Direct Analysis of the Kinetic Profiles of Organophosphate−Acetylcholinesterase Adducts by MALDI-TOF Mass Spectrometry†

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ABSTRACT: A sensitive matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry procedure has been established for the detection and quantitation of acetylcholinesterase (AChE) inhibition by organophosphate (OP) compounds. Tryptic digests of purified recombinant mouse AChE (mAChE) were fractionally inhibited by paraoxon to form diethyl phosphoryl enzyme. The tryptic peptide of mAChE that contains the active center serine residue resolves to a molecular mass of 4331.0 Da. Phosphorylation of the enzyme by paraoxon results in covalent modification of the active center serine and a corresponding increase in molecular mass of the tryptic peptide by 136 Da. The relative abundance of AChE peptides containing a modified active center serine strongly correlates with the fractional inhibition of the enzyme, achieving a detection range of phosphorylated to nonphosphorylated enzyme of 5−95%. Modifications of AChE by OP compounds resulting in dimethyl, diethyl, and diisopropyl phosphoryl adducts have been monitored with subpicomole amounts of enzyme. The individual phosphorylated adducts of AChE that result from loss of one alkyl group from the inhibited enzyme (the aging reaction) and the reappearance of unmodified AChE (spontaneous reactivation) have been resolved by the kinetic profiles and relative abundance of species. Further, the tryptic peptide containing the active center serine of AChE, isolated from mouse brain by anion-exchange and affinity chromatography, has been monitored by mass spectrometry. Native brain AChE, purified from mice treated with sublethal doses of metrifonate, has demonstrated that enzyme modifications resulting from OP exposure can be detected in a single mouse brain. For dimethyl phosphorylated AChE, OP exposure has been monitored by the ratio of tryptic peptide peaks that correspond to unmodified (uninhibited and/or reactivated), inhibited, and aged enzyme.

Acetylcholinesterase (AChE;[1] EC 3.1.1.7) is a serine hydrolase that regulates cholinergic neurotransmission in the peripheral and central nervous systems by hydrolyzing acetylcholine with a remarkably high catalytic efficiency. Mammalian AChE is encoded by a single gene that is alternatively spliced to yield molecular isoforms that differ in tissue-specific expression, solubility, and mode of membrane attachment (*1*, *2*). The catalytic triad of AChE contains aligned serine, histidine, and glutamate residues. It is well established that the catalytic activity of AChE is inhibited by organophosphates (OPs), a diverse group of compounds commonly used as pesticides as well as chemical warfare agents. The inhibition of AChE by OP compounds involves phosphorylation of the active site serine residue and the formation of stable phosphoryl AChE adducts (*3*). Subsequent reactions that occur spontaneously with OP−AChE conjugates include dephosphorylation of AChE to generate reactivated enzyme and dealkylation of phosphorylated AChE, by a unimolecular process referred to as aging (*4*, *5*), to result in enzyme that is irreversibly inactivated.

Mechanisms of phosphorylation, reactivation, and aging of AChE by OP compounds have been extensively studied. Site-directed mutagenesis, molecular modeling, and kinetic studies have been used to determine reaction rate constants and elucidate important structural determinants for the interaction of OPs with AChE (references cited in ref *6*). Aged phosphoryl enzyme conjugates have been directly observed of AChE inhibited by soman, sarin, methyl phosphonofluoridate, and DFP using NMR studies (*7*) and X-ray crystallography (*8*). Mass spectrometric approaches have been used to investigate the reaction pathways for OP-mediated AChE inhibition. The reaction products of AChE phosphorylation and aging of the OP−AChE conjugates with tabun and analogues, methamidophos, and other methyl phosphonofluoridates have been resolved (*9*−*11*). The mechanism of AChE inhibition by (1*S*,3*S*)-isomalathion (*12*) and

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[1] Abbreviations: AChE, acetylcholinesterase; mAChE, mouse acetylcholinesterase; ACP, active center peptide of acetylcholinesterase; ATCh, acetylthiocholine; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; OPs, organophosphates; DFP, diisopropyl fluorophosphate; paraoxon, diethyl *p*-nitrophenyl phosphate; DDVP, *O*,*O*-dimethyl *O*-(2,2,-dichlorovinyl) phosphate; metrifonate, (2,2,2-trichloro-1-hydroxyethyl)phosphonic acid dimethyl ester; BSA, bovine serum albumin.

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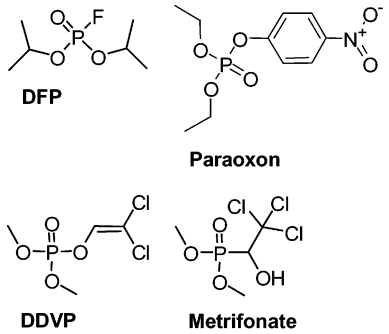
Chart 1: Structures of Organophosphates[a]



[a] Chemical formulas of the organophosphate AChE inhibitors: DFP, diisopropyl fluorophosphate; paraoxon, diethyl *p*-nitrophenyl phosphate; DDVP, *O*,*O*-dimethyl *O*-(2,2-dichlorovinyl) phosphate; metrifonate, (2,2,2-trichloro-1-hydroxyethyl)phosphonic acid dimethyl ester.

products of aged soman-inhibited AChE (*13*) have also been reported.

In the current work, a MALDI-TOF mass spectrometric procedure has been established for the sensitive detection and quantitation of AChE inhibition by DDVP, paraoxon, and DFP (Chart 1). The quantitation of analytes by MALDI-TOF MS has been reported for proteins and peptides (*14*−*16*) as well as low molecular mass species as substrates and products of biocatalytic reactions (*17*). In this study, the relative signal intensities for the tryptic peptides of AChE that contain the active center serine residue, unmodified or phosphorylated by OP treatment, have been monitored kinetically, quantitated, and compared to activity measurements as determined by standard methods (*18*). Modifications of the active center serine of AChE by OPs have been observed with subpicomole amounts of enzyme, and the analysis has proved a reliable means to determine the fractional inhibition of enzyme activity. It is demonstrated that AChE modifications by OPs can be quantified by MALDI-TOF MS. Moreover, the relative abundance of OP-inhibited AChE that has spontaneously reactivated and phosphoryl conjugates that have undergone aging have been resolved at specific time intervals.

The study of OP−AChE conjugates has been extended to an in vivo system, where endogenous AChE isolated from mouse brain tissue has been analyzed by mass spectrometry to detect phosphoryl enzyme adducts that result from exposure of mice to OPs. In previous studies, endogenous AChE has been purified from vertebrate brain tissue of human and calf (*19*), rabbit (*20*), rat (*21*), pig (*22*), quail (*23*), and chicken (*24*) to investigate structure−activity relationships for the enzyme with substrates and inhibitors and establish the distribution of AChE molecular isoforms. The significant advance reported here is that native and modified AChE from a single mouse brain has been isolated and characterized. The detection sensitivity of MALDI-TOF MS analysis is sufficient to resolve discrete modifications of AChE and has facilitated the analysis of brain enzyme isolated by a strategy that does not require pooling of tissue or purification of enzyme from multiple animals.

MATERIALS AND METHODS

Chemicals and Reagents. ATCh, DTNB, DFP, paraoxon, metrifonate, and DEAE-Tris-acryl resin were purchased from Sigma (St. Louis, MO). DDVP was purchased from ICN Biomedical Inc. (Irvine, CA). Sequence-grade modified trypsin was purchased from Promega (Madison, WI). Acridinium-conjugated affinity resin was synthesized as previously described (*19*, *25*, *26*). CHCA matrix (α-cyano-4-hydroxycinnamic acid) was purchased from Agilent Technologies (Palo Alto, CA).

Hazardous Procedures. OPs are highly toxic and should be handled with caution by trained personnel in a well-ventilated hood. OP chemicals are hydrolyzed by dilution into 4 M NaOH to render them inactive as cholinesterase inhibitors.

Recombinant Mouse Acetylcholinesterase (mAChE). Recombinant AChE was produced by transfection of an expression plasmid encoding the 547 N-terminal amino acid residues of mouse AChE into human embryonic kidney cells (HEK-293). Soluble monomeric mAChE, secreted in the culture medium of stable cell clones expressing high levels of recombinant enzyme, was purified by affinity chromatography and characterized as previously described (*2*, *27*). The purity of recombinant mAChE used in experiments was confirmed by SDS−polyacrylamide gel electrophoresis and silver stain of native protein and protein that had been enzymatically deglycosylated with peptide−*N*-glycosidase F (PNGase F). The specific activity of recombinant mAChE preparations was calculated from AChE activity measurements and protein content, as determined by the methods of Ellman (*18*) and Bradford (*28*), respectively.

Organophosphate Treatment of Mouse Acetylcholinesterase (in Vitro). To achieve fractional inhibition of recombinant mAChE by OP treatment, samples were prepared by adding paraoxon to mAChE at various enzyme-to-inhibitor molar ratios. At an initial concentration of 10 μM enzyme in 100 mM sodium phosphate buffer, pH 7.0, mAChE was treated with freshly prepared substocks of paraoxon at various concentrations from 0 to 12 μM. Following incubation at room temperature for 45 min, an aliquot was removed to measure the fractional activity by Ellman assay. Activity measurements were performed in the presence of 100 mM sodium phosphate buffer, pH 7.0, 0.1 mg/mL BSA, 0.3 mM DTNB, and 0.5 mM ATCh. Residual OP and other reaction byproducts were removed from the incubation mixture, and the buffer was exchanged to 50 mM ammonium bicarbonate, pH 7.4, by spin filtering using Microcon-30 devices (Millipore, Bedford, MA). Samples were evaporated to dryness by vacuum centrifugation and redissolved in 50 mM ammonium bicarbonate buffer to a protein concentration of 0.7 mg/mL (Bradford assay) for subsequent digestion by trypsin.

To resolve modifications of mAChE that result from extended exposure to OP compounds, time course experiments were conducted with DFP, paraoxon, and DDVP. A 1.5-fold molar excess of OP was added to mAChE (10 μM enzyme in 100 mM sodium phosphate, pH 7.0, buffer) and incubated at room temperature. At various time intervals, an aliquot was removed to measure AChE activity, then frozen, and stored at −80 °C. The OP-inhibited mAChE samples were thawed and prepared in parallel for mass spectrometric analysis, in the same manner as that described for fractional inhibition studies.

Trypsin Proteolysis of mAChE. mAChE (5−15 μg) was subjected to proteolytic digestion by incubation with sequence-grade modified trypsin (1:50 protein ratio of trypsin:mAChE) at 37 °C for 3 h. The tryptic peptides from mAChE

fractionally inhibited by paraoxon were lyophilized and dissolved in 0.1% (v/v) trifluoroacetic acid (TFA) for analysis by mass spectrometry. In other instances, the total peptides from tryptic digests of mAChE were fractionated prior to mass spectrometric analysis. Trypsinized mAChE was adsorbed to reverse-phase resin using a ZipTip-C4 (Millipore) and eluted with solutions containing 0.1% (v/v) TFA and increasing concentrations of acetonitrile in a step-increment gradient of 20%, 25%, 30%, 35%, 40%, and 60% (v/v) acetonitrile, as previously described (*11*).

Purification of Acetylcholinesterase from Mouse Brain. Mice (male, 7−8 weeks old, 20−25 g) were treated with saline or acute sublethal doses of metrifonate (200 or 400 mg/kg) by ip injection. The animals were sacrificed 45 min after treatment, and the brains were removed, frozen on liquid nitrogen, and stored at −80 °C. The purification procedure was performed at 4 °C, and all buffer solutions were ice cold. Unless indicated otherwise, buffer solutions contained 0.02% (w/v) NaN_3 as a preservative and the following protease inhibitors, added immediately prior to use: 5 mg/L leupeptin, 5 mg/L aprotinin, 5 mg/L pepstatin A, 10 mg/L bacitracin, 15 mg/L benzamidine, and 40 mg/L soybean trypsin inhibitor. Aliquots were taken at each stage of the purification procedure to measure AChE activity and protein content.

Enriched membrane extracts were prepared from brain tissue that was partially thawed and homogenized by Polytron (Brinkmann Instruments, Westbury, NJ) in a hypotonic buffer solution of 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA. The homogenate was centrifuged at 150000*g* in a Beckman 60 Ti rotor (40000 rpm) for 1 h at 4 °C. The pellet was resuspended in an extraction buffer of 20 mM sodium phosphate, pH 7.0, 0.15 M NaCl, 1 mM EDTA, and 0.5% (v/v) Triton X-100 and ultracentrifuged (60 Ti rotor, 40000 rpm, 2 h, 4 °C). The supernatant, containing detergent-solubilized membranes, was removed, frozen on liquid nitrogen, and stored at −80 °C.

The solubilized membrane fraction was further enriched by anion-exchange chromatography on DEAE-Tris-acryl resin. The extract from a mouse brain was diluted into binding buffer [20 mM bis-Tris propane hydrochloride, pH 7.0, 0.05% (v/v) Triton X-100] and adsorbed to a 10 mL DEAE-Tris-acryl gel bed previously equilibrated with binding buffer. Following a wash with two bed volumes of binding buffer, a protein fraction containing the majority of AChE activity (about 80%) was eluted with 20 mM piperazine hydrochloride, pH 5.0, 0.2 M NaCl, and 0.05% (v/v) Triton X-100.

Endogenous AChE was purified from the enriched protein fraction by affinity chromatography on *N*-methylacridinium-conjugated Sepharose 4B resin, with modifications to procedures previously reported (*19*). A 25:75 slurry of acridinium resin in buffer was added to the DEAE-Tris-acryl eluate (1 mL of slurry per 10 mL of eluate) and incubated in batch with gentle mixing at 4 °C for 2−3 h. The mixture was poured into a small column, and the resin was washed with 20 bed volumes of buffer A [10 mM sodium bicarbonate, pH 8.0, 0.1 M NaCl, 40 mM $MgCl_2$, 0.05% (v/v) Triton X-100], followed by a second wash with buffer A containing an additional 0.1 M NaCl. AChE was eluted from the acridinium resin by incubation with 5 mM decamethonium hydrochloride in buffer A, without protease inhibitors, for 1 h with gentle mixing. The eluate was diluted with 50 mM ammonium bicarbonate, pH 7.4, buffer, to reduce the concentration of Triton X-100 to 0.005% (v/v; below the CMC value), prior to being concentrated and buffer exchanged by spin-filtering using a Centricon-30 device (Millipore). Preparation of endogenous mAChE for analysis by mass spectrometry was performed in a manner similar to that described for recombinant mAChE samples.

Mass Spectrometry. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on a PE Biosystems Voyager DE-STR Biospectrometry workstation (Framingham, MA). Mass spectra were acquired in positive-ion linear mode under delayed extraction conditions, using an acceleration voltage of 25 kV and laser intensity of 2000−2400 V with a 337 nm pulsed nitrogen laser. External calibration was performed using ACTH peptide (amino acid residues 7−38) and bovine insulin, with average masses of 3657.93 and 5734.59 Da, respectively. Trypsinized mAChE or tryptic peptides of mAChE that were fractionated by adsorption to C4 reverse-phase resin were mixed with a matrix of 5 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) in 50% (v/v) acetonitrile and 0.3% (v/v) TFA, pH 2.2. A 1 μL aliquot of the peptide−matrix mixture was spotted, in duplicate, on a polished MALDI-TOF MS target plate and dried by semifast evaporation at 50 °C. Analyte−matrix cocrystals appeared homogeneous in nature. The mass spectra shown are the average of 256 laser scans collected from multiple locations on the target spot and monitored by a digital oscilloscope during acquisition. Laser intensity was adjusted such that ion intensity did not exceed 60−70% of the maximum threshold value. The data were processed and quantified with the PE Biosystems Grams 3.0 software program.

Analytical Procedures. SDS−polyacrylamide gel electrophoresis, silver stain, and immunoblotting techniques were performed by standard procedures. Affinity-purified polyclonal antibodies to mAChE were produced by Bethyl Laboratories (Montgomery, TX) by immunization of rabbits with purified recombinant mAChE protein in soluble monomeric form. The antibodies were characterized in our laboratory, based on strict criteria, and shown to react with high avidity and specificity for mAChE protein in immunoblotting and immunoprecipitation procedures.

RESULTS

Fractional Inhibition of mAChE by Paraoxon: Detection and Quantitation by MALDI-TOF MS. The catalytic triad of mAChE contains the active center serine at position 203. Proteolytic digestion of mAChE with trypsin generates a 42 amino acid peptide extending from Leu 178 to Arg 219 that contains the active center serine residue. The tryptic peptide, subsequently referred to as active center peptide (ACP), has a theoretical average mass of 4331.0 Da. MALDI-TOF mass spectra acquired in positive-ion linear mode from trypsinized recombinant mAChE resolve the ACP with an observed average mass of 4331.0 (±0.5) Da.

Paraoxon-mediated inhibition of mAChE results in diethyl phosphorylated enzyme. Complete AChE inhibition is associated with loss of unmodified ACP (4331.0 Da) and the appearance of modified peptide (4467.0 Da), corresponding to the expected mass increase of 136 Da from the diethyl

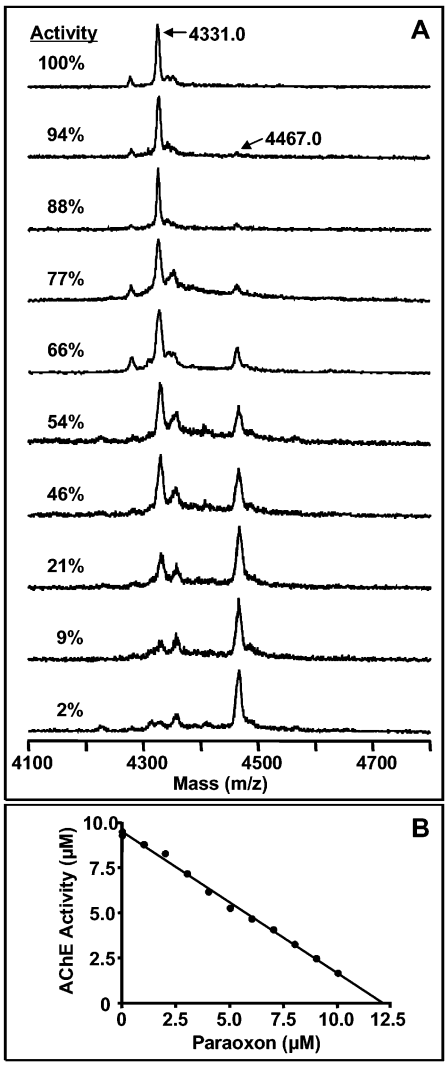


FIGURE 1: Fractional inhibition of mAChE by paraoxon. (A) Representative MALDI-TOF mass spectra acquired from total peptides of mAChE tryptic digests fractionally inhibited by paraoxon (0−12 μM). Residual enzyme activity for each sample, as determined by Ellman assay, is indicated. Paraoxon-mediated inhibition of mAChE results in the diethyl phosphoryl enzyme adduct and a corresponding increase of 136 Da in the molecular mass of the active center peptide (ACP), from 4331.0 to 4467.0 Da. AChE inhibition is associated with a reduction in the signal intensity of unmodified ACP and a corresponding increase in diethyl phosphorylated ACP. (B) Activity measurements following treatment of mAChE by various concentrations of paraoxon, demonstrating that a stoichiometric ratio of OP is required to achieve fractional inhibition of AChE activity.

phosphorylated ACP adduct. Mass spectra acquired from tryptic digests of mAChE, incubated with 0−12 μM concentrations of paraoxon to achieve a range of fractionally inhibited enzyme, are shown in Figure 1, panel A. Residual enzyme activity was determined by Ellman assay, and the data shown in Figure 1, panel B, confirm that a stoichiometric ratio of paraoxon was required to achieve fractional inhibition of enzyme activity. As the amount of inhibited mAChE enzyme increases in each sample, the signal intensity for unmodified ACP in mass spectra is reduced in a manner that parallels a larger signal intensity for the diethyl phosphoryl ACP adduct.

The relative amount of unmodified ACP and the diethyl phosphorylated ACP conjugate was determined, in multiple

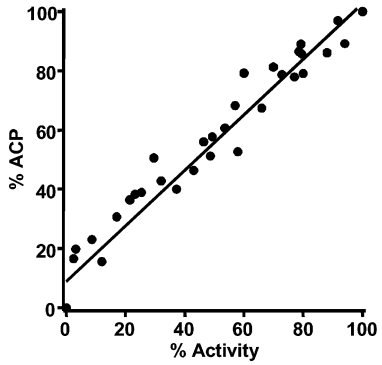


FIGURE 2: The relative abundance of unmodified ACP, determined by ion intensity from MALDI-TOF mass spectra, strongly correlates ($r^2 = 0.96$) with the residual activity of mAChE following paraoxon treatment. The relative abundance of unmodified ACP (4331.0 Da), referred to as % ACP, was calculated by the fraction of unmodified ACP compared to total ion intensity of the unmodified and diethyl phosphoryl ACP adduct (4467.0 Da) acquired from tryptic digests of paraoxon-inhibited mAChE. Representative mass spectra are shown in Figure 1. Data are compiled from six independent experiments.

trials, from ion intensity in mass spectra acquired from trypsinized, paraoxon-inhibited enzyme (Figure 2). The phosphorylation stoichiometry (percent ACP) was calculated from peak ion intensity by dividing unmodified ACP by the total of unmodified ACP and the diethyl phosphoryl ACP adduct. The percent ACP and residual enzyme activity measurements are both normalized to 100%. The relative abundances of unmodified ACP strongly correlate with the residual activities of mAChE following paraoxon inhibition. Therefore, the relative signal intensity of unmodified and phosphorylated ACP is directly related to the abundance of the OP−mAChE conjugate. The fractional inhibition studies demonstrate that MALDI-TOF mass spectrometry can be used as a quantitative measure to determine the degree of enzyme modification by OP compounds. It is evident that, in some samples where no residual AChE activity was measured, a minor fraction of unmodified ACP was resolved. It is likely that a small amount (less than 10%) of inactive enzyme is present in some preparations of recombinant mAChE and may account for the minor deviation seen in the correlation plot of activity versus ACP abundance.

Time-Resolved Organophosphate Modifications of Recombinant mAChE. Inhibition of mAChE by the OP compounds, paraoxon, DDVP, and DFP, resulting in diethyl, dimethyl, and diisopropyl phosphorylation of the enzyme, respectively, has been monitored with subpicomole amounts of enzyme. The relative abundance of phosphoryl ACP adducts that result from enzyme inhibition, loss of one alkyl group from the inhibited enzyme (the aging reaction), and dephosphorylation to give unmodified mAChE (reactivation) has been resolved at various time intervals. For each of the OPs, the expected mass increase for the ACP upon phosphorylation of mAChE and mass reduction for ACP phosphoryl adducts upon aging are given in Table 1.

Treatment of mAChE with paraoxon results in enzyme inhibition via diethyl phosphorylation of the active center serine. The inhibited enzyme can reactivate or undergo aging, whereby loss of one ethyl group renders the enzyme nonreactivatable. At various times after the addition of a molar excess of paraoxon to mAChE, aliquots of treated enzyme were removed for tryptic digestion and mass

Table 1: Theoretical Masses for mAChE Active Center Peptides following Modification with Organophosphates[a]

organo-phosphate	phosphoryl adduct	ACP (unmodified)	Δ mass from inhibition	inhibited ACP adduct	Δ mass from aging	aged ACP adduct
DFP	diisopropyl	4331.0	+164	4495.0	−42	4453.0
paraoxon	diethyl	4331.0	+136	4467.0	−28	4439.0
DDVP	dimethyl	4331.0	+108	4439.0	−14	4425.0

[a] The average theoretical mass for unmodified active center peptide (ACP) from trypsinized mAChE is 4331.0 Da. The phosphoryl ACP adducts of mAChE that result from treatment by the OP compounds, DFP, paraoxon, and DDVP, reflect dialkylphosphoryl adducts and are given as average mass (Da). The "aged" phosphoryl ACP adducts are calculated to reflect the loss of one alkyl group from the inhibited enzyme−OP conjugate. Mass values reflect the loss of hydrogen from the Ser residue upon inhibition by the OP compound and addition of hydrogen to the oxyanion following the aging reaction.

spectrometric analysis. The reactions of inhibition, aging, and reactivation of AChE require a conformationally stable enzyme and the unique configuration of the active center, comprised of several subsites (*29*). Therefore, the relative amounts of mAChE phosphoryl species resulting from OP-mediated inhibition for specific time intervals will not vary following tryptic digestion of intact enzyme. mAChE tryptic peptides were fractionated by adsorption to C4 reverse-phase resin, with the ACP and phosphoryl ACP adducts isolated by elution in 60% (v/v) acetonitrile and 0.1% (v/v) TFA solution. In control experiments, where mAChE was labeled by [^{3}H]DFP and monitored by scintillation counting, greater than 95% of the ACP from trypsinized mAChE eluted from the C4 reverse-phase resin in the fraction containing 60% (v/v) acetonitrile.

The relative abundance of ACP phosphoryl species was determined from ion intensity in acquired mass spectra (Figure 3) and shown, with comparison to enzyme activity measurements, in Figure 4. Since paraoxon treatment of mAChE results in complete and rapid enzyme inhibition, diethyl phosphorylated ACP (4467.0 Da) is predominant at early time points. A small fraction of aged enzyme is indicated by the monoethyl phosphoryl ACP adduct (4439.0 Da) at 8 h. The relative abundance of aged enzyme increases with time, as evident by the larger peak intensity of the aged ACP adduct. With extended incubation, some unmodified ACP reappears (48 h) and is indicative of hydrolysis of the phosphoryl serine bond and enzyme reactivation. This was confirmed by activity measurements. The abundance of the diethyl phosphorylated enzyme decreases in a manner that parallels the reactivation and aging time course. The fraction of mAChE that has aged is stable and does not reactivate. A minor amount of mAChE in this preparation is presumed to be catalytically inactive, since unmodified ACP (less than 12%) is evident in samples that have no detectable activity by Ellman assay.

The relative abundance of phosphoryl species can be fit to a scheme of parallel first-order reactions, allowing for some residual reinhibition that may occur at early time points due to an initial excess of OP inhibitor. The rate constant for diethyl phosphoryl adducts ($k_{overall}$) would represent the sum of the rate constants for formation of monoethyl phosphoryl adducts (k_{aging}) and unmodified ACP ($k_{reactivation}$). Rate constants of $k_{overall} = 0.0161$ h^{-1} ($t_{1/2} \sim 43$ h), determined by the disappearance of the diethyl phosphoryl adduct, $k_{aging} = 0.0099$ h^{-1} ($t_{1/2} \sim 70$ h), determined by the formation of the monoethyl phosphoryl adduct, and $k_{reactivation} = 0.0070$ h^{-1} ($t_{1/2} \sim 99$ h), determined by the regeneration of the free serine, were estimated from the profiles in Figure 4.

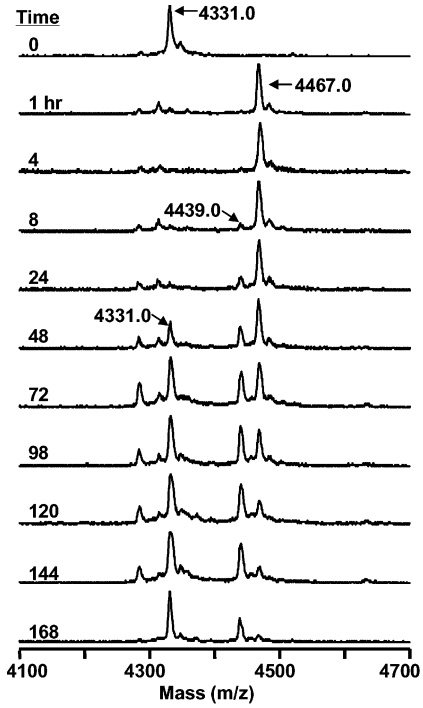


FIGURE 3: ACP conjugates of mAChE, resolved by time, following enzyme inhibition with paraoxon in molar excess. At various time intervals, an aliquot of paraoxon-treated mAChE was taken for mass spectrometric analysis. Samples were trypsinized, and the peptides were fractionated by reverse-phase chromatography to enrich for ACP adducts, as described in the text. Enzyme inhibition results in the diethyl phosphoryl ACP adduct (4467.0 Da). The aging reaction, which is defined as the loss of one ethyl group from the OP−enzyme conjugate, is represented by the monoethyl phosphoryl ACP adduct (4439.0 Da). The reappearance of unmodified ACP (4331.0 Da) is indicative of enzyme reactivation. MALDI-TOF mass spectra are representative of three experiments. In this and subsequent figures, a small shoulder on peak is observed of approximately 16 mass units above the parent peak. We presume this represents oxidation of a methionine in the ACP to a sulfoxide.

In a similar time course study, mAChE was inhibited by DDVP, where loss of enzyme activity is associated with dimethyl phosphorylation of the active center serine. As shown in Figure 5, the inhibited enzyme and aged conjugate are resolved from the unmodified and reactivated enzyme as dimethyl and monomethyl phosphoryl ACP adducts of 4439.0 and 4425.0 Da, respectively. Enzyme reactivation is evident at 4 h by the reappearance of unmodified ACP (4331.0 Da). In comparison to that observed with paraoxon, the aged conjugate appears more rapidly with DDVP-inhibited mAChE. The aging half-time of dimethyl phosphoryl AChE is significantly shorter than for diethyl phosphoryl AChE; dimethyl phosphorylated AChE has an

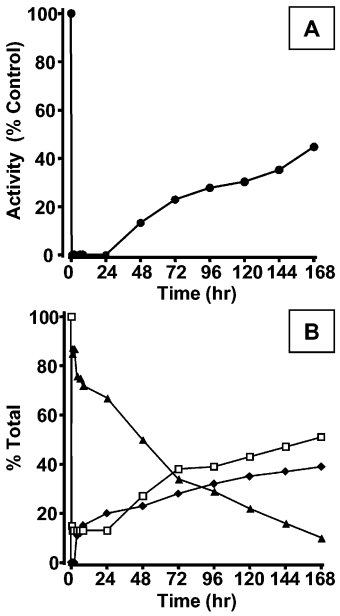


FIGURE 4: Relative abundance of ACP and phosphoryl adducts in mass spectra acquired from mAChE at various times following paraoxon inhibition of mAChE. (A) mAChE activity (●) was measured by Ellman assay at various time periods after the addition of a molar excess of paraoxon to the enzyme. (B) The ratio of ACP adducts at each time point was calculated from the signal intensity in mass spectra acquired from fractionated tryptic digests of paraoxon-inhibited mAChE samples. Unmodified ACP (□), diethyl phosphoryl ACP adduct (▲), and monoethyl phosphoryl ACP adduct (◆) represent control/reactivated, inhibited, and aged AChE enzyme, respectively.

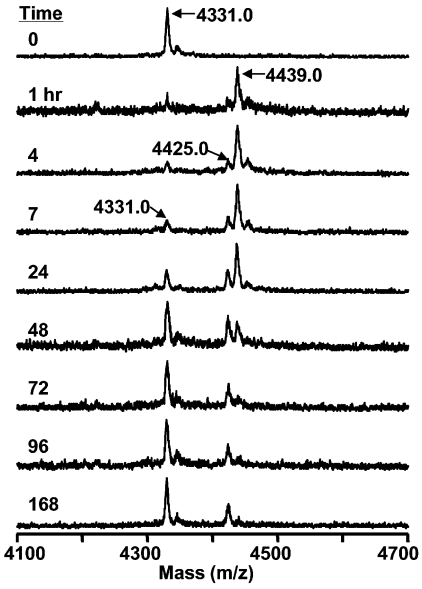


FIGURE 5: OP conjugates of mAChE following inhibition with a molar excess of DDVP, monitored by MALDI-TOF MS. Mass spectra were acquired from samples taken at various times, as indicated, and prepared for analysis by tryptic digestion and reverse-phase fractionation. Inhibition of mAChE by DDVP results in dimethyl phosphorylated enzyme and an increase in molecular mass of the ACP to 4439.0 Da. Aging of the OP−enzyme conjugate is demonstrated by the appearance of the monomethyl phosphoryl ACP adduct (4425.0 Da). Reactivation of the dimethyl phosphoryl mAChE conjugate is evident by the reappearance of unmodified ACP (4331.0 Da).

aging half-time of about 4 h (*30*, *31*). Moreover, spontaneous reactivation of dimethyl phosphoryl enzyme proceeds more rapidly than aging, and the reappearance of unmodified ACP is evident at early time intervals.

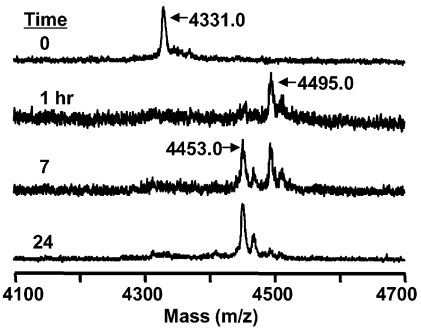


FIGURE 6: ACP and OP adducts of mAChE resulting from treatment with DFP in molar excess. MALDI-TOF mass spectra were acquired from fractionated tryptic peptides prepared from samples taken at time intervals as indicated. DFP-inhibited mAChE and the aged OP−enzyme conjugate are represented by diisopropyl (4495.0 Da) and monoisopropyl (4425.0 Da) phosphoryl-ACP adducts, respectively. mAChE reactivation (unmodified ACP) is not evident, as treatment with a molar excess of DFP results in nearly complete aging of the inhibited enzyme in a 24 h period.

Inhibition of mAChE by DFP leads to the formation of a diisopropyl phosphoryl conjugate with the active center serine and a corresponding ACP adduct with mass of 4495.0 Da (Figure 6). DFP-modified enzyme readily undergoes the aging reaction, as demonstrated by the substantial abundance of the ACP adduct at 7 h with mass of 4453.0 Da, representing the loss of one isopropyl group from the complex. The propensity of the diisopropyl phosphoryl conjugate of mAChE to undergo the aging reaction results in complete monoisopropyl phosphorylated ACP within 24 h, indicating virtually no spontaneous reactivation occurs.

Endogenous mAChE Purified from Brain. Endogenous AChE, purified from the brain tissue of a single mouse, has been resolved by SDS−PAGE (Figure 7, panel A). A protein with an apparent mass of about 68 kDa is evident, with a diffuse banding pattern suggesting that AChE is heterogeneously glycosylated. It migrates with a similar electrophoretic mobility to recombinant mAChE. In crude homogenate from a single mouse brain, on average, the specific abundance (molar ratio) of AChE is 0.5 pmol/mg of total protein, and the total abundance of AChE is about 15 pmol or 1 μg of protein. Since the molecular mass of mAChE is about 65 kDa, pure enzyme would have a specific abundance of 15 nmol/mg of protein. Therefore, a purification of AChE from mouse brain to near homogeneity would represent an approximate 30000-fold enrichment. The immunoblot in Figure 7 (panel B) shows endogenous AChE samples purified from the brain of a mouse treated with saline or different doses of metrifonate. It is evident that the relative yields of protein from the control and metrifonate-treated mice are similar, indicating that unmodified and phosphorylated AChE purifies with equal efficiency by acridinium affinity chromatography.

Recombinant mAChE was used in control experiments to determine the relative yields of acridinium affinity purification for unmodified AChE and enzyme fully inhibited by OPs. Dimethyl phosphorylated mAChE purified with efficiency similar to that of the unmodified enzyme, while the diethyl phosphorylated enzyme gave a significantly reduced yield. Given that acridinium interacts near the acyl pocket

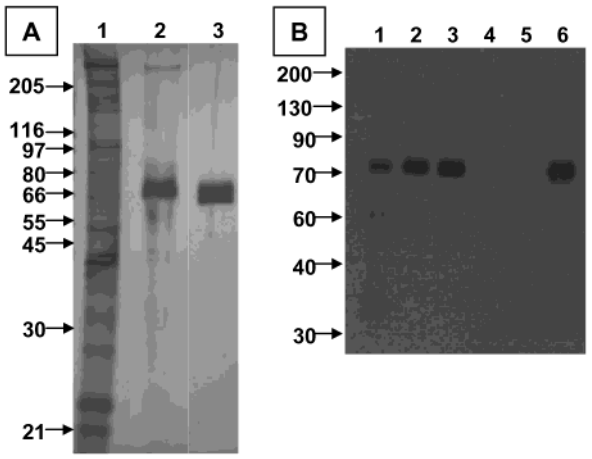


FIGURE 7: Endogenous AChE purified from mouse brain. (A) Silver stain of SDS−polyacrylamide gel electrophoresis. Lanes: 1, enriched membrane extract from mouse brain; 2, endogenous AChE purified from mouse brain as described in the text (the AChE from mouse brain migrates with an apparent molecular mass of approximately 68 kDa); 3, recombinant soluble monomeric mAChE (50 ng). (B) Immunoblot with rabbit anti-mAChE affinity-purified polyclonal antibodies of endogenous AChE purified from the brain of (lane 1) control mouse, (2, 3) mice treated with metrifonate (200 and 400 mg/kg ip), respectively, (4) total AChE knockout mouse, (5) blank, and (6) recombinant mAChE protein (20 pg). The migration of molecular mass markers is indicated by arrows and mass given in kDa.

of the AChE active site, the size of the alkyl group at the phosphorylated serine appears to be a crucial determinant for purification efficiency. The larger diethyl phosphoryl modification likely interferes, by steric hindrance, with acridinium−AChE interactions, where the smaller dimethyl phosphorylated adduct does not. Further, using recombinant mAChE inhibited by DDVP, it was determined that the relative abundances of inhibited, aged, and reactivated species did not progress during the procedures required for purification of endogenous enzyme.

No reactive protein is evident in samples prepared from brain tissue of mouse that is a total knockout for AChE (*32*), demonstrating high specificity of the purification procedure. Some proteolytic products are seen in AChE from control animals but not in AChE isolated from mice that were treated with OP, a likely indication of proteolysis mediated by serine proteases consequentially inhibited by OP.

Endogenous AChE isolated from mouse brain was trypsinized, fractionated by reverse-phase resin, and resolved by MALDI-TOF mass spectrometry to elucidate the peptide containing the active center serine (Figure 8). Metrifonate spontaneously hydrolyses in vivo to DDVP and thereby inhibits AChE through modification of the active center serine to a dimethyl phosphoryl adduct. For control mice, the active center peptides from endogenous brain AChE resolve to the expected mass for unmodified ACP, 4331.0 Da. In mice treated with acute doses of metrifonate, AChE modifications are indicated by the dimethyl and monomethyl phosphoryl ACP adducts, with masses of 4439.0 and 4425.0 Da, respectively. In both the 200 and 400 mg/kg metrifonate treatments, a significant fraction of the phosphorylated AChE enzyme has undergone the aging reaction and is thereby predicted to be irreversibly inhibited. Further, a larger fraction of unmodified ACP is evident in the animal exposed to a lower dose of metrifonate, representing enzyme that may be

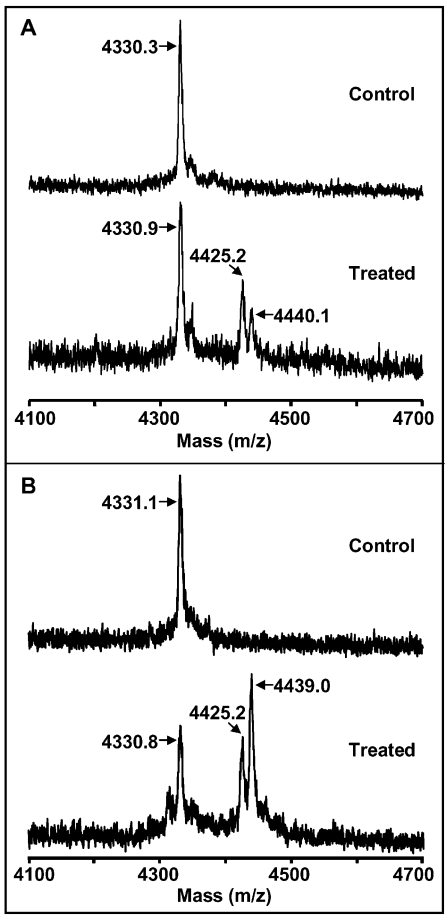


FIGURE 8: Endogenous mouse brain AChE. Mass spectra were acquired from AChE purified from mouse brain, digested with trypsin, and fractionated by reverse-phase resin. (A) Saline-injected control and mouse treated with 200 mg/kg metrifonate. (B) Control and 400 mg/kg metrifonate-treated mouse. Animals were sacrificed 45 min after ip injection. Unmodified ACP (4331.0 Da) of endogenous AChE from control and OP exposed mice is observed. OP−AChE conjugates are observed in metrifonate-treated animals that correspond to inhibited and aged enzyme, dimethyl and monomethyl phosphoryl ACP adducts (4439.0 and 4425.0 Da), respectively. Representative mass spectra from three independent experiments are shown.

uninhibited, spontaneously reactivated, or catalytically inactive.

DISCUSSION

The analysis of trypsinized AChE by MALDI-TOF MS has shown that specific chemical modifications that result from enzyme inhibition mediated by OP compounds can be detected with exquisite sensitivity and their relative abundance determined. It has been generally believed that quantitation of proteins and peptides from ion intensity on mass spectra acquired by MALDI-TOF MS is limited by variability due to sample preparation, inhomogeneous crystallization methods, and a phenomenon referred to as ion suppression (*33*). We have overcome such limitations by establishing a procedure to acquire mass spectra of the tryptic peptide of AChE containing the active center serine that has proved to be robust and highly reproducible. In multiple experiments conducted with mAChE inhibited by paraoxon, the relative abundances of AChE peptides containing a modified active center serine strongly correlate with the

fractional inhibition of the enzyme, achieving a wide detection range of phosphorylated to nonphosphorylated enzyme.

A comprehensive analysis by mass spectrometry has elucidated distinct chemical modifications of AChE inhibited by OPs that form diisopropyl, diethyl, and dimethyl phosphoryl AChE adducts. A simple fractionation of trypsinized AChE by reverse-phase chromatography gives an enrichment of the peptide containing the active center serine that enhanced the detection sensitivity of the MALDI-TOF MS procedure to subpicomole levels. The relative abundance of phosphoryl AChE conjugates that occur with time following inhibition by OPs has been observed and gives a measure of the propensity of the inhibited enzyme to spontaneously reactivate or undergo the aging process. Mass spectrometric analysis of AChE, as described in this report, allows for the direct observation of the abundance of reactivated enzyme and aged conjugate following inhibition by OPs. This approach offers advantages compared to classical methods that have relied on indirect measures of catalytic activity determined by nucleophilic reactivation of OP-inhibited AChE (*30*, *31*, *34*).

The abundance of aged enzyme for DFP-treated mAChE monitored by monoisopropyl phosphoryl adducts at 7 h following inhibition is about 50%, consistent with the aging half-time ($t_{1/2}$) of 6.7 h reported by others for AChE inhibited by DFP (*34*). For paraoxon-mediated inhibition of AChE, a reactivation $t_{1/2}$ of 83 h (*34*) and aging $t_{1/2}$ of 31−60 h have been observed (*30*, *34*). In this study, the fractions of reactivated and aged species of mAChE that resolve following treatment with paraoxon are about equal and similarly increase in abundance with time. Since the presence of excess OP and reinhibition would initially suppress observation of reactivated enzyme, and giving consideration to the reported rate constants for the diethyl phosphorylated enzyme, the fraction of aged species should exceed that of reactivated enzyme. Our results suggest that diethyl phosphoryl AChE conjugates may undergo aging at a rate slower than previously determined by nucleophile reactivation studies. The rates of reactivation and aging for dimethyl phosphoryl enzyme conjugates that result from OP-inhibited AChE were recently studied by measuring activity and susceptibility to oxime nucleophiles (*31*). Spontaneous hydrolysis of the phosphoryl serine bond occurred with a $t_{1/2}$ of 0.7 h. In the presence of excess inhibitor, the $t_{1/2}$ of aging was 4.2 h (*31*). The relative amounts of reactivated and aged enzyme determined here by mass spectrometric analysis are consistent with these rate constants. The aged enzyme adduct appears early in the time course, when reactivation is masked by reinhibition. As excess inhibitor is depleted, spontaneous hydrolysis predominates, and a large abundance of reactivated enzyme is observed. Concomitantly, a plateau is observed for the fraction of aged enzyme conjugate. The ability to quantitate the complement of OP−AChE adducts and hydrolytic products by MALDI-TOF MS has enabled us to resolve kinetic profiles for the separate processes of inactivation, aging, and reactivation of AChE as an isolated enzyme and demonstrate its potential for exposure detection in intact tissue.

Native AChE purified from brain tissue of mice treated with an acute dose of metrifonate has shown that phosphorylation of endogenous enzyme resulting from OP exposure can be detected in a single mouse brain. Moreover, the relative abundance of the unmodified enzyme and phosphoryl AChE conjugates evident after treatment with different doses of metrifonate confirms that subtle differences in AChE modifications can be detected. The amount of phosphorylated AChE in the animal exposed to 200 mg/kg (ip) metrifonate is clearly less than for the 400 mg/kg treated mouse. In fact, it is noteworthy that a significant fraction of OP-inhibited AChE in the metrifonate-treated animals is observed as the monomethyl phosphoryl conjugate, corresponding to aged enzyme. The half-time of reactivation and aging for dimethyl phosphorylated human AChE, as determined by in vitro studies, is reported as 0.7 and 4.2 h, respectively (*31*). The relative abundance of aged AChE conjugates within the 45 min time interval of OP exposure in these animals would not have been predicted to occur for the dimethyl phosphorylated enzyme. However, with in vivo treatment regimens of OP exposure, it is difficult to estimate the amount of inhibitor available to interact with brain AChE in the treated mice. The presence of excess inhibitor, perhaps sequestered in tissue, would suppress observation of spontaneous reactivation and thereby lead to a progressive accumulation of aged enzyme.

Most of the OP insecticides in current worldwide use form either the dimethyl or diethyl phosphoryl conjugates with AChE. We show here that these conjugates exhibit progressive spontaneous hydrolysis and aging, but at different rates. Since the aged species can no longer be reactivated, either by oxime or spontaneously, and the nonaged species will be susceptible to hydrolysis and subsequent reinhibition, aged species should accumulate with the multiple inactivation−reactivation events occurring upon chronic exposure. Accordingly, the simultaneous measurement of the complement of the three species should provide an indication of the duration of exposure to the OP.

Substantial variability of AChE activity levels in the various tissues of animal species within defined populations has been reported, even among litter mate siblings from inbred mouse colonies (*32*). With the aim to develop a technology to correlate endogenous AChE inactivation with biological end points, the sensitive detection of the phosphoryl AChE by MALDI-TOF MS provides a direct measure of the covalent modification of enzyme in animals exposed to OPs. This approach of measuring the conjugate directly thereby circumvents the limitations of biological variation when reductions in AChE activity from a range of standard values determined from control animals are monitored.

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REFERENCES

1. Li, Y., Camp, S., Rachinsky, T. L., Getman, D., and Taylor, P. (1991) *J. Biol. Chem. 266*, 23083−23990.
2. Rachinsky, T. L., Camp, S., Li, Y., Ekstrom, T. J., Newton, M., and Taylor, P. (1990) *Neuron 5*, 317−327.
3. Taylor, P. (2001) in *Goodman and Gilman's, the Pharmacological Basis of Therapeutics* (Limbird, L. E., Ed.) pp 175−192, McGraw-Hill, New York.
4. Michel, H. O., Hackley, B. E., Jr., Berkowitz, L., List, G., Hackley, E. B., Gillilan, W., and Pankau, M. (1967) *Arch. Biochem. Biophys. 121*, 29−34.
5. Benschop, H. P., and Keijer, J. H. (1966) *Biochim. Biophys. Acta 128*, 586.
6. Ordentlich, A., Barak, D., Kronman, C., Benschop, H. P., De Jong, L. P., Ariel, N., Barak, R., Segall, Y., Velan, B., and Shafferman, A. (1999) *Biochemistry 38*, 3055−3066.
7. Segall, Y., Waysbort, D., Barak, D., Ariel, N., Doctor, B. P., Grunwald, J., and Ashani, Y. (1993) *Biochemistry 32*, 13441−13450.
8. Millard, C. B., Kryger, G., Ordentlich, A., Greenblatt, H. M., Harel, M., Raves, M. L., Segall, Y., Barak, D., Shafferman, A., Silman, I., and Sussman, J. L. (1999) *Biochemistry 38*, 7032−7039.
9. Barak, D., Ordentlich, A., Kaplan, D., Barak, R., Mizrahi, D., Kronman, C., Segall, Y., Velan, B., and Shafferman, A. (2000) *Biochemistry 39*, 1156−1161.
10. Barak, R., Ordentlich, A., Barak, D., Fischer, M., Benschop, H. P., De Jong, L. P., Segall, Y., Velan, B., and Shafferman, A. (1997) *FEBS Lett. 407*, 347−352.
11. Elhanany, E., Ordentlich, A., Dgany, O., Kaplan, D., Segall, Y., Barak, R., Velan, B., and Shafferman, A. (2001) *Chem. Res. Toxicol. 14*, 912−918.
12. Doorn, J. A., Gage, D. A., Schall, M., Talley, T. T., Thompson, C. M., and Richardson, R. J. (2000) *Chem. Res. Toxicol. 13*, 1313−1320.
13. Viragh, C., Kovach, I. M., and Pannell, L. (1999) *Biochemistry 38*, 9557−9561.
14. Desiderio, D. M., Wirth, U., Lovelace, J. L., Fridland, G., Umstot, E. S., Nguyen, T. M., Schiller, P. W., Szeto, H. S., and Clapp, J. F. (2000) *J. Mass Spectrom. 35*, 725−733.
15. Hensel, R. R., King, R. C., and Owens, K. G. (1997) *Rapid Commun. Mass Spectrom. 11*, 1785−1793.
16. Tang, X., Sadeghi, M., Olumee, Z., Vertes, A., Braatz, J. A., McIlwain, L. K., and Dreifuss, P. A. (1996) *Anal.Chem. 68*, 3740−3745.
17. Kang, M. J., Tholey, A., and Heinzle, E. (2000) *Rapid Commun. Mass Spectrom. 14*, 1972−1978.
18. Ellman, G. L., Courtney, D., Valentino, A. J., and Featherstone, R. M. (1961) *Biochem. Pharmacol. 7*, 88−95.
19. Vallette, F. M., Marsh, D. J., Muller, F., Massoulie, J., Marcot, B., and Viel, C. (1983) *J. Chromatogr. 257*, 285−296.
20. Mintz, K. P., and Brimijoin, S. (1985) *J. Neurochem. 44*, 225−232.
21. Rakonczay, Z., Mallol, J., Schenk, H., Vincendon, G., and Zanetta, J. P. (1981) *Biochim. Biophys. Acta 657*, 243−256.
22. Reavill, C. A., and Plummer, D. T. (1978) *J. Chromatogr. 157*, 141−151.
23. Son, J. Y., Shin, S., Choi, K. H., and Park, I. K. (2002) *Int. J. Biochem. Cell Biol. 34*, 204−210.
24. Rotundo, R. L. (1984) *J. Biol. Chem. 259*, 13186−13194.
25. Rosenberry, T. L., and Richardson, J. M. (1977) *Biochemistry 16*, 3550−3558.
26. Webb, G., and Clark, D. G. (1978) *Arch. Biochem. Biophys. 191*, 278−288.
27. Marchot, P., Ravelli, R. B., Raves, M. L., Bourne, Y., Vellom, D. C., Kanter, J., Camp, S., Sussman, J. L., and Taylor, P. (1996) *Protein Sci. 5*, 672−679.
28. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
29. Ordentlich, A., Barak, D., Kronman, C., Ariel, N., Segall, Y., Velan, B., and Shafferman, A. (1996) *J. Biol. Chem. 271*, 11953−11962.
30. Worek, F., Backer, M., Thiermann, H., Szinicz, L., Mast, U., Klimmek, R., and Eyer, P. (1997) *Hum. Exp. Toxicol. 16*, 466−472.
31. Worek, F., Diepold, C., and Eyer, P. (1999) *Arch. Toxicol. 73*, 7−14.
32. Xie, W., Stribley, J. A., Chatonnet, A., Wilder, P. J., Rizzino, A., McComb, R. D., Taylor, P., Hinrichs, S. H., and Lockridge, O. (2000) *J. Pharmacol. Exp. Ther. 293*, 896−902.
33. Sechi, S., and Oda, Y. (2003) *Curr. Opin. Chem. Biol. 7*, 70−77.
34. Aldridge, W. N., and Reiner, E. (1972) *Enzyme inhibitors as substrates. Interactions of esterases with esters of organophosphorus and carbamic acids*, American Elsevier, New York.

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