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Aging of Mipafox-Inhibited Human Acetylcholinesterase Proceeds by Displacement of Both Isopropylamine Groups to Yield a Phosphate Adduct

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Aging of phosphylated serine esterases, e.g., acetylcholinesterase (AChE) and neuropathy target esterase (NTE), renders the inhibited enzymes refractory to reactivation. This process has been considered to require postinhibitory side group loss from the organophosphorus moiety. Recently, however, it has been shown that the catalytic domain of human NTE inhibited by *N*,*N*′-diisopropylphosphorodiamidofluoridate (mipafox, MIP) ages by deprotonation. For mechanistic understanding and biomarker development, it would be important to know the identity of the MIP adduct on target esterases after inhibition and aging occurred. Accordingly, the present study was performed to determine if MIP-inhibited human AChE ages by side group loss or an alternate method, e.g., deprotonation. Diisopropylphosphorofluoridate (DFP), the oxygen analogue of MIP, was used for comparison, because DFP-inhibited AChE is known to age by net loss of an isopropyl group. Kinetics experiments were done with DFP and MIP against AChE to follow the time course of inhibition, reactivation, and aging for each inhibitor. MS studies of tryptic digests from kinetically aged DFP-inhibited AChE revealed a mass shift of 122.8 ± 0.7 Da for the active site peptide (ASP) peak, corresponding to the expected monoisopropylphosphoryl adduct. In contrast, the analogous mass shift for kinetically aged MIP-inhibited AChE was 80.7 ± 0.9 Da, corresponding to a phosphate adduct. Because this finding was unexpected, the identity of the phosphoserine-containing ASP was confirmed by immunoprecipitation followed by MS. The results indicate that aging of MIP-inhibited AChE proceeds by displacement of both isopropylamine groups. Further research will be required to elucidate the detailed mechanism of formation of a phosphate conjugate from MIP-inhibited AChE; however, knowledge of the identity of this adduct will be useful in biomarker studies.

Introduction

Certain organophosphorus (OP)[1] compounds inhibit acetylcholinesterase (AChE), which leads to cholinergic toxicity in the central and peripheral nervous systems (*1*). Inhibition of AChE by OP compounds occurs by organophosphylation of the AChE active site Ser residue. The hydroxyl oxygen of the active site Ser attacks the OP compound at the phosphorus atom, displacing the primary leaving group, forming a covalent bond with the phosphyl moiety. The activity can be restored only by cleavage of the Ser−phosphyl bond and can be either spontaneous (using water as the nucleophile) or mediated by other nucleophiles, such as the oxime, pyridine-2-aldoxime methiodide (2-PAM), or fluoride ion. Reactivation proceeds by S_N2 displacement of the enzyme from the phosphyl moiety, regenerating enzymatically active AChE. However, the phosphylated serine may first undergo a postinhibitory aging process to yield an adduct that does not reactivate, either spontaneously or after oxime treatment. Aging is mechanistically defined as the creation of a negative charge on the phosphylated serine moiety. Aging usually involves scission of a side group from the phosphylated enzyme to yield the anionic species, although this may also occur by deprotonation (*2*). Aging of AChE inhibited by OP compounds of the phosphate and phosphonate classes has classically been thought to proceed by an S_N1 mechanism transiently yielding a carbocation (*3*, *4*).

Diisopropylphosphorofluoridate (DFP) is a thoroughly studied OP compound; the aging of DFP-inhibited enzymes has been shown to yield an anionic monosubstituted phosphyl ligand on the active site serine for AChE (*5*, *6*), butyrylcholinesterase (BChE) (*7*), neuropathy target esterase (NTE) (*5*, *8*, *9*), and human recombinant NTE esterase domain (NEST) (*2*). *N*,*N*′-Diisopropylphosphorodiamidofluoridate (mipafox, MIP), the phosphoramidate analogue of DFP, is expected to interact with AChE in the same way as DFP does. MIP-inhibited AChE has been shown to be reactivated quickly in vitro by treatment with potassium fluoride after inhibition if reactivated within 18 h of inhibition (*10*). This suggests that conventional, time-dependent aging does occur for mipafox-inhibited enzymes in a manner similar to that for DFP-inhibited esterases. Furthermore, it is known that some *N*-alkyl phosphoramidates age by fission of the phosphorus−nitrogen bond (*11*, *12*). The possibility of phosphorus−nitrogen bond fission suggests that the aging of mipafox-inhibited AChE may occur by the hydrolytic loss of an alkylamino group. However, the mechanism of mipafox-inhibited BChE or AChE aging needs to be investigated further; recent evidence has established that MIP-inhibited NEST ages by proton loss, yielding an intact *N*,*N*′-diisopropylphospho-

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[1] Abbreviations: 2-PAM, pyridine-2-aldoxime methiodide; AChE, acetylcholinesterase; ASP, active site peptide; ATCh, acetylthiocholine; BChE, butyrylcholinesterase; DFP, diisopropylphosphorofluoridate; DTNB 5,5′-dithio-bis(2-nitrobenzoic acid); MALDI, matrix-assisted laser desorption/ionization; MH^+, protonated molecule; MIP, *N*,*N*′-diisopropylphosphorodiamidofluoridate, mipafox; NEST, human recombinant NTE esterase domain; NTE, neuropathy target esterase; OP, organophosphorus; SELDI, surface-enhanced laser desorption/ionization; TOF, time-of-flight.

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rodiamido group, not by isopropylamine loss as expected (*2*). The present study was, therefore, undertaken to determine the mechanism of aging of MIP-inhibited AChE.

Experimental Procedures

Chemicals and Enzymes. Human recombinant AChE (EC 3.1.1.7) (stock C1682, lot 062K1391), alkaline phosphatase (stock P6772, lot 052K7048), trypsin (stock T8658, lot 101K51042), and DFP (98% pure by GC) were purchased from Sigma (St. Louis, MO). Monoclonal antibodies to phosphoserine (ALX-850-02, lot L10533) were purchased from Alexis Biochemicals (Montreal, Quebec). Mipafox was purchased from ChemSyn Laboratories (Lenexa, KS) (99% pure by HPLC). All other chemicals were the highest purity available and obtained from commercial sources.

Bimolecular Rate Constant of Inhibition (k_i). The residual activity of inhibited AChE was assayed in 0.10 M sodium phosphate buffer (pH 8.0 at 25 °C) using a modification of the method of Ellman et al. (*13*). Inhibitors were dissolved in acetone and diluted in phosphate buffer. The final acetone concentration was ≤1% (v/v), a solvent concentration determined not to affect AChE activity. Enzyme and inhibitor were preincubated for various measured times. At the end of each period, substrate solution containing acetylthiocholine (ATCh) and 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) (final concentrations: ATCh, 1.0 mM; DTNB, 0.32 mM) was added. The residual activity was determined by measuring the change in absorbance at 412 nm and 25 °C over a 1−2 min period using a SPECTRAmax 340 microplate reader (Molecular Devices Corporation, Sunnyvale, CA). The apparent k_i values for DFP and mipafox against AChE were determined as previously described (*14*).

Reactivation Rate Constants (k_3). Reactivation of inhibited AChE was carried out by incubating AChE with inhibitors at concentrations required to yield ∼90% inhibition in 10 min (MIP, 15.7 μM; DFP, 0.134 μM). Reactions were performed at 25 °C in pH 8.0 phosphate buffer. An aliquot of the enzyme solution was diluted 1:100 (v/v) in buffer (25 °C) alone or buffer containing 2-PAM or KF (final concentrations, 200 μM). Aliquots were withdrawn at timed intervals from 0 to 18 h and assayed for activity as described above for AChE (*15*). The apparent k_3 values for spontaneous and oxime-mediated reactivation were determined according to the method of Doorn et al. (*16*, *17*).

Rate Constants of Aging (k_4). Aging was carried out similarly to reactivation by incubating enzymes with inhibitors at 25 °C for 10 min in pH 8.0 phosphate buffer to achieve ∼90% inhibition. Aliquots were then diluted 1:100 (v/v) with phosphate buffer effectively to stop the inhibition reaction. Inhibition and dilution buffers were without 2-PAM. The inhibited enzyme was then allowed to age for timed intervals from 0 to 18 h. At the end of each timed interval, an aliquot of inhibited enzyme solution was removed and incubated with 2-PAM (final concentration, 100 μM) for 20 min at 25 °C before determining the residual enzyme activity. In parallel, the residual activity in the absence of oxime was determined to serve as a control. Reactions were performed at 25 °C. Apparent k_4 values were determined as described by Clothier et al. (*18*) and Jianmongkol et al. (*15*).

Mass Spectrometry. AChE was incubated in 50 mM ammonium bicarbonate buffer, pH 8.0, with concentrations of inhibitors (DFP or mipafox) determined from kinetics studies to yield greater than 90% inhibition (*16*, *17*). Control samples of AChE were incubated with buffer only. One set of inhibited samples was subjected immediately after inhibition or control incubation to tryptic digestion (see below) in order to attempt detection of nonaged adducts. Another set of control or inhibited samples was allowed to incubate for 0.5, 1, 2, 4, 8, 24, and 168 h.

Control, inhibited, and aged samples were subjected to tryptic digestion as described by Doorn et al. (*16*). The sequence for human AChE was obtained from the SWISS-PROT database (http://www.expasy.ch/sprot/sprot-top.html) (*19*). The average masses of the tryptic digests of each sequence were predicted using the MS-Digest feature of ProteinProspector version 4.0.4 (http://www.prospector.ucsf.edu/mshome4.0.htm) (*20*). Using these tools, the tryptic digest peptide containing the esterase site serine (shown in boldface) and its associated average *m*/*z* value for the protonated molecule (MH^+) were predicted to be as follows: LALQWVQENVAAFGGDPTSVTLFGE**S**AGAASVGMHLLSPPSR, 4270.84. Adduction of this active site serine with DFP, monoisopropyl DFP, mipafox, and monoisopropyl mipafox would result in positive average *m*/*z* shifts of 165.15, 123.07, 163.18, and 122.08, respectively.

Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) is a modification of matrix-assisted laser desorption/ionization (MALDI)-TOF MS. The SELDI or ProteinChip technique combines MALDI-TOF MS with retentive chromatographic separation on the MALDI plate (*21*). Aliquots of nondiluted 1:10 and 1:100 (v/v) dilutions of the peptide mixture in 50 mM ammonium bicarbonate were deposited (1−3 μL) on gold, H4, and NP1 ProteinChip plates (Ciphergen Biosystems, Inc., Fremont, CA) for treatment or no treatment. No treatments of the gold plate were made. Samples for treatment were deposited on H4 plates prewashed with acetonitrile and incubated at room temperature in a humidity chamber for 20 min. The samples were then washed three times with 5 μL of 20% (v/v) acetonitrile. Samples for treatment were deposited on NP1 plates and incubated for 30 min at room temperature in a humidity chamber. The samples were then washed three times with 2 μL of water. All samples were dried at 50 °C, and 1 μL of a 50% (v/v) acetonitrile/50% (v/v) 1% trifluoroacetic acid (w/v) solution saturated with the matrix, α-cyano-4-hydroxycinnamic acid, was plated on top of the samples and dried at 50 °C. A PBS-II SELDI-TOF-MS instrument (Ciphergen Biosystems, Inc.) equipped with a nitrogen laser (337 nm, 4 ns pulse width) was used to acquire mass spectra. Analysis was carried out with an acceleration voltage of 20 kV, and 100−200 laser shots were averaged for each spectrum. The instrument was externally and internally calibrated before each experiment in the following manner. A solution of 1 mg/mL human recombinant AChE was digested with trypsin, and the predicted peptide map was obtained as described as above. Aliquots of 1 μL of AChE peptide digest were plated as described as above. After drying, the sample spot was spiked with 1 μL of a solution containing 1 mg/mL dynorphin A and 1 mg/mL human angiotensin-I (Ciphergen Biosystems, Inc.) in 50 mM ammonium bicarbonate, pH 8.0, and analyzed as described above. Coverage of 70−80% of predicted peptides for digested AChE was obtained (data not shown). These peaks, along with peaks corresponding to dynorphin A and human angiotensin-I, were used to obtain the most accurate calibration for the AChE experimental samples. Subsequently, internal calibration was performed using trypsin and AChE peaks other than the active site peptide (ASP).

Immunoprecipitation. To confirm the presence of a phosphoserine on the ASP in the MIP-treated sample, DFP-inhibited AChE digests, MIP-inhibited AChE digests, and MIP-inhibited AChE digests treated with bovine alkaline phosphatase were subjected to immunoprecipitation with anti-phosphoserine antibodies. Digests for phosphatase treatments were incubated for 30 min with bovine alkaline phosphatase at a ratio of 20 μg of sample protein to 0.1 units of phosphatase at pH 8.0 and 25 °C.

All incubations and washes were performed at 25 °C. For all samples, protein G was incubated with Trap-eZe affinity beads (Ciphergen Biosystems, Inc.) in 50 mM acetate buffer, pH 5.0, for 17 h on a rotator. The supernatant was removed, and the pellet was washed once with 500 μL of acetate buffer, pH 5.0. The pellet was then incubated in 600 μL of 0.5 M Tris-HCl, pH 9.0, at 25 °C, 0.1% (v/v) Triton X-100 for 2 h on a rotator. The supernatant was removed, and the pellet was washed with 500 μL of PBS four times. Two microliters of the antibody solution (clones 162, 163, or 164) was added with 50 μL of 200 mM $NaHCO_3$, pH 9.2, at 25 °C and incubated, while rotating for 1.5 h. The supernatant was removed, and the pellet was washed once with 200 μL of 200 mM $NaHCO_3$, pH 9.2, at 25 °C. MIP-treated AChE samples (25 μL) shown by mass spectrometry data possibly to contain a phosphoserine on the active site were added and incubated, rotating, for 1 h. Supernatant was removed, and the pellet was washed once with

Table 1. Kinetic Values for MIP- and DFP-Treated AChE Samples[a]

inhibitor	k_i (M^{-1} min^{-1})	k_3 (min^{-1})[b]	k_4 (min^{-1})
MIP	$(1.47 \pm 0.07) \times 10^4$	$(2.39 \pm 1.09) \times 10^{-4}$	0.0192 ± 0.0013
DFP	$(1.72 \pm 0.07) \times 10^6$	$(14.2 \pm 9.2) \times 10^{-4}$	0.0213 ± 0.0016

[a] Values are means ± SEM ($n = 4$). [b] Values for spontaneous reactivation.

Table 2. MH^+ (Average Mass) for Unreacted and DFP- or MIP-Reacted AChE ASPs[a]

		Δm			
		DFP		MIP	
data type	unreacted	intact	aged	intact	aged[b]
observed	4270.8 ± 0.3	165.0 ± 0.9	122.8 ± 0.7	163.4 ± 1.0	80.7 ± 0.9
theoretical	4270.8	164.2	122.1	162.2	80.0

[a] Means ± SEM ($n \geq 4$). [b] The aged peak corresponds to the "doubly" aged peak (H_2PO_3 adduct). No peak corresponding to a singly aged adduct was detected.

500 μL of PBS for 5 min and twice with 200 μL of 50 mM Tris buffer, pH 7.2, at 25 °C, 1.0 M urea, 0.1% (w/v) CHAPS, and 0.5 M NaCl for 5 min. The sample was then washed once with HPLC grade water. Ten microliters of 50% (v/v) acetonitrile and 0.3% (w/v) TFA was added to the sample and incubated for 60 min. One microliter of the supernatant was spotted on gold and H4 plates along with 0.5 μL of a 1:1 mixture of 0.1 μg/μL human β-endorphin, 3465.0 *m*/*z*, and 0.1 μg/μL bovine insulin, 5733.6 *m*/*z*, and analyzed by mass spectrometry as described above.

Statistical Analyses. All plots, along with their regressions and other statistical analyses, were done using GraphPad version 3.02 for Windows, GraphPad Software, Inc. (San Diego, CA).

Results

The results of the inhibitory and postinhibitory kinetic determinations are displayed in Table 1. MIP was found to be approximately 275 times less potent than DFP as an inhibitor of AChE. Reactivation rate constants for MIP-inhibited AChE were 0.000239 ± 0.000109, 0.0109 ± 0.00312, and 0.00889 ± 0.00014 min^{-1} for spontaneous, KF-, and 2-PAM-mediated reactivation, respectively. Reactivation rate constants for DFP-inhibited AChE were 0.00142 ± 0.00092, 0.0539 ± 0.0098, and 0.0462 ± 0.0017 min^{-1} for spontaneous, KF-, and 2-PAM-mediated reactivation, respectively. The rate constants of aging for MIP- and DFP-inhibited AChE were found not to be significantly different (*t*-test, $p > 0.1$).

Average mass data from mass spectrometry for unreacted and DFP- or MIP-reacted AChE ASPs are displayed in Table 2. Sample spectra from mass spectrometry for unreacted and DFP- or MIP-reacted AChE ASPs are displayed in Figure 1.

Mass spectrometry data of MIP-inhibited AChE show two time-dependent shifts in the peak corresponding to the AChE ASP (4270.8 *m*/*z*, Figures 1A and 3). The first peak (4434.2 *m*/*z*; Figure 1B) was seen at 0, 0.5, and 1 h time points and corresponds to an ASP adducted by the intact MIP inhibition product, an *N*,*N*′-diisopropylphosphorodiamido moiety. The second new peak (4352.1 *m*/*z*; Figure 1B) appeared at time points from 0.5 to 168 h. This second peak corresponds to an AChE active site adducted by H_2PO_3 or, in other words, a doubly aged MIP-inhibited ASP.

Mass spectrometry data of DFP-inhibited AChE show two time-dependent shifts in the peak corresponding to the AChE ASP (4270.8 *m*/*z*; Figure 1A). The first peak (4435.8 *m*/*z*; Figure 1C) was seen at 0, 0.5, and 1 h time points and corresponds to an ASP adducted by the intact DFP moiety, a diisopropyl phosphate adduct. The second new peak (4393.6 *m*/*z*; Figure 1C) appeared at time points from 0.5 to 168 h. This second peak corresponds to an aged DFP-inhibited AChE ASP, a monoisopropyl phosphate-adducted ASP.

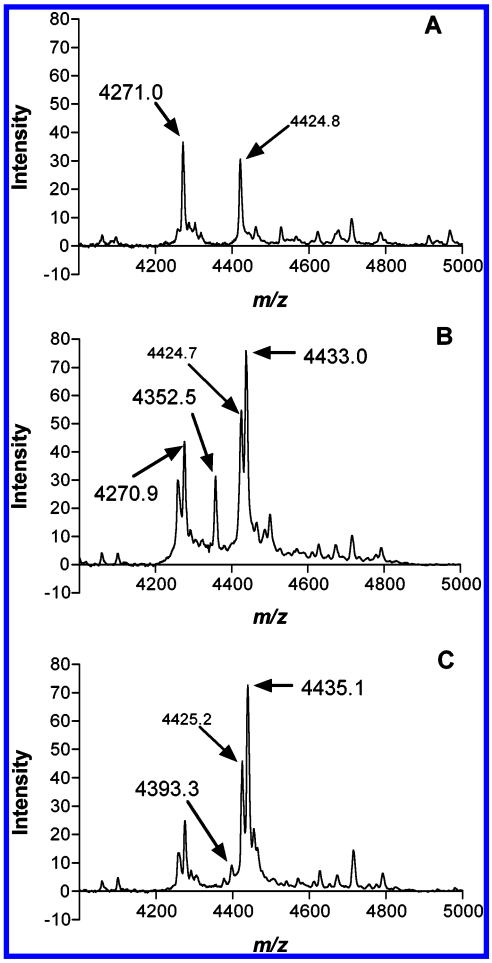

Figure 1. Representative SELDI-TOF MS spectra of tryptic digest of AChE samples. (A) Control AChE sample. The peak with *m*/*z* of 4271.0 represents the unmodified ASP containing the catalytic Ser. (B) AChE sample modified by MIP. The peak with *m*/*z* of 4433.0 represents the ASP with an *N*,*N*′-diisopropylphosphorodiamido adduct. The peak with *m*/*z* of 4352.5 represents the ASP with a phosphate (H_2PO_3) adduct. (C) AChE sample modified by DFP. The peak with *m*/*z* of 4435.1 represents the ASP with a diisopropyl phosphate adduct. The peak with *m*/*z* of 4393.3 represents the ASP with a monoisopropyl phosphate adduct. All *m*/*z* values are average values. Peptides other than those containing the active site serine are also labeled for comparison.

Mass spectrometry peak coverage for corresponding peptides of digested AChE was 68% (data not shown). No treatment-dependent mass shifts were found in any mass spectrometry results, aside from those mentioned above. Peptides other than those containing the active site serine are labeled for comparison (Figure 1).

Sample spectra from mass spectrometry from immunoprecipitation experiments are displayed in Figure 2. A peak corresponding to a phosphorylated AChE ASP was found after immunoprecipitation with an anti-phosphoserine immunoglobulin and was performed on digested MIP-inhibited AChE. No peptides were found in postimmunoprecipitation DFP-inhibited AChE samples or in phosphatase-treated MIP-inhibited AChE samples.

Discussion

The kinetic results show time-dependent inhibition, reactivation, and aging for MIP- and DFP-treated human AChE. This study is the first to report these values for human recombinant

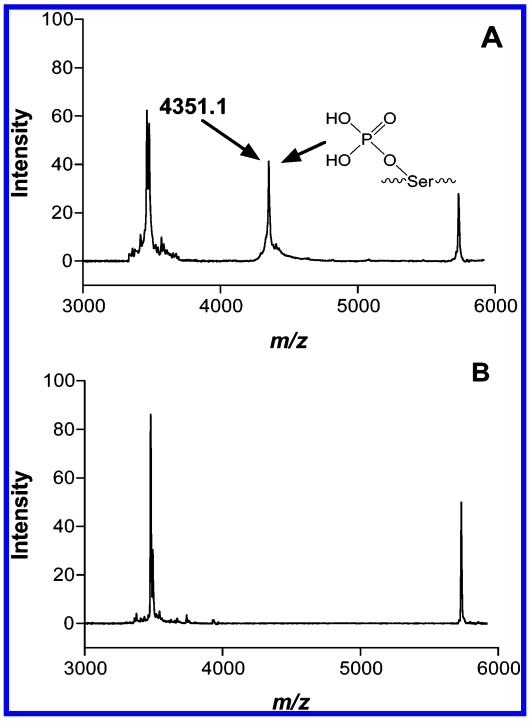

Figure 2. Representative SELDI-TOF MS spectra of antiphosphoserine immunoprecipitation-digested AChE samples. (A) Immunoprecipitation of MIP-treated AChE peptides. The peak with an *m*/*z* of 4351.1 corresponds to an AChE ASP adducted by a phosphate (H_2PO_3) group. The two other peaks correspond to the calibration peptides, human β-endorphin at 3465.0 *m*/*z* and bovine insulin at 5733.6 *m*/*z*. All *m*/*z* values are average values. (B) Immunoprecipitation of nontreated AChE. Calibration peptides human β-endorphin at 3465.0 *m*/*z* and bovine insulin at 5733.6 *m*/*z* were the only peaks found corresponding to sample and calibration peptides. All *m*/*z* values are average values.

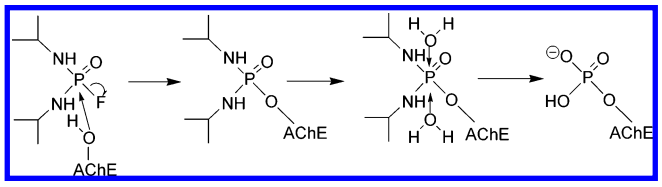

Figure 3. Proposed mechanism of inhibition and aging of MIP on AChE proceeds by net loss of isopropylamine groups. The exact mechanism (S_N1 or S_N2) of aging is not known but is shown here as S_N2. The aged moiety is shown in its expected ionization state at pH 8.0.

AChE against DFP and MIP. Values presented here are within 10% of previous findings for AChE species for which data were available (*5*, *10*, *22*).

Mass spectrometry of trypsinized DFP-treated AChE revealed aging by net loss of an isopropyl group. Peaks corresponding to the peptide containing the AChE active site were found to be nonadducted in controls, adducted by a diisopropyl phosphate group in DFP-treated AChE at early time points, and adducted by a monoisopropyl phosphate group at later time points. The peak corresponding to the monoisopropyl phosphate group first appears in the DFP-treated AChE sample at the 0.5 h time point. This result agrees with the k_4 reported here for DFP-inhibited AChE, which corresponds to a $t_{1/2}$ of 32.5 min. This result also agrees with the identification of a monoisopropyl phosphate group on aged mouse AChE inhibited by DFP (*23*).

Contrary to expectation, in mass spectrometry experiments of MIP-treated AChE, no peak was found corresponding to an ASP aged by net loss of an isopropylamine group (4392.9 *m*/*z*). From the k_4 reported here for MIP-inhibited AChE, corresponding to a 36.1 min $t_{1/2}$, one would expect this peak to appear in the 0.5 h sample. Although no peak appeared at 4392.9 *m*/*z*, a peak at 4351.1 *m*/*z* did appear, beginning at the 0.5 h time point in MIP-treated AChE samples. This 4351.1 *m*/*z* peak corresponds to a phosphate (H_2PO_3) adduct on the ASP, which would represent a doubly aged species derived from the MIP conjugate. The mass shift is 80.0 *m*/*z*, because two hydrogens would be expected to be present on the phosphoric acid moiety in the acidic conditions of the mass spectrometry experiments (pK_a of the second hydrogen is 5.65), and the serine hydrogen is lost upon inhibition by MIP. Phosphoserine immunoprecipitation confirmed the presence of the doubly aged moiety. The proposed inhibition and aging mechanisms are shown in Figure 3.

The phosphoserine adduct present after aging of MIP-inhibited AChE indicates aging by P−N scission as reported for AChE inhibited by tabun (ethyl *N*,*N*′-dimethylphosphoramidocyanidate) (*11*). It is not known how this bond scission occurs, but it is most likely by acid-catalyzed hydrolysis. This reaction may proceed by one of two ways (*12*). The first is a direct S_N2 substitution of the isopropylamine group by water. The second is a two-step S_N1 addition−elimination reaction with a proton addition to the nitrogen to release the isopropylamine group and subsequent attack on the phosphorus by water, which leads to the elimination of the isopropylamine group. In either case, the isopropylamine group is replaced by water as confirmed by immunoprecipitation of the aged product with antiphosphoserine immunoglobulin, making it difficult to determine the exact mechanism. It is more likely that the scission occurs by direct S_N2 substitution due to the nature of the P−N bond and the plausibility provided by the recent evidence of a S_N2 substitution breaking the P−O bond for echothiophate-inhibited human BChE (*24*).

This is the first known demonstration of a double side group loss of an OP compound-inhibited enzyme. It is not known if this is a rare event, as very few OP compound adducts have been directly characterized, and one would not be able to distinguish between singly, doubly, or alternatively aged inhibited esterases by kinetic methods alone. However, such discrimination can be accomplished by pairing kinetics with MS detection of the inhibited and aged species as done in the present study and in a recent report of paraoxon-inhibited AChE (*23*). In view of the conventional single aging of the diisopropyl phosphate adduct shown here and the inability of phosphinates, sulfonates, and carbamates to age (*25*, *26*), it is not expected that aging by double side group loss is common except, perhaps, for *N*,*N*′-dialkylphosphorodiamido adducts.

This work brings up many questions regarding the aging mechanism of MIP-inhibited AChE. One would assume that the aging is a two-step process, i.e., that there are two distinct isopropylamine loss reactions. It is possible that certain residues stabilize the aged moiety by forming a salt bridge as shown for the *Torpedo californica* AChE residue His^{440} and phosphonates (*27*). Such stabilization may allow for the scission of the second P−N bond but would presumably not allow for a similar scission of the P−O bond for DFP-inhibited AChE. This suggests either that the activation energy for C−O scission is greater than P−N scission due to the nature of the NH moiety vs O for these compounds or that the orientation of the isopropylamido group of MIP-inhibited AChE in the active site is more favorable for P−N scission than the orientation of the isopropyl group of DFP-inhibited AChE, or both. It is relatively certain that the presence of the phosphoramidate P−N bond results in aging mechanisms that are significantly different across OP compounds and esterases given the evidence presented here and elsewhere (*2*, *11*). It is unclear, though, whether the P−N or

P−O bond scissions investigated here are S_N1 or S_N2. There is evidence for both types of aging of other OP compounds (*3*, *4*, *7*, *16*, *24*, *28*), although the mechanisms have not been directly investigated in each case.

In view of the unexpected and different results for MIP-inhibited AChE and NEST aging, it would be of interest to determine the mechanism of inhibition of aging of MIP on other serine hydrolases, e.g., BChE, carboxylesterase, or cholesterol esterase. It is difficult to predict what would be expected from MIP-inhibited BChE aging. Given the data presented here for AChE and the similarities in *T. californica* AChE and human BChE structure (*29*), one might anticipate that MIP-inhibited BChE could age in the same manner as MIP-inhibited AChE, but this would require experimental verification. Additional logical future work would involve using other phosphoramidates as inhibitors of a given esterase and carrying out combined kinetics and mass spectrometry experiments to determine the specific structure−activity relationships that lead to double side group loss vs other modes of aging. Now that the X-ray crystal structures of *T. californica* AChE and human BChE are known (*29*), investigation by crystallography and/or molecular modeling of DFP- and MIP-inhibited enzymes would also likely yield significant information about the aging process.

Because the rate of aging is usually determined operationally by kinetic methods, which would not differentiate between aged moieties created by single or double side group loss, the present results do not have immediate implications for the clinical treatment of OP poisoning (*1*). However, these findings do highlight the importance of the use of mass spectrometry in mechanistic studies and as a biomarker tool (*2*, *11*, *16*, *17*, *23*, *30*, *31*). It will be important to determine the intact and aged products of OP compound inhibition for each target enzyme of interest (AChE, BChE, NTE, or other esterases), as these products may be significantly different among enzymes and/or compounds. Understanding the basis for different modes of aging among inhibited serine hydrolases also affords new opportunities to use phosphoramidates and their analogues as mechanistic probes of the active sites of these enzymes.

In summary, whereas DFP-inhibited human AChE ages by net loss of a single isopropyl group and MIP-inhibited NEST ages by deprotonation, MIP-inhibited human AChE does not age by either deprotonation or displacement of a single isopropylamine group but instead undergoes displacement of two isopropylamine groups to yield a phosphate adduct on the active site serine.

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References

(1) Thompson, C. M., and Richardson, R. J. (2004) Anticholinesterase insecticides. In *Pesticide Toxicology and International Regulation* (Marrs, T., and Ballantyne, B., Eds.) pp 89−127, John Wiley and Sons Ltd., Chichester.

(2) Kropp, T. J., Glynn, P., and Richardson, R. J. (2004) The mipafox-inhibited catalytic domain of human neuropathy target esterase ages by reversible proton loss. *Biochemistry 43*, 3716−3722.

(3) Coult, D. B., and Marsh, D. J. (1966) Dealkylation studies on inhibited acetylcholinesterase. *Biochem. J. 98*, 869−873.

(4) Michel, H. O., Hackley, B. E., Berkowitz, L., List, G., Hackley, E. B., Gillilan, W., and Pankau, M. (1967) Aging and dealkylation of soman (pinacolylmethylphosphonofluoridate)-inactivated eel cholinesterase. *Arch. Biochem. Biophys. 121*, 20−34.

(5) Clothier, B., and Johnson, M. K. (1979) Rapid aging of neurotoxic esterase after inhibition by di-isopropyl phosphorofluoridate. *Biochem. J. 177*, 549−558.

(6) 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) Crystal structures of aged phosphonylated acetylcholinesterase: Nerve agent reaction products at the atomic level. *Biochemistry 38*, 7032−7039.

(7) Masson, P., Fortier, P. L., Albaret, C., Froment, M. T., Bartels, C. F., and Lockridge, O. (1997) Aging of di-isopropyl-phosphorylated human butyrylcholinesterase. *Biochem. J. 327*, 601−607.

(8) Williams, D. G. (1983) Intramolecular group transfer is a characteristic of neurotoxic esterase and is independent of the tissue source of the enzyme. A comparison of the aging behaviour of di-isopropyl phosphorofluoridate-labeled proteins in brain, spinal cord, liver, kidney and spleen from hen and in human placenta. *Biochem. J. 209*, 817−829.

(9) Williams, D. G., and Johnson, M. K. (1981) Gel-electrophoretic identification of hen brain neurotoxic esterase, labeled with tritiated di-isopropyl phosphorofluoridate. *Biochem. J. 199*, 323−333.

(10) Milatovic, D., and Johnson, M. K. (1993) Reactivation of phosphorodiamidated acetylcholinesterase and neuropathy target esterase by treatment of inhibited enzyme with potassium fluoride. *Chem.-Biol. Interact. 87*, 425−430.

(11) Elhanany, E., Ordentlich, A., Dgany, O., Kaplan, D., Segall, Y., Barak, R., Velan, B., and Shafferman, A. (2001) Resolving pathways of interaction of covalent inhibitors with the active site of acetylcholinesterases: MALDI-TOF/MS analysis of various nerve agent phosphyl adducts. *Chem. Res. Toxicol. 14*, 912−918.

(12) Eto, M. (1979) *Organophosphorus Pesticides: Organic and Biological Chemistry*, CRC Press, Cleveland.

(13) Ellman, G., Courtney, K. D., Andres, V., Jr., and Featherstone, R. M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol. 7*, 88−95.

(14) Kropp, T. J., and Richardson, R. J. (2003) Relative inhibitory potencies of chlorpyrifos oxon, chlorpyrifos methyl oxon, and mipafox for acetylcholinesterase versus neuropathy target esterase. *J. Toxicol. Environ. Health A 66*, 1145−1157.

(15) Jianmongkol, S., Marable, B. R., Berkman, C. W., Tally, T. T., Thompson, C. M., and Richardson, R. J. (1999) Kinetic evidence for different mechanisms of acetylcholinesterase inhibition by (1*R*)- and (1*S*)-stereoisomers of isomalathion. *Toxicol. Appl. Pharmacol. 155*, 43−53.

(16) Doorn, J. A., Gage, D. A., Schall, M., Talley, T. T., Thompson, C. M., and Richardson, R. J. (2000) Inhibition of acetylcholinesterase by (1*S*,3*S*)-isomalathion proceeds with loss of thiomethyl: Kinetic and mass spectral evidence for an unexpected primary leaving group. *Chem. Res. Toxicol. 13*, 1313−1320.

(17) Doorn, J. A., Schall, M., Gage, D. A., Talley, T. T., Thompson, C. M., and Richardson, R. J. (2001) Identification of butyrylcholinesterase adducts after inhibition with isomalathion using mass spectrometry: Difference in mechanism between (1*R*)- and (1*S*)-stereoisomers. *Toxicol. Appl. Pharmacol. 176*, 73−80.

(18) Clothier, B., Johnson, M. K., and Reiner, E. (1981) Interaction of some trialkyl phosphorothiolates with acetylcholinesterase: Characterization of inhibition, aging and reactivation. *Biochim. Biophys. Acta 660*, 306−316.

(19) Boeckmann, B., Bairoch, A., Apweiler, R., Blatter, M. C., Estreicher, A., Gasteiger, E., Martin, M. J., Michoud, K., O'Donovan, C., Phan, I., Pilbout, S., and Schneider, M. (2003) The SWISS-PROT protein knowledgebase and its supplement TrEMBL in 2003. *Nucleic Acids Res. 31*, 365−370.

(20) Clauser, K. R., Baker, P. R., and Burlingame, A. L. (1999) Role of accurate mass measurement (±10 ppm) in protein identification strategies employing MS or MS/MS and database searching. *Anal. Chem. 71*, 2871.

(21) Issaq, H. J., Veenstra, T. D., Conrads, T. P., and Felshow, D. (2002) The SELDI-TOF MS approach to proteomics: Protein profiling and biomarker identification. *Biochem. Biophys. Res. Commun. 292*, 587−592.

(22) Aldridge, W. N., and Reiner, E. (1972) *Enzyme Inhibitors as Substrates: Interactions of Esterases with Esters of Organophosphorus and Carbamic Acids*, North-Holland Publishing Company, Amsterdam.

(23) Jennings, L. L., Malecki, M., Komives, E. A., and Taylor, P. (2003) Direct analysis of the kinetic profiles of organophosphate-acetylcholinesterase adducts by MALDI-TOF mass spectrometry. *Biochemistry 42*, 11083−11091.

(24) Nachon, F., Asojo, O. A., Borgstahl, G. E. O., Masson, P., and Lockridge, O. (2005) Role of water in aging of human butyrylcholinesterase inhibited by echothiophate: The crystal structure suggests two alternative mechanisms of aging. *Biochemistry 44*, 1154−1162.

(25) Johnson, M. K. (1975) Structure−activity relationships for substrates and inhibitors of hen brain neurotoxic esterase. *Biochem. Pharmacol. 24*, 797−805.
(26) Davis, C. S., Johnson, M. K., and Richardson, R. J. (1985) Organophosphorus compounds. In *Neurotoxicity of Industrial and Commercial Chemicals*, Vol. II, pp 1−23, CRC Press, Boca Raton.
(27) Bencsura, A., Enyedy, I., and Kovach, I. M. (1995) Origins and diversity of the aging reaction in phosphonate adducts of serine hydrolase enzymes: What characteristics of the active site do they probe? *Biochemistry 34*, 8989−8999.
(28) Saxena, A., Viragh, C., Frazier, D. S., Kovach, I. M., Maxwell, D. M., Lockridge, O., and Doctor, B. P. (1998) The pH dependence of dealkylation in soman-inhibited cholinesterases and their mutants: Further evidence for a push−pull mechanism. *Biochemistry 37*, 15086−15096.
(29) Nicolet, Y., Lockridge, O., Masson, P., Fontecilla-Camps, J. C., and Nachon, F. (2003) Crystal structure of human butyrylcholinesterase and of its complexes with substrate and products. *J. Biol. Chem. 42*, 41141−41147.
(30) Doorn, J. A., Thompson, C. M., Christner, R. B., and Richardson, R. J. (2003) Stereoselective interaction of *Torpedo californica* acetylcholinesterase by isomalathion: Inhibitory reactions with (1*R*)- and (1*S*)-isomers proceed by different mechanisms. *Chem. Res. Toxicol. 16*, 958−965.
(31) Peeples, E. S., Schopfer, L. M., Dysen, E. G., Spaulding, R., Voelker, T., Thompson, C. M., and Lockridge, O. (2005) Albumin, a new biomarker of organophosphorus toxicant exposure, identified by mass spectrometry. *Toxicol. Sci. 83*, 303−312.

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