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Resolving pathways of interaction of mipafox and a sarin-analog with human acetylcholinesterase by kinetics, mass spectrometry and molecular modeling approaches

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Abstract

The hydroxyl oxygen of the catalytic triad serine in the active center of serine hydrolase acetylcholinesterase (AChE) attacks organophosphorus compounds (OPs) at the phosphorus atom to displace the primary leaving group and to form a covalent bond. Inhibited AChE can be reactivated by cleavage of the Ser-phosphorus bond either spontaneously or through a reaction with nucleophilic agents, such as oximes. At the same time, the inhibited AChE adduct can lose part of the molecule by progressive dealkylation over time in a process called aging. Reactivation of the aged enzyme has not yet been demonstrated. Here our goal was to study oxime reactivation and aging reactions of human AChE inhibited by mipafox or a sarin analog (Flu-MPs, fluorescent methylphosphonate). Progressive reactivation was observed after Flu-MPs inhibition using oxime 2-PAM. However, no reactivation was observed after mipafox inhibition with 2-PAM or the more potent oximes used. A peptide mass fingerprinted mass spectrometry (MS) method, which clearly distinguished the peptide with the active serine (active center peptide, ACP) of the human AChE adducted with OPs, was developed by MALDI-TOF and MALDI-TOF-TOF. The ACP was detected with a diethyl phosphorylated adduct after paraoxon inhibition, and with an isopropylmethyl phosphonylated and a methyl phosphonylated adduct after Flu-MPs inhibition and subsequent aging. Nevertheless, nonaged nonreactivated complexes were seen after mipafox inhibition and incubation with oximes, where MS data showed an ACP with an NN diisopropyl phosphoryl adduct. The kinetic experiments showed no reactivation of activity. The computational molecular model analysis of the mipafox-inhibited hAChE plots of energy versus distance between the atoms separated by dealkylation showed a high energy demand, thus little aging probability. However with Flu-MPs and DFP, where aging was observed in our MS data and in previously published crystal structures, the energy demand calculated in modeling was lower and,

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Conflict of interest

The authors declare that there are no conflicts of interest.

consequently, aging appeared as a more likely reaction. We document here direct evidence for a phosphorylated hAChE refractory to oxime reactivation, although we observed no aging.

INTRODUCTION

Organophosphorus compounds (OPs) are a diverse group of chemicals used as both pesticides and chemical warfare agents. It has been well-established that progressive inhibition of serine (Ser) hydrolases, such as cholinesterases (ChEs) or neuropathy target esterase (NTE), by OPs involves the phosphorylation of the active site. The hydroxyl oxygen of active site serine attacks the organophosphate at the phosphorus atom to displace the primary leaving group and to form a covalent bond (Figure 1, **reaction 1**). The activity of initially formed OP conjugates can be reactivated by the cleavage of the phosphorus–Ser bond either spontaneously by water or through a reaction with nucleophilic agents, such as fluoride ions, hydroximates or oximes (Taylor; 1994; Sultatos., 1994). Pralidoxime [N-methyl-(2-hydroxyaminoformylpyridinium or 2-PAM) is currently the oxime most frequently used as an antidote of OP poisoning. Nevertheless, more potent oximes are currently being developed (Taylor and Radi ., 1994; Sit et al., 2014; Figure 1, **reaction 3**). In some conjugated OPs, one of the P-O alkyl groups can undergo a “dealkylation” reaction termed “aging” (Figure 1, **reaction 2**). Aged ChEs are resistant to reactivation either spontaneously or by adding a nucleophilic agent such as 2PAM (Figure 1. **reaction 4**). Serine esterases aging is of considerable toxicological significance due to (1) the major limitation of the effectiveness of reactivation therapy in OP poisoning cases, and (2) the aging of NTE, this being the mechanism proposed for the development of organophosphorus-induced delayed neuropathy (OPIDN; Sultatos., 1994). A better understanding of the molecular basis of aging could, therefore, help in design reactivators of ChEs.

Over the years, substantial efforts have been made to understand the molecular mechanism of aging and to explain the nonreactivability of aged ChEs. It has been suggested that a new bond is formed between the aged adduct and the active center residue to thus prevent reactivation (Hobbiger., 1963). Later it was proposed that the negatively charged oxygen on the phosphorus atom formed an electrostatic shield against the nucleophilic attack of dissociated oxime hydroxyls toward the phosphorus atom (Harris et al., 1966). NMR studies and crystal structures have, in the meantime, shown a formation of a salt bridge between the protonated catalytic histidine and the negatively charged oxygen of the dealkylated conjugate (Masson et al., 1997). Hence it has been generally accepted that aging is the consequence of the dealkylation of the OP-enzyme conjugate. Dealkylation of a phosphorylated ChE is a unimolecular SN1 reaction that involves a carbocationic intermediate (Masson et al., 2010). However, other types of chemical deteriorations of phosphorylated serine may occur. Currently, aged phosphoryl enzyme conjugates of AChE inhibited by soman, sarin and DFP have been directly observed using NMR and X-ray crystallography studies (Segall et al., 1993; Millard et al., 1999; Carletti et al., 2010). Resistance to reactivation has been attributed mainly to two possible mechanisms: (1) the formation of the salt bridge between the catalytic protonated histidine and the negatively charged oxygen of the

monophosphylate moiety; and (2) aged ChEs may electrostatically repulse oximes as a result of the excessive negative charge generated (Masson et al., 1997).

Mechanisms of phosphylation, reactivation and aging of AChE by OPs have been extensively studied by site-directed mutagenesis, mass spectrometry (MS), computational molecular modeling and kinetic studies. These approaches have been used to determine reaction rate constants and to elucidate the detailed mechanistic pathways of the interaction between OPs and some serine esterases. Protein MS has been used with some OPs as a valuable tool to identify OP-conjugated proteins by employing peptide mass fingerprinting to match isolated proteins to protein sequences by analyzing the OP-protein conjugates of AChE and BuChE (Grigoryan et al., 2009; Doorn et al., 2001; Jennings et al., 2003; Kropp and Richardson., 2005; 2006). The advances made in protein MS have proved most useful for identifying not only the adduction of different OPs compounds to isolated proteins, but also post-inhibition processes, such as spontaneous reactivation or aging reactions. Hence understanding the reaction pathways of the phosphylation, reactivation and “aging” of AChE with toxic OPs appears to be a biochemical and pharmacological challenge.

In the present study, reactivation of human AChE inhibited by mipafox and Flu MP s was studied using several oximes under variable experimental conditions. No reactivation was observed after mipafox inhibition in any of the cases. An MS method, which clearly distinguishes the OP inhibited conjugate of human AChE, from the aged and reactivated enzyme, was developed and tested upon human AChE inhibition with paraoxon, Flu-MPs and mipafox. Although it was not possible to reactivate the conjugate of hAChE with mipafox, no aged adduct was observed. Some computational molecular modeling has shown energetically preferred aging in the cases of OP-AChE conjugates formed by Flu-MPs and DFP as opposed to those formed by mipafox.

METHODS

Chemicals and Reagents

Acetylthiocholine (ATCh), 5-5'-dithiobis nitrobenzoic acid (DTNB) was purchased from Sigma (St. Louis, MO, USA). Sequence grade-modified trypsin was acquired from Promega (Madison, WI, USA). The CHCA matrix (R-cyano-4-hydroxycinnamic acid) was obtained from Agilent Technologies (Palo Alto, CA, USA).

Enzyme

Recombinant human acetylcholinesterase (hAChE). Highly purified monomeric hAChEs was prepared as described earlier (Cochran et al., 2011). All the given enzyme concentrations refer to the concentration of catalytic sites; i.e. monomers.

OPs

The chemical structures of the OPs used in this work are presented in Figure 2. Paraoxon was purchased from Sigma-Aldrich and US Biological. Mipafox (mipafox, purity >98%) was acquired from Lark Enterprise (Webster, MA, USA). The now-toxicity nonvolatile Flu-MP (fluorescent methylphosphonate) used in the *in vitro* experiments as an analog of nerve

agent sarin (Amitai et al., 2007) were kindly provided by Dr. Gabriel Amitai of the Israel Institute for Biological Research, Ness-Ziona, Israel. This Flu-MP differs from Sarin by only the structure of the respective leaving group. Inhibition of hAChE by Flu-MP results in the OP–hAChE covalent conjugate, which is identical to that formed upon inhibition with nerve agent sarin.

Enzyme assay

Cholinesterase activity was measured by a spectrophotometric assay (Ellman et al., 1961). To this end, the enzyme preparation was incubated with 1.0 mM substrate ATCh (acetylthiocholine) and 0.3 mM DTNB (dithiobis (2-nitrobenzoic acid) at 25°C in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.01% BSA.

Oximes

2PAM (2-pyridinealdoxime methiodide) and HI-6 (dichloride) were purchased from Sigma-Aldrich and US Biological. MMB4 was kindly provided by Dr. Tony Shih [USAMRICD (US Army Medical Research Institute of Chemical Defense), Aberdeen Proving Ground, MD, USA]. Aldoximes RS-194B and RS2-33A (oxime “3”) were prepared as previously described (Radi et al., 2012; Sit et al., 2014).

Reactivation screen of mipafox–hAChE by oximes *in vitro*

MPs or mipafox-inhibited hAChE were/was prepared by incubating 10 µl of 51 µM purified hAChE with 200 µM mipafox or 100 µM Flu-MPs in 100 mM sodium phosphate buffer, pH 7.4, for 5 min. Residual OP and other reactions by products were removed from incubation by spin filtering using Microcon-30 devices (Millipore, Bedford, MA) at 4°C and diluting to a protein concentration of 10 µM. The oxime reactivation experiments were performed as previously described (Sit et al., 2011). For this purpose, the inhibited enzyme was incubated with the oxime (at 37°C in the 0.1 M sodium phosphate buffer (pH 7.4) that contained 0.01% BSA] and the 10-µl aliquots were taken for the enzyme activity measurements at different times up to 1000 min.

Treatment of hAChE for the mass spectrometry analysis

Preparation of inhibited hAChE for MS peptide fingerprinting—The samples of purified recombinant 10 µM hAChE prepared in 100 mM sodium phosphate buffer at pH 7.4 were incubated with 100 µM paraoxon, 100 µM mipafox or 50 µM Flu-MP. The control samples of AChE were incubated only with buffer. Following incubation at room temperature for 3–5 min, an aliquot was removed to measure fractional activity by the Ellman assay and by the above-described procedure. Immediately afterward, buffer was exchanged and samples were denatured as described below.

Buffer exchange and denaturing—Residual OP and other reactions by products were removed from the incubation mixture. The buffer was exchanged to 50 mM ammonium bicarbonate buffer, pH 7.4, to a protein concentration of 10 µM by spin filtering using Microcon-30 devices (Millipore, Bedford, MA) at 4°C. Samples were denatured by

incubating at 95°C for 15 min and were subjected to trypsin proteolytic digestion (see the procedure below).

Preparation of aged hAChE for MS peptide fingerprinting—The purified recombinant 10 μ M hAChE samples prepared in 100 mM sodium phosphate buffer, pH 7.4, were incubated with either 100 μ M mipafox or 50 μ M Flu-MPs. The control hAChE samples were incubated only with buffer. Following incubation at room temperature for 5 min, an aliquot was removed to measure activity by the Ellman assay. After 5 min, one set of samples was subjected immediately to buffer exchange and denaturation, as described above. The other set of samples was incubated for 30 min at 37°C, and the final set of samples was incubated for 2 h.

Preparation of reactivated hAChE with oximes for MS peptide fingerprinting—The purified recombinant 10 μ M hAChE samples prepared in 100 mM sodium phosphate buffer, pH 7.4, were incubated with either 100 μ M mipafox or 50 μ M Flu-MPS. The control hAChE samples were incubated only with buffer. Following incubation at room temperature for 5 min, an aliquot was removed to measure fractional activity by the Ellman assay. Buffer was exchanged and residual OP and other reaction products were removed from the incubation mixture by spin filtering (as explained above). Oximes RS194B or 2PAM at the 2 mM concentration in ammonium bicarbonate were added to the inhibited samples and were incubated for 10 min. Aliquots were removed to measure activity by the Ellman assay and to be subjected to tryptic digestion

Trypsin proteolysis of hAChE

A volume of 50 μ l of 10 μ M inhibited, aged or reactivated hAChE in 50 mM ammonium bicarbonate, pH 7.4, was subjected to proteolytic digestion by overnight incubation with sequence-grade modified trypsin 1 μ l, 10 μ M (1:50 protein ratio of trypsin:hAChE) at room temperature.

The tryptic peptides from hAChE were lyophilized and dissolved in 20% ACN, 0.1% (v/v), trifluoroacetic acid (TFA) for the mass spectrometry analysis.

Prediction of peptides from tryptic digestion

The complete monomeric protein is composed of a FLAGpeptide (DYKDDDK), a linker (LEVLFQGPL) and the native coding sequence of human AChE (deposited in the SWISS-PROT database <http://www.expasy.ch/sprot/sprot-top.html> under accession code P22303) formed by 547 amino acids. The catalytic triad is predicted to contain the active center serine at position 203 in the native coding sequence.

In silico protein digestion was performed using the MS digest feature of Protein Prospector, version 4.04 (<http://www.prospector.ucsf.edu/mshome4.0.htm>), and the sequence for human AChE obtained from the SWISS-PROT database (P22303).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis

The trypsinized-treated samples of hAChE were analyzed on a PE Biosystems Voyager DE-STR Biospectrometry workstation (Framingham, MA, USA). Mass spectra were acquired in the positive ion linear mode under delayed extraction conditions using an acceleration voltage of 25 kV and a laser intensity of 2000–2400 V with a 337nm pulsed nitrogen laser. External calibration was performed with the ACTH peptide (amino acid residues 7–38) and bovine insulin, with average masses of 3657.93 and 5734.59 Da, respectively.

Trypsinized hAChE was mixed with a matrix of 5 mg/mL R-cyano-4 hydroxycinnamic acid (CHCA) in 50% (v/v) acetonitrile and 0.3% (v/v) TFA (trifluoroacetic acid), pH 2.2. An 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, monitored by a digital oscilloscope during acquisition. Laser intensity was adjusted so that ion intensity did not exceed 60–70% of the maximum threshold value. Data were processed and quantified with the PE Biosystems Grams 3.0 software program.

Tandem mass spectrometry (MALDI-TOF-TOF) analysis

The trypsinized-treated samples of hAChE were analyzed in an ABI 4800 MALDI TOF-TOF analyzer (Applied Biosystems Inc., Framingham, MA, USA). Trypsinized hAChE was mixed with a matrix of 5 mg/mL CHCA in 50% (v/v) acetonitrile and 0.3% (v/v) TFA, pH 2.2. An 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. MALDI spectra were recorded in the positive ion mode in the 4800 MALDI TOF/TOF analyzer, equipped with an Nd:YAG 355 nm laser. An accelerating voltage of 20kV was applied and the reflectron voltage was 25kV. Each spectrum represented the cumulative average of 400 profiles from each well. Data were processed and quantified with the Data Explorer software program (ABI).

Computational analysis

Density functional studies—The crystallographic structure of human AChE inhibited by tabun and complexed with fasciculin II was downloaded from the PDB database (Carletti et al., 2010) under code 2X8B. The fasciculin II structure was removed and the tabun structure was modified for the structures of mipafox, DFP or sarin, using the Accelrys Discovery Studio 3.5 software (Biovia, San Diego, USA). Molecular structures were optimized by the Insight II modeling suite (Accelrys, San Diego, USA).

QM calculations were done with the Spartan (version 10)[®] software (Hehre et al, 1999) and the Gaussian 03 package (Frisch et al 2004). The QM regions consisted in hAChE residues Asp74, Tyr86, Gly121, Tyr133, Glu202, Ala204, Trp236, Phe 295, Phe297, Glu334, Tyr337, Tyr341, His447, and OP-conjugated Ser203. The initial coordinates for all the studied systems were aligned with those from aged AChE from the crystallographic structures of *Torpedo californica* AChE/DFP and of mouse AChE/DFP. To perform the mechanistic study

with the mipafox structure, the O–P bond of OP (N–P for MPX) was broken and the distance between these two atoms varied from 1 600 to 3 300 Å in increments of 0.1 Å. After each change in the O–P distance, relative energy was calculated. The energy profile at the selected DFT geometries along the reaction pathway was computed at the B3LYP level of theory using the 6-311G basis set of the Gaussian 03 software (Frisch et al, 2004).

After each optimization, the nature of all the stationary points was established by calculating and diagonalizing the Hessian matrix (the force constant matrix). The unique imaginary frequency associated with the transition vector (TV) (Ramalho and Taft., 2005), i.e., the eigenvector associated with the unique negative eigenvalue of the force constant matrix, was characterized. All the minima connected by a given TS were confirmed by intrinsic reaction coordinate (IRC) driving calculations (Gonzalez and Schlegel., 1989) (in mass-weighted coordinates), as implemented in the Gaussian 03 program. (Frisch et al., 2004) The solvent effect was evaluated with the utilization of polarized continuum model (PCM) solvation calculations, initially proposed by Barone et al. (Baroni, 1998), using functional B3LYP and basis set 6-311G**. Herein the solute cavity may be specified as any set of overlapping spheres. By representing atoms as spheres, a more realistic cavity shape is produced for extended molecules, unlike another solvation model (Ramalho et al., 2008). The calculations of energy differences and energy barriers refer to the enthalpy, entropy and Gibbs-free energy terms, corrected for zero-point energy (ZPE) at 298.15 K.

The plots of energy versus the distance for each aged OP were constructed with the Sigma Plot software, version 12 (Systat Software Inc., Chicago, USA).

3. RESULTS

3.1 ENZYMATIC ACTIVITY ANALYSIS

Reactivation of mipafox-hAChE—More than 95% of enzymatic activity was inhibited by incubating the enzyme preparation with 100 µM mipafox within 5 min. Then the inhibitor was removed as described in Methods section. After adding oxime 2PAM (0.5, 1 or 5 mM) to the inhibited enzyme, the incubation performed at 37°C recovered activity, measured at 0, 3, 11, 25, 65, 80 and 960 min, and was always lower than 5% (Figure 3A).

During the procedure for removing the inhibitor, the residual mipafox concentration was estimated to be well below 50 nM. In order to rule out any possible reinhibition, an experiment was done by adding 100 nM mipafox and 5 mM 2PAM to the enzyme for times up to 1000 min. Activity was measured at 0, 10, 18, 30 and 1000 min. and was 110%, 97%, 97%, 103% and 97% respectively, compared to a control without mipafox. Hence no inhibition of activity was observed with 100 nM mipafox.

The alternative prolonged reactivation of mipafox inhibited hAChE (more than 95% inhibited hAChE by 100 µM mipafox) was tested with 1 mM of alternative oximes as follows: HI6 (65 min of reactivation) or RS194B, RS186B or RS41A (150 min of reactivation). Reactivation was always lower than 3% (data not shown).

Reactivation of sarin analog-inhibited hAChE—As positive controls, reactivation experiments were run with sarin analogs. For this purpose, hAChE was inhibited to more than 99% by 100 μ M Flu-MP for 5 min. Reactivation by 0.5 mM 2PAM for 40 min recovered around 40% of the initially inhibited hAChE activity (Figure 3B).

3.2. Mass spectrometry analysis

In silico prediction of the proteolytic digestion of hAChE: prediction of the active center peptide (ACP)—*In silico* prediction of the proteolytic digestion of hAChE was performed by considering peptides from 800.0 m/z to 5000.0 m/z using the Protein prospector proteomic tool, which predicted 54 amino acid peptide sequences. The active center peptide (ACP), which contains serine at position 203, was predicted from Leucine 178 to Arginine 219 as LALQWVQENVAAFGGDPTSVTLFGESAGAASVGMHLLSPPSR, with an associated average m/z of 4270.8. The adduction by organophosphorylation in the active serine (in bold) resulted in the positive average m/z shown in Table 1 of the Results section.

Peptide mass fingerprinting data of native hAChE—Coverage of >80% of the predicted peptides for digested AChE was obtained in the MALDI TOF. These peaks were used to obtain the most accurate calibration for the AChE experimental samples. Subsequently, internal calibration was performed using AChE peaks other than the ACP.

A peak of 4270.8 m/z was detected and identified as the peptide that corresponded to the ACP in unreacted hAChE, according to the prediction in Protein Prospector (Figure 4, **Plot A**).

Peptide mass fingerprinting data of paraoxon-inhibited hAChE—The ACPs of hAChE inhibited by paraoxon or Flu-MPS were predicted as shown in Table 1. The MS spectra of the samples, where more than 95% of the h-AChE activity was inhibited by paraoxon, showed a peak of 4406.7 m/z, which represents the ACP with an increase in mass 135.9 Da over the native ACP corresponding to the diethyl-phosphorylated ACP (Figure 4, **plot B**, Table 1).

Peptide mass fingerprinting data of sarin analog-inhibited hAChE—The MS spectra of the samples, where more than 95% of hAChE activity was inhibited by sarin analog Flu-MP, showed two peaks: one with 4391.1 m/z and the other with 4350.6 m/z. The former showed an increase of 120.3 Da over the native ACP and was identified as the methyl ethyl-phosphorylated ACP. The latter showed an increase of 79.8 Da over the native ACP and was identified as the ethyl-phosphorylated ACP, which corresponded to loss of a diethyl group during the aging reaction (Figure 4, **Plot C** Table 1). The other peaks corresponded to other *in silico*-predicted peptides (e.g. 3805.3 or 3867.3 m/z). Therefore, the applied methodology was able to detect the molecular structure after organophosphorylation and aging.

Peptide mass fingerprinting data of hAChE treated with mipafox—The MS spectra of the samples, where more than 95% of hAChE activity was inhibited by mipafox in

5 min, showed a peak of 4432.8–4433.3 m/z, which was detected and identified as the ACP with an N,N'-diisopropylphosphorodiamido adduct and an increase of 162–162.5 Da over the native ACP (Figure 5). When these samples were incubated for 30 minutes or 2 h, the same MS spectra were observed and no any other peaks were detected within the 3000–5000 m/z range (Figure 5). Hence no aged peptide was observed in any of the experiments.

To confirm the negative observation of the aged peptide, the samples inhibited and incubated for 30 min and 2 h were also analyzed in an ABI 4800 MALDI TOF-TOF mass spectrometer. The same number and mass fingerprinting peaks were detected. The enzyme was sequenced with 90% coverage from the data obtained with the ABI 4800 MALDI TOF-TOF (data not shown). This was considered an internal validation of the methodology employed (the enzyme used and the capability of detecting peptides in the instrument), which confirms the conclusion drawn of a negative observation of an aged peptide.

Serine phosphorylation identification by Tandem Mass Spectrometry—For a further detailed analysis, the ACP of the samples of hAChE unreacted and treated with mipafox was subjected to a second ionization process performed in the ABI 4800 MALDI TOF-TOF proteomics analyzer.

The MS fingerprinting spectra of the native ACP of unreacted hAChE (composed of the sequence of amino acids LALQWVQENVAAFGGDPTSVTLFGESAGAASVGMHLLSPPSR) showed a peak of 1550.8 m/z (Figure 6), which was identified as the ion composed of the sequence of amino acids AGAASVGMHLLSPPSR and a peak of 1637.84 m/z, identified as the ion composed of amino acids SAGAASVGMHLLSPPSR, where the first S is the serine of the active catalytic site.

The MS fingerprinting mass spectra of inhibited mipafox hAChE, left to age for 2 h, showed a peak of 1550.8 m/z, identified as the ion composed of the sequence of amino acids AGAASVGMHLLSPPSR, and also a peak of 1799.84 m/z with an increase of 162 Da over the 1637.84 m/z peak of the native ACP; it was identified as the ion composed of amino acids SAGAASVGMHLLSPPSR with an N,N'-diisopropylphosphorodiamido adduct. No unreacted peptide and non-aged peptide were observed in the samples left to age, which supports the notion that neither spontaneous reactivation nor aging was observed.

MS analysis of the inhibited active center peptide treated with oximes for reactivation—Figure 7 presents the MALDI-TOF mass spectral data acquired for the tryptic digests of the mipafox-hAChE and Flu-MPs-hAChE conjugate samples, which contained less than 5% of the initial hAChE activity, and which were incubated with either 5 mM 2PAM or 5 mM 194B for 10 minutes at 37°C.

Reactivation experiments with sarin analog-inhibited hAChE: A peak of 4389.6–4391.8 m/z was observed in the sarin analog-hAChE conjugate samples, which was identified as the methyl ethyl-phosphorylated ACP with an increase of 120.3 Da over the native ACP (Figure 7; Panels D, E, F).

A peak with a mass of 4270.9 was observed in the sarin- analog-hAChE conjugate samples incubated with either 2PAM or 194B (Figure 7; **Panels E, F**), which represented the unmodified ACP and was identified as the reactivated ACP.

Reactivation experiments with mipafox-inhibited hAChE: A peak of 4432.4–4433.9 m/z was observed in the spectra of all the mipafox-inhibited hAChE samples. This peak was identified as the ACP modified with an N,N'- diisopropylphosphorodiamido adduct and with an increase of 162–162.5 Da over the native ACP (Figure 7; **Panels A, B, C**). However neither a peak corresponding to the native ACP nor an aged peptide peak was detected after mipafox inhibition (Figure 7; **Panels B, C**).

3.3. COMPUTATIONAL MOLECULAR MODELING ANALYSIS

The molecular structures of the aged mipafox, DFP and sarin-analog inhibited hAChE were modeled as explained in the Methods section by applying the Gaussian 03 software. The crystal structure of the aged Tabun-inhibited hAChE was used as a template (Figure 8). The QM regions were considered to include amino acids Asp74, Tyr86, Gly121, Tyr133, Glu202, Ala204, Trp236, Phe 295, Phe297, Glu334, Tyr337, Tyr341, His447, and the corresponding OP conjugated at Ser203.

The distances between atoms separated by dealkylation were fitted in the Spartan software (Hehre, 1999). The energy calculated is shown in Figure 9. Only the relationships for the mipafox-hAChE conjugate showed a significant discontinuous increase of energy.

4. DISCUSSION

This work provides evidence that mipafox-inhibited hAChE is reductant to reactivation by oximes, while the MS analysis demonstrated the phosphorylated adduct, but not the aged adduct, after being left to age for 2 h. This negative observation is confirmed in the experiments with two different mass spectrometers, and also when a second ionization was performed in MALDI-TOF-TOF. With the same experimental conditions and methodology, aged adducts were observed in a positive control with a sarin analog. The MS analysis was also able to detect more than 90% of the total protein sequence, which suggests no significant loss of detectable peaks. The computational modeling of the aged mipafox-hAChE also indicated a high energy demand for aging, so probably not facilitated energetically. Therefore, this work indicates that aging in the reaction does not significantly occur under our tested condition with human AChE after left 2 h to age. The *in vitro* kinetics data of this work show a time-dependent reactivation for MPs-inhibited hAChE of around 40% with oxime 2-PAM in 40 min. However, no progressive reactivation of activity was observed for mipafox-inhibited hAChE a few reactivation (2–5 % of activity) was observed, but was no progressive when the enzyme was incubated overnight with the oxime (Figure 3). Several more potent and structurally diverse oximes (HI6, RS194B, RS186B and RS41A; Radic et al., 2012) did not show any reactivation after mipafox inhibition. No reactivation in this experimental situation could be explained by three alternatives: (A) a very fast aging reaction (while preparing the inhibited enzyme and removing the inhibitor); (B) an experimental artifact in removing mipafox, so reactivation and reinhibition occurred at the same time. So we observed the result of both kinetic reactions while measuring enzymatic

activity; and (C) the mipafox-inhibited enzyme is very stable and refractory to reactivation by oximes. Since no inhibition was observed during the simultaneous incubation of native hAChE with 2PAM and mipafox, option B can be ruled out.

The MS studies of paraoxon-inhibited hAChE and sarin analog-inhibited hAChE revealed that our MS method is able to detect the inhibited and aged active center peptide (ACP) (Figures 4). However, the mass spectra of the mipafox-inhibited hAChE conjugate incubated for 30 min or 2 h after inhibition (to allow aging, if required) were identical and revealed only an ACP adducted with a mass of 4332–4333 Da. These results indicate the absence of post-inhibitory reactions following the mipafox inhibition of hAChE within 2 h of the experiment. No peak of the native ACP was detected, which suggests that no significant spontaneous reactivation occurred within 2 h.

The differential influence of oximes 2PAM and RS-194B on the tryptic peptides profile in the MS analysis of the sarin/hAChE-inhibited conjugates (Figure 7) was detected, but not after mipafox inhibition. This suggests that mipafox-inhibited hAChE is largely resistant to reactivation, and reveals only the inhibited form with an N,N'-diisopropylphosphorodiamido adduct. These data demonstrate the resistance of a phosphorylated, but not aged, serine esterase to reactivation by efficient structurally diverse oxime reactivators of a variety of other OP-hAChE conjugates. However, Milatovich and Johnson (1993) reported the reactivation of mipafox-inhibited AChE and NTE by prolonged treatment with aqueous potassium fluoride (KF), and they did not detect aging either. Other authors have shown that Tabun-ChE conjugates display extraordinary resistance to most oximes. The crystal structures of non-aged and aged forms of tabun-inhibited hBChE have clearly shown that the aging reaction proceeds through O-dealkylation of tabun. In this case, however, the MS analysis of aged tabun-inhibited hBChE revealed that both the dimethylamine and ethoxy side chains were missing from phosphorus (Carletti et al., 2008).

It should also be highlighted that mipafox is the OP model of OPIDN, whose mechanism is assumed to be the inhibition and aging of enzyme NTE. Kropp et al., (2006) observed similar results in a MS analysis of NEST (the recombinant NTE esterase domain) inhibited by mipafox and DFP. These authors detected an adducted ACP with a mass of 162.7, which they interpreted as an ACP with an N,N'-diisopropylphosphorodiamido adduct, which could have been aged by reversible proton loss. These authors were unable to reactivate any enzymatic activity within 18 h of using KF or KCl. Similar conclusions of their study and ours indicate that mipafox-inhibited enzymes can be very stable and refractory to nucleophilic reactivation.

However, the data presented in this work are not in line with previous studies by Kropp and Richardson (2005). In an MS analysis of mipafox-inhibited AChE, they detected a peptide with a mass of 4352.5. They attributed it to be the ACP with an increased mass of 81.5, which they identified to be the adduction of mipafox after losing both isopropylamine groups. We can assume several reasons for this apparent discrepancy: (1) a different mechanism in the post-inhibitory reaction in different preparations of the hAChE enzymes used; (2) a very small percentage of the enzyme can be aged and we were unable to detect it; or (3) there was an MS artifact in which addition of a phosphate group was observed during

electrospray ionization, which could occur in the different equipments used to various extents (Schroeder et al., 2004).

Alternative MS equipment, with a second ionization process and a last-generation tandem mass spectrometer, was also used in our work to elucidate this discrepancy, and no aged peptide was observed in any sample. The enzyme was also sequenced with more than 90% coverage. This allowed us to validate our enzyme and the methodology employed.

With all the information we have obtained to date, it seems plausible that mipafox hAChE is not reactivatable in spite of an aging reaction being absent, as shown with NEST. As the aging rate is usually determined operationally by kinetic methods, which do not differentiate between aged and inhibited moieties that are reactivatable, an MS method was required to elucidate the exact mechanism of interaction between mipafox and serine esterases.

Plots of energy versus the distance of the atoms being separated during dealkylation in the molecular modeling experiments may possibly indicate that for mipafox, eventual aging would imply high energy demand. With sarin and DFP however, for which aging was observed in MS, the energy demand was lower and, consequently, aging was more likely. This is the first observation reported of an AChE inhibited by OPs where no reactivation has been observed in nonaging OP-inhibited AChE.

These results will be useful for understanding resistance to oxime reactivation. Knowledge of the three-dimensional structure of noninhibited, inhibited and aged cholinesterases will allow us to understand the intimate irreversible enzyme inhibition mechanism. Modeling the enzyme structure in the presence of effectors is essential for finding out new therapeutic agents against organophosphate poisoning.

This work raises many questions about the aging mechanism of OPs. One could assume that aging is not energetically beneficial and is not produced, and it remains unclear why no reactivation occurred after mipafox inhibition with the more potent structurally diverse oximes. The approach of combining a kinetics analysis with the mass spectrometry and molecular modeling presented herein has proven useful for elucidating the inhibitory and post-inhibitory reactions of diverse OPs with serine esterases, and could be applied in research with other irreversible enzyme inhibitors. The MS and molecular modeling methods presented in this work will be useful for conducting further research. Research by crystallography could also provide relevant information about the aging process and its role in both toxicity and therapeutic approaches of OP intoxications.

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Figure 1.
Inhibition, aging and reactivation of a serine esterase by an organophosphorus compound. 1. A serine esterase interacts with an organophosphorus compound. 2. The enzyme is inhibited. 3 The enzyme is aged. 4. Pralidoxime (2-PAM) attacks the inhibited enzyme, the enzyme is reactivated and OP binds to 2-PAM. 5. The aged enzyme is unable to be reactivated.

Figure 2.

Chemical formulas of organophosphate AChE inhibitors. A Mipafox (N, N ϵ -diisopropylphosphorodiamidofluoridate), **B** paraoxon (diethyl 4-nitrophenyl phosphate) and **C Flu MP** (Fluorescent methylphosphonofluoridate).

Figure 3.

Progressive reactivation of mipafox hAChE and Flu MP hAChE by oxime 2PAM. A.

Enzymatic activity of mipafox-inhibited hAChE recovered after removing the inhibitor and adding 2PAM for times up to 1000 min. The black triangles shows the reactivation with 5 mM, white circles with 1 mM 2PAM and black circles with 0.5 mM 2PAM. B. Enzymatic activity of sarin-inhibited hAChE recovered after removing the inhibitor and adding 5 mM 2PAM for times up to 40 min.

Figure 4. Representative MALDI-TOF mass spectra acquired from the total peptides of hAChE tryptic digests (3000– 5000 m/z). **A.** Control hAChE sample. **B.** hAChE with more than 95% of the activity inhibited by paraoxon **C.** More than 95% of the activity inhibited by Flu-MPs. The peak of 4270. m/z 8 represents the ACP that contains catalytic serine. The peak of 4406.7 m/z represents the diethyl-phosphorylated ACP with a corresponding increase of 135.9 Da in the molecular mass of the ACP. The peak of 4350.6 m/z represents the methyl ethyl-phosphorylated ACP with a corresponding increase of 120.3 Da in the

molecular mass of the ACP. The peak of 4391.1 m/z represents the ethyl-phosphorylated ACP with a corresponding increase of 79.8 Da in the molecular mass of the ACP.

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Figure 5.

Representative MALDI-TOF mass spectra acquired from the total peptides of hAChE tryptic digests. A. Control hAChE sample. B. hAChE modified by mipafox. C. hAChE modified by mipafox and left to “age” for 30 min. D. hAChE modified by mipafox and left to “age” for 2 h. The peak of 4270.8 m/z represents the ACP that contains catalytic serine. The peak of 4432.8–4433.3 m/z represents the modified ACP with an N,N'-diisopropylphosphorodiamido adduct and a corresponding increase of 162–162.5 Da in the molecular mass of the ACP.

Figure 6. Representative MALDI-TOF TOF mass spectra using the 4800 proteomics analyzer in the positive reflectron mode (A) hAChE unreacted (B) hAChE inhibited (C) hAChE inhibited and left to age for 30 min (D) hAChE inhibited and left to age for 2 h. The peak of 1550. m/z 8 represents the sequence of amino acids AGAASVGMHLLSPPSR. The peak of 1799.84 m/z. The peak of 1637.84 m/z represents the sequence of amino acids SAGAASVGMHLLSPPSR, where the first S is the serine of the active catalytic site. The peak of 1799.84 m/z represents the ion composed of amino acids

SAGAASVGMHLLSPPSR with an N,N'- diisopropylphosphorodiamido adduct and a corresponding increase of 162 Da.

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Figure 7.

Representative MALDI-TOF mass spectra acquired from the total peptides of hAChE tryptic digests inhibited by 100 μ M mipafox for 50 min or 50 μ M Flu-MPs for 2 min and incubated with 2 mM 2PAM or 2 mM 194B oximes for 10 min. In all cases, inhibition was higher than 98% of activity. **A. hAChE inhibited by mipafox and incubated with 2PAM for 10 min. **B.** A. hAChE inhibited by mipafox and incubated with 194B for 10 min. **C.** hAChE inhibited by Flu-MPs and incubated with 2PAM for 10 min. **D.** hAChE inhibited by Flu-MPs and incubated with 194B for 10 min. The peak of 4270.9 m/z represents the unmodified ACP. The peak of 4432.4–4433.9 m/z represents the modified ACP with an N,N'-diisopropylphosphorodiamido adduct and a corresponding increase of 162–162.5 Da in the molecular mass of the ACP. The peak 4389.6–4390.7 m/z represents the modified ACP with a methylphosphonylated adduct and a corresponding increase of 121 Da in the molecular mass of the ACP. The experimental conditions used to prepare the enzyme and to generate spectra are described Methods.**

Figure 8. Molecular structures of hAChE with mipafox, DFP and sarin, which were age-modeled. hAChE inhibited and aged by mipafox (A, B), DFP (C) and sarin (D) were modeled by homology using the crystal structure of hAChE aged by Tabun in Spartan as a template. The QM regions consisted of amino acids Asp74, Tyr86, Gly121, Tyr133, Glu202, Ala204, Trp236, Phe 295, Phe297, Glu334, Tyr337, Tyr341, His447, and the corresponding OP conjugated at Ser203.

Figure 9.

Variation of relative energy with the distance between O(Ser203)-P(OP) for the aging mechanisms of DFP and Sarin, and the possible aging mechanisms of mipafox. Black circles (sarin aging), white circles (mipafox aging), gray triangles (mipafox at both sites) and white triangles (DFP). The QM calculations were obtained with the Spartan software (Hehre, 1999) using the initial coordinates for all the studied systems, including those from AChE aged from the crystallographic structures of AChE/DFP and AChE/sarin. The distance between the O—P bond of the OP with Ser varied from 1600 to 3300 Å at intervals of 0.1 Å, and relative energy was calculated.

Table 1

Masses of theoretical and observed MH⁺ ions unmodified and modified active center peptides of human AChE with OPs paraoxon, mipafox and Flu-MPs.

MH ⁺ (average m/z)		
<i>Peptide</i>	<i>Theoretical</i>	<i>Observed</i>
<i>ACP unmodified</i>	4270.8	4270.8–4270.9
<i>ACP inhibited by mipafox</i>	4432.8	4432.8–4433.9
<i>ACP aged by mipafox that lost two isopropylamine groups</i>	4349.8	not observed
<i>ACP aged by mipafox and lost an isopropylamine group</i>	4396.8	not observed
<i>ACP inhibited by paraoxon</i>	4406.8	4406.7
<i>ACP inhibited by Flu-MPs or sarin</i>	4391.8	4390.6–4391.8
<i>ACP inhibited and aged by Flu-MPs or sarin</i>	4350.8	4350.6–4350.9