9).

In this study, we sought to utilize the exquisite selectivity of antibody-antigen interactions to differentiate between AChE and AChE modified by reactive phosphorus compounds. These mechanism-based antibodies can then serve as probes of the phosphorylation/dephosphorylation process and possible indicators of enzyme reaction rate. And since the resultant OP conjugate typically correlates directly with the OP structure, an antibody-based analysis could improve upon the current, end point-based colorimetric method. To test the hypothesis that OP-AChE conjugates can be distinguished from native, uninhibited AChE, polyclonal antiserum (anti-AChE<sub>10S</sub>) was raised against a linear decapeptide corresponding to the native mouse and human AChE sequence (Fig. 1, 4a) that includes the catalytic serine residue. To represent OP-modified AChE and to serve as an antigen distinct from native protein, a phosphoserine (Ser-OPO<sub>3</sub><sup>2-</sup>) decapeptide (Fig. 1, 4b) was used to generate a second polyclonal antiserum (anti-AChE<sub>10SP</sub>). The selection of decapeptide serine phosphate as an antigen is consistent with the proposed inhibition of AChE by phosphoryl oxychloride (POCl<sub>3</sub>) (10). The initial OP-AChE conjugate resulting from reaction of POCl<sub>3</sub> with AChE is a chlorophosphate (10). The initial OP-AChE conjugate was hypothesized to better react with aged AChE than did anti-AChE<sub>10S</sub>, and overall, the two antisera should differentiate between native, inhibited, and aged AChE. A critical aspect of biomolecular recognition of the catalytic serine of AChE is its location at a deep 18-Å gorge within the protein (11) and is largely inaccessible to many traditional forms of analysis unless the protein is denatured. The experiments described here involve reacting the intact, catalytically active enzyme with OP, measuring the activity of the enzyme, and then denaturing the enzyme to expose the active site to antibodies. This approach permits the study of an “inaccessible,” catalytic peptide sequence and allows the corresponding covalent phosphorylation by OP that will enable the determination of mechanism and reaction progress.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Recombinant mouse AChE (rMoAChE) was purified as described previously (12). Electric eel AChE (Type V-S) and horse butyrylcholinesterase were used as purchased (Sigma). Anti-mAChE antiserum is an affinity-purified polyclonal antiserum raised against monomeric rMoAChE enzyme. POCl<sub>3</sub> and DFP were purchased from Aldrich, and paraoxon was purchased from Chem Service Inc. (West Chester, PA). The S,S-stereoisomer of isomalathion was synthesized as described previously (13). Reagents for immunofluorescence purification were purchased from Bio-Rad.

**Polynic Antiser Generaion and Purification**—Anti-AChE<sub>10S</sub> and anti-AChE<sub>10SP</sub> antisera were generated by conjugating peptides AChE<sub>10S</sub> 4a and AChE<sub>10SP</sub> 4b (Fig. 1) to keyhole limpet hemocyanin. Rabbits were immunized using standard procedures (SynPep Corp., Dublin, CA). Antisera were equilibrated into TSSA buffer (20 mM Tris-HCl, pH 8.0, 25 mM NaCl, 0.02% NaN<sub>3</sub>) with Ecospan 10D columns. Contaminants were removed by chromatography over a DEAE Affi-Gel Blue column. Peptides 4a and 4b were conjugated to Affi-Gel 15 beads in anhydrous isopropanol for 24 h at 4 °C. Antisera were purified over the peptide-conjugated column by eluting less specific antisera with 1 M NaSCN. Peptide-specific antisera were eluted with 0.1 M glycine-HCl (pH 1.8) and immediately buffered to pH 8.0. All eluted fractions were analyzed by enzyme-linked immunosorbent assay for recognition of keyhole limpet hemocyanin, AChE<sub>10S</sub> and AChE<sub>10SP</sub> to ensure high specificity recognition (14).

**Reaction of Organophosphates with rMoAChE**—rMoAChE was diluted in 0.1 M phosphate buffer (pH 7.6) (final concentration, 110 nM) prior to OP addition. OP compounds were diluted in appropriate vehicle, and either vehicle or OP were added. Final concentrations of OP and their respective vehicles used in experiments were as follows: DFP (136 μM in isopropanol), POCl<sub>3</sub> (3 μM in tetrahydrofuran), isomalathion (526 μM in ethanol), and paraoxon (750 μM in acetonitrile). All vehicles were added to <5% final volume and had no significant effect on rMoAChE activity or reactivity with anti-mAChE antisera (data not shown).

**Western Immunoblot Assays**—Western immunoblot assays were performed as described (15) with the following minor modifications. Primary antibodies were diluted 1:10,000 in blocking buffer (2% bovine serum albumin, phosphate-buffered saline, 0.1% Tween 20). Secondary antibodies were 1:100 in blocking buffer (2% bovine serum albumin, phosphate-buffered saline, 0.1% Tween 20). Secondary antibodies were diluted 1:10,000 in blocking buffer. Densitometric measurements were performed using a Bio-Rad GS800 densitometer and Quantity One software.

**AChE Activity Assays**—rMoAChE activity was measured colorimetrically as described previously (6, 15, 16). Kinetic activity assays were performed by incubation of AChE samples with 5,5-dithiobis(2-nitro-
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RESULTS

Anti-AChE108 Recognition of Cholinesterases Anti-AChE108 was tested for reactivity with cholinesterases from several different sources. The decapeptide used as the antigen to generate anti-AChE108 is 100% homologous with rMoAChE and horse butyrylcholinesterase (Fig. 1, 4a) and differs from electric eel AChE by one amino acid (Leu → Ile). Anti-AChE108 recognized rMoAChE, electric eel AChE, and horse butyrylcholinesterase (Fig. 2A), and the molecular mass of the band in the rMoAChE sample was ~60 kDa, which corresponds with the correct molecular mass due to truncation at the carboxyl terminus of the cDNA at amino acid 548 (12). Anti-AChE108 detected two bands in the electric eel AChE sample. One band at ~65 kDa corresponds to the correct mass for intact protein (19); however, the major band recognized by anti-AChE108 migrates at ~43 kDa. This has been observed by our laboratory in other antibody analyses of eel AChE (15) and presumably corresponds to partially degraded enzyme. The band detected in the horse butyrylcholinesterase samples migrates at ~65 kDa, which corresponds to the predicted size (20).

Anti-AChE108 Antiserum Recognition of Native, Uninhibited rMoAChE and Anti-AChE108 Recognition of POCl3-treated rMoAChE—To characterize the specificity of the antiserum, rMoAChE was first treated with POCl3 to reduce the AChE activity to less than 5% of control activity (Table II). The inhibited rMoAChE samples were then analyzed by immunoblot. Anti-AChE108 clearly recognized native and vehicle-treated rMoAChE but only weakly recognized the inactive, POCl3-treated rMoAChE (Fig. 2B, top panel), the latter recognition likely due to partially reversible or incomplete interaction of POCl3 with rMoAChE. This result demonstrated that anti-AChE108 recognized the native active site and that phosphorylation of the active site serine is sufficient to disrupt anti-AChE108 recognition. Anti-AChE108SP recognized only POCl3-treated rMoAChE (Fig. 2B, bottom panel), which indicates that the phosphorylation of serine is required for anti-AChE108SP recognition.
**Anti-AChE$_{10SP}$ Recognition Is Specific for POCl$_3$-treated rMoAChE**—To further characterize the specificity of anti-AChE$_{10SP}$, OP-AChE conjugates were generated from the reaction of rMoAChE with three additional OP compounds, and the recognition was tested. rMoAChE was reacted with isomalathion, paraoxon, or DFP, and rMoAChE activity was determined to be less than 5% of control (Table II). Inhibited rMoAChE samples were analyzed for antibody recognition by immunoblot analysis. Anti-AChE$_{10S}$ recognized the vehicle-treated rMoAChE but did not recognize any of the OP-AChE conjugate samples (Fig. 2C, top panel), although it slightly recognized rMoAChE treated with POCl$_3$ (as indicated previously). These results demonstrate that regardless of the OP ester ligands, the covalently OP-modified active sites of rMoAChE disrupted anti-AChE$_{10S}$ recognition. Anti-AChE$_{10SP}$ recognized the rMoAChE treated with POCl$_3$ (Fig. 2C, middle panel) but not native rMoAChE or rMoAChE inhibited by the other OPs. As a positive control to determine the relative protein amount and integrity, immunoblots were probed with a polyclonal anti-mAChE antiserum (Fig. 2C, bottom panel). The anti-mAChE antiserum showed equivalent recognition of all samples and also supports the conclusion that OP treatment did not affect the integrity of the enzyme.

**Anti-AChE$_{10SP}$ Recognition Is Specific for the Phosphorylated (Dianionic) Active Site of rMoAChE**—Because POCl$_3$ can act as a non-selective phosphorylating agent and potentially react with AChE at multiple residues (21), the possibility existed that anti-AChE$_{10SP}$ recognized phosphorylated residues other than the catalytic serine. To address this, a competition experiment was conducted in which rMoAChE was reacted first with DFP, which phosphorylates only the catalytic serine (22, 23). Next POCl$_3$ was added to the DFP-inhibited rMoAChE to phosphorylate any other reactive residues. If anti-AChE$_{10SP}$ is capable of recognizing phosphorysereine residues other than the active site, recognition of this sample preparation should occur. Conversely rMoAChE was reacted stepwise with POCl$_3$ and then DFP to form the expected phosphorysereine group at the active site. For DFP to fully protect the active site serine from POCl$_3$, it was necessary to allow the diisopropyl phosphorylated rMoAChE, the DFP-AChE conjugate, to age (4, 5). Therefore, the length of time of the pretreatment was increased to allow a maximum amount of DFP-AChE conjugates to age as determined colorimetrically. After the initial exposure, DFP and POCl$_3$ inhibited rMoAChE activity to less than 5% of either control (data not shown). After reactivation of the remaining DFP-AChE population with 2-PAM, the DFP-treated rMoAChE reactivated slightly (<5%), and the POCl$_3$-treated rMoAChE did not reanimate, indicating maximum aging (data not shown). Immunoblots of these pairwise competition experiments showed that anti-AChE$_{10SP}$ recognized both rMoAChE treated with POCl$_3$ followed by vehicle and rMoAChE treated with POCl$_3$ followed by DFP (Fig. 3). However, anti-AChE$_{10SP}$ did not recognize rMoAChE treated with DFP followed by vehicle or, more importantly, rMoAChE treated with DFP followed by POCl$_3$ (Fig. 3). This result supports our hypothesis that anti-AChE$_{10SP}$ is specific for the phosphoserine (Y = O−) in the active site and not a random phosphoserine or other phosphorylated group resulting from reaction with POCl$_3$.

**Anti-AChE$_{10SP}$ Recognition of Oxime-reactivated rMoAChE**—When possible, OP-AChE conjugates can reanimate, a process resulting in restored AChE activity. Reactivation occurs via scission of the phosphoserine ester bond either spontaneously or promoted chemically and kinetically with oxime antidotes (e.g. 2-PAM). Since anti-AChE$_{10S}$ antibody specifically recognizes uninhibited rMoAChE and not OP-modified rMoAChE, it was of interest to determine whether reanimated enzymatic activity could be correlated with anti-AChE$_{10SP}$ recognition.

To study the reactivation of OP-AChE conjugates, rMoAChE was treated with paraoxon, and the inhibition- reactivation processes were monitored versus time using the anti-AChE$_{10S}$ antibody. Paraoxon was used for this experiment instead of POCl$_3$ because the reactivation of paraoxon-inhibited AChE is known and the postinhibitory rate of aging for paraoxon-inhibited AChE is slow (α$_{1/2}$ = 24 h) compared with POCl$_3$ (α$_{1/2}$ = 5, 10, 24). To achieve the maximum reactivation of the paraoxon-AChE conjugate and test the specificity of anti-AChE$_{10S}$, rMoAChE was inhibited to less than 5% of the original activity with paraoxon (as described previously) and treated with increasing concentrations (100–400 µM) of 2-PAM versus time. The addition of 2-PAM at 100 µM showed only a slight effect on reactivating paraoxon-inhibited AChE, whereas the addition of 2-PAM at 200–400 µM concentrations afforded proportionally greater percent reactivation to a maximum of 70% of the original activity. The time course of the paraoxon-AChE conjugate reactivation shows a large initial reactivation within 10 min that accounts for about 75% of the reactivation and another modest increase after 70 min (Fig. 4). The return of over 70% of the original activity at 90 min shows that a significant population of inhibited rMoAChE can be regenerated following near complete inhibition of the enzyme. Although the addition of 100 µM 2-PAM did not restore much reactivity, the possibility exists that 2-PAM removed the enzyme to its native active form due to denaturation or other consequence of the experiment. Therefore, in these experiments a small percentage of rMoAChE may be inactive yet contain the native sequence that is recognized by anti-AChE$_{10S}$.

**To test the hypothesis that anti-AChE$_{10S}$ is capable of quantifying total populations of active and reactivated AChE enzyme, rMoAChE was inhibited by paraoxon,** and the reactivation process was monitored at 15 and 90 min kinetically via the colorimetric assay and probed mechanically via recognition with anti-AChE$_{10S}$. After inhibition with paraoxon, samples...
were reactivated with 2-PAM (100–300 μM) for 15 min and simultaneously measured for AChE activity and recognition by anti-AChE10S. The relative band density as recognized by anti-AChE10S was plotted against the percent reactivation and, as evaluated by linear regression, showed an excellent correlation ($R^2 = 0.902$) with slope $2.10$. The paraoxon-inhibited rMoAChE samples reactivated to 8–24% of control activity when treated with 2-PAM, but without 2-PAM no reactivation occurred. Recognition by anti-AChE10S of these same samples increased from near zero for inhibited rMoAChE (no 2-PAM treatment) to 21–52% of control when treated with 2-PAM. Analysis of the samples with anti-mAChE was conducted to ensure equal sample loading (Fig. 5A).

The correlation between the colorimetric assay and anti-AChE10S recognition was next examined after a 90-min reactivation period anticipating an increase in percent reactivation and a corresponding correlation with anti-AChE10S recognition. rMoAChE was inhibited with paraoxon and treated with 2-PAM (100–350 μM), and the reactivation was allowed to proceed for 90 min whereupon the percent reactivation increased to 56–103% of control. Recognition by anti-AChE10S also increased to 49–158% of control (Fig. 5B). Similar to the 15-min reactivation data, a strong correlation ($R^2 = 0.819$) with slope = 2.10 was found linking the kinetic data and anti-AChE10S recognition.

**DISCUSSION**

In this report, we describe the rationale for and characterization of two polyclonal antisera raised against native and phosphate-modified active sites of AChE. As presented previously, OPs can vary in chemical composition leading to a panel of possible OP-inhibited AChE structures. Moreover each individual OP compound can lead to more than one OP-AChE conjugate structure by virtue of aging or other postinhibitory processes. On this basis, a number of possible OP-AChE structures are possible, yet little work has been done to develop methods or tools capable of measuring or differentiating between native AChE and the OP-AChE conjugate structures. Critical questions in this study were whether or not antibodies could be generated against a catalytic active site sequence of an enzyme, whether these antibodies would differentiate between native and modified enzyme, and finally would analysis on a denaturing gel allow for investigation of active site modifications that are buried deep within a protein structure. In sum, the development of these mechanism-based antibodies was envisioned to help identify OP-AChE conjugate structures, differentiate OP-AChE conjugates, and monitor reaction (inhibition/reactivation) progress.

Antibodies raised against native and OP-inhibited AChE have been used to probe biochemical properties of AChE including (a) the three-dimensional structure of AChE (25), (b) allosteric influences over catalytic activity (26–28), and (c) structural characteristics between isoforms of AChE (29, 30). In one of these studies, antibodies were raised against a synthetic peptide corresponding to the active site sequence of *Torpedo californica* AChE for the purpose of determining whether the active site was present on external or internal domains of the enzyme (25).
Anti-AChE10S antisera was found to differentiate between native, uninhibited rMoAChE and rMoAChE that had been reacted with several types of OP compounds. The only "nonspecific" interaction noted was in the modest recognition of POCI₃-treated rMoAChE by anti-AChE10S. Among the many reasons for this nonspecific interaction, one possibility is that the phosphate group may have been ejected, thereby reactivating the AChE and allowing recognition by anti-AChE10S. Alternatively the phosphoserine group could have been eliminated to form a dehydroalanine, which may allow recognition by anti-AChE10S. In support of the latter outcome, anti-AChE10S recognition of peptide 4a was compared with a decapeptide that has the identical sequence of 4a except that it contains an alanine substitution for the serine residue (4aAsn). By enzyme-linked immunosorbent assay, anti-AChE10S was capable of significant recognition of 4aAsn, albeit less than the recognition of 4a (data not shown). This indicates that anti-AChE10S may be capable of recognizing AChE with a dehydroalanine in the active site.

Anti-AChE10S is also capable of recognizing rMoAChE that has been previously inhibited and reactivated with an oxime. Importantly the restoration of enzyme function and anti-AChE10S recognition of the reactivated enzyme correlated very well. The slopes of both linear regression plots (Fig. 4) are greater than 1, suggesting that the antibody-based analysis better evaluates the molecular state of the active site than the colorimetric activity assays measure. This may be due to the fact that although scission of the phosphoryl group from the active site serine (reactivation) affords the correct epitope for antibody recognition, removal of the phosphoryl group may not necessarily restore the enzyme activity due to protein denaturation or other disruptive pathway. These results demonstrate that a strong correlation can be achieved between AChE activity and antibody recognition and also that antibodies modified to modified active site sequences can complement colorimetric enzyme assays in understanding the nature and extent of the inhibition process.

Although the colorimetric assay used to evaluate OP exposure provides rapid and reliable data on cholinesterase activity, it fails to identify the structure of the insulating OP agent. Further the colorimetric assay is not routinely used to ascertain the reactivation potential of the enzyme-OP complex although it can be used to evaluate reactivation rate. The reactivation of OP-AChE conjugates (Fig. 1) is significant because this time-dependent process varies greatly with both OP structure and stereochemistry (5, 31). Antibody assays that can discriminate between populations of native, inhibited, or reactivated AChE molecules have the potential to provide more specific mechanistic evidence that the reactivation event occurred in part or in full. Further the ability of the antisera to recognize AChE that had previously been inhibited provides structural information of the reactivated enzyme active site.

The anti-AChE10S antisera specifically recognized rMoAChE that had been inhibited to yield a dianion phosphoserine in the active site but not rMoAChE that had been inhibited with other OP compounds including those that age to afford mononic phosphoryl groups (DFP and isomaltol). This result originally surprised us because we had hypothesized that anti-AChE10S would recognize rMoAChE containing any phosphate ion group at the catalytic serine even though the antibody was generated against the phosphate dianion. We hypothesize that the differences in charge on the phosphate and/or presence of an alkyl ester group may negate anti-AChE10S recognition. Based on this result, it is anticipated that antibodies can be raised to specifically recognize individual OP-AChE conjugates based on the precise chemical modification imposed by a specific reactive OP compound (Table I).

The anti-AChE10S and anti-AChE10Sp antibodies generated for this study aided the measurement of active and inactive (covalent modification by OPs) rMoAChE. Moreover these antibodies were helpful in identifying the structure of the OP group attached to the active site serine of rMoAChE. This level of structural analysis and mechanistic detail is essential to understanding the direction and extent of postinhibitory processes, namely, reactivation and/or aging. For example, the presence of a charged phosphate group at the catalytic serine of AChE is far less likely to reactivate than an active site serine bearing a neutral phosphorus ester group. Such key mechanistic information could be useful in the clinical setting in which the identity of the OP-AChE conjugate is needed to evaluate therapeutic choices including the probability of oxime efficacy.

Our results establish native and modified enzyme active sites of AChE as differential epitopes for antibody recognition. Even subtle differences in the OP modifying group conferred clear differences in the selectivity and specificity of these mechanism-based antibodies toward native and modified rMoAChE. Antibody recognition also correlated well with existing methods of enzyme analysis (colorimetric assay of activity) thereby allowing for a more powerful combined approach to better understand the mechanism of action of OP compounds.

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