

# Mass Spectrometry Method to Identify Aging Pathways of *Sp*- and *Rp*-Tabun Adducts on Human Butyrylcholinesterase Based on the Acid Labile P-N Bond

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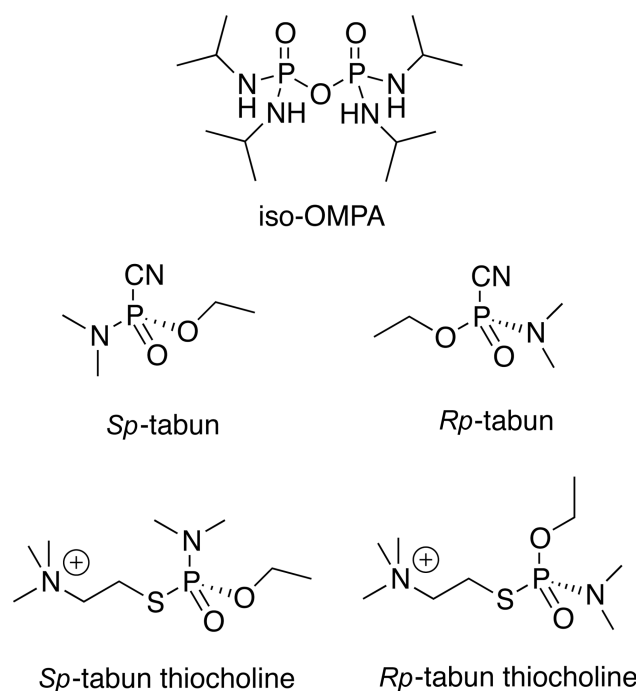
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The phosphoramidate nerve agent tabun inhibits butyrylcholinesterase (BChE) and acetylcholinesterase by making a covalent bond on the active site serine. The adduct loses an alkyl group in a process called aging. The mechanism of aging of the tabun adduct is controversial. Some studies claim that aging proceeds through deamination, whereas crystal structure studies show aging by O-dealkylation. Our goal was to develop a method that clearly distinguishes between deamination and O-dealkylation. We began by studying the tetraisopropyl pyrophosphoramidate adduct of BChE because this adduct has two P-N bonds. Mass spectra showed that the P-N bonds were stable during trypsin digestion at pH 8 but were cleaved during pepsin digestion at pH 2. The P-N bond in tabun was also acid labile, whereas the P-O bond was stable. A scheme to distinguish aging by deamination from aging by O-dealkylation was based on the acid labile P-N bond. BChE was inhibited with *Sp*- and *Rp*-tabun thiocholine nerve agent model compounds to make adducts identical to those of tabun with known stereochemistry. After aging and digestion with pepsin at pH 2, peptide FGES<sub>198</sub>AGAAS from *Sp*-tabun thiocholine had a mass of 902.2 m/z in negative mode, indicating that it had aged by deamination, whereas peptide FGES<sub>198</sub>AGAAS from *Rp*-tabun thiocholine had a mass of 874.2 m/z in negative mode, indicating that it had aged by O-dealkylation. BChE inhibited by authentic, racemic tabun yielded both 902.2 and 874.2 m/z peptides, indicating that both stereoisomers reacted with BChE and aged either by deamination or dealkylation.

**Key Words:** iso-OMPA; tabun; aging; dealkylation; deamination; mass spectrometry.

The nerve agents sarin, soman, cyclosarin, VX, and VR make adducts on serine 198 of butyrylcholinesterase (BChE) that lose an alkyl group in a process called aging. The aged adducts of these nerve agents are stable during digestion with pepsin at pH 2 (Fidder *et al.*, 2002; Sporty *et al.*, 2010). That is, the organophosphorus molecule does not lose additional groups of atoms during digestion with pepsin. The alkoxy

groups of sarin, soman, cyclosarin, VX, and VR are attached to phosphorus through an oxygen atom. By contrast, all alkyl groups in tetraisopropyl pyrophosphoramidate (iso-OMPA) are attached to phosphorus through nitrogen atoms (Fig. 1).



**FIG. 1.** Chemical structures and stereochemistry of the molecules described in this study. Tabun has a center of chirality about phosphorus and can exist as two stereoisomers. Upon binding to BChE, the cyanide leaving group in authentic *Sp*- and *Rp*-tabun points toward the mouth of the active site gorge. In contrast, the thiocholine leaving group in *Sp*- and *Rp*-tabun thiocholine model compounds points toward the choline-binding site. The orientation of authentic tabun in human BChE and AChE has been established by crystal structure analysis. The orientation of the tabun model compounds is based on the results of this work but has not yet been confirmed by crystal structure studies.

In this report, we demonstrate that the P-N bonds in the iso-OMPA adduct of BChE are hydrolyzed at pH 2 during pepsin digestion. Similarly, the P-N bond in tabun-inhibited BChE is acid labile (Carletti *et al.*, 2008; van der Schans *et al.*, 2008). Knowledge of the acid labile nature of the P-N bond was applied to a study of the mechanism of aging of tabun-inhibited BChE. The tabun adduct on BChE contains an ethyl group attached to phosphorus through an oxygen atom and dimethylamine attached to phosphorus through a nitrogen atom. Only the P-N bond is labile at pH 2. Based on the observation that only the P-N bond is acid labile, we identified which alkyl group is present after the tabun adduct on BChE has undergone an enzyme-catalyzed dealkylation.

Mass spectrometry results of aged tabun adducts on BChE and acetylcholinesterase (AChE) have been equivocal because the mass difference between a tabun adduct that has aged by O-dealkylation and an adduct that has aged by deamination is 1 Da. The first studies on the mechanism of aging of tabun-inhibited AChE concluded that aging proceeds by deamination (Barak *et al.*, 2000; Elhanany *et al.*, 2001). However, this conclusion was reversed in crystal structure studies which showed that aging of tabun-inhibited AChE proceeds by O-dealkylation (Carletti *et al.*, 2008, 2010). The controversy regarding the mechanism of aging of tabun adducts reveals the need for a method that clearly distinguishes between the two possible mechanisms of aging of phosphoramidyl adducts. In the present work, we provide such a method. The mass spectrometry method described herein is based on the observation that the P-N bond, but not the P-O bond, is acid labile. We applied this method to human BChE inhibited by stereoisomers of tabun thiocholine (model compounds of tabun) and to BChE inhibited by authentic racemic tabun. Our results support the conclusion that the *Rp*-tabun thiocholine adduct ages by O-dealkylation, whereas the *Sp*-tabun thiocholine adduct ages by deamination. Both stereoisomers of authentic racemic tabun make adducts with BChE, and consequently, the aging products represent both *Rp*- and *Sp*-tabun.

## MATERIALS AND METHODS

The following were from Sigma-Aldrich (St Louis, MO). iso-OMPA (Sigma T1505) was dissolved in 95% ethanol to 25mM. A 20mg/ml solution of 2, 5-dihydroxybenzoic acid (DHB) (Fluka 85707) in 50% acetonitrile, 1% trifluoroacetic acid was stored at  $-20^{\circ}\text{C}$ . Porcine pepsin (Sigma P-6887) was dissolved in 5% formic acid about 30 min before use. Stereoisomers of a tabun nerve agent model compound, (dimethylamino(ethoxy)phosphoryl) thiocholine, were stereoselectively synthesized at the Human BioMolecular Research Institute (San Diego, CA) (Barakat *et al.*, 2009). Stereoisomers were purified by silica gel column chromatography. Optical rotation was measured on a Jasco P-1010 polarimeter (Jasco, Easton, MD). Both the *Sp* and *Rp* stereoisomers gave a single peak when analyzed by  $^{31}\text{P}$  NMR, suggesting that the compounds are > 95% pure (Barakat *et al.*, 2009). The leaving group in the tabun model compounds was thiocholine rather than cyanide (Barakat *et al.*, 2009). The *Sp* stereoisomer was dissolved in dimethylsulfoxide. The *Rp* stereoisomer was used as a solid. These tabun nerve agent model compounds afford the same adduct on human BChE as authentic tabun, as measured by mass spectrometry

(Gilley *et al.*, 2009). Authentic racemic tabun (CEB, Vert-le-Petit, France) was used in a surety laboratory to inhibit BChE. A saturated solution of  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) matrix (Applied Biosystems, Foster City, CA) was prepared in 50% acetonitrile, 0.1% trifluoroacetic acid. Sequencing grade-modified trypsin (Promega V5113), trifluoroacetic acid (A11650), and acetonitrile (BP1170) were from Fisher Scientific (Fair Lawn, NJ). BChE was purified from outdated human plasma as described (Lockridge *et al.*, 2005).

**Assay of BChE activity.** BChE activity was measured with 1mM butyrylthiocholine and 0.5mM 5, 5'-dithiobis (2-nitrobenzoic acid) in 0.1M potassium phosphate buffer pH 7.0 at  $25^{\circ}\text{C}$ . The change in absorbance at 412 nm was monitored in a Gilford spectrophotometer in quartz cuvettes. Units of activity were calculated using an extinction coefficient of  $13,600\text{M}^{-1}\text{cm}^{-1}$  (Ellman *et al.*, 1961). One unit of BChE activity was defined as the amount that hydrolyzes 1  $\mu\text{mole}$  of butyrylthiocholine in 1 min. The concentration of BChE protein was calculated from units per milliliter using 720 units/mg as the specific activity of pure BChE (Lockridge *et al.*, 2005). For example, a BChE solution with an activity of 170 units/ml has a BChE protein concentration of 0.236 mg/ml.

**Inhibition of BChE activity by iso-OMPA, *Sp*- and *Rp*-tabun nerve agent model compounds, and authentic racemic tabun.** A 1-ml aliquot of 2.8  $\mu\text{M}$  BChE (170 units/ml) in 10mM  $\text{NH}_4\text{HCO}_3$  0.01% sodium azide (pH 8.1) was treated with 10  $\mu\text{l}$  of 25mM iso-OMPA at  $37^{\circ}\text{C}$  for 3 days. A 0.5-ml aliquot of 2.8  $\mu\text{M}$  BChE in 10mM  $\text{NH}_4\text{HCO}_3$  0.01% sodium azide (pH 8.1) was incubated with approximately 20  $\mu\text{M}$  *Sp*- or *Rp*-tabun thiocholine at  $37^{\circ}\text{C}$  for 4 days to allow the adducts to age. BChE (50  $\mu\text{l}$  of 1.32 mg/ml) inhibited with 5  $\mu\text{M}$  authentic racemic tabun was aged for 4 days at room temperature and stored for 4 years at  $-20^{\circ}\text{C}$ . BChE activity was completely inhibited at the end of the incubation periods.

**Pepsin digestion of inhibited BChE.** The pH of a 70- $\mu\text{l}$  aliquot of inhibited BChE containing a total of 16.5  $\mu\text{g}$  BChE protein was adjusted to pH 2 by adding 0.7  $\mu\text{l}$  of 25% trifluoroacetic acid. A fresh solution of 2 mg/ml pepsin in 5% formic acid was prepared just before use. The BChE was digested with 4  $\mu\text{l}$  pepsin (8  $\mu\text{g}$ ) at  $37^{\circ}\text{C}$  for 2 h. Digestion was stopped by incubating the reaction mixture 5 min in a boiling water bath. A 1- $\mu\text{l}$  aliquot was analyzed by MALDI-TOF mass spectrometry. The remaining digest of iso-OMPA-treated BChE was enriched on a Titansphere tip in preparation for analysis on a 4800 MALDI-TOF/TOF mass spectrometer.

Pepsin-digested samples were also purified by HPLC. Samples intended for HPLC purification contained 0.4 ml of inhibited BChE (236  $\mu\text{g}$ ) digested with 100  $\mu\text{g}$  pepsin.

**Trypsin digestion of iso-OMPA-inhibited BChE.** Excess iso-OMPA was removed from 70  $\mu\text{l}$  of iso-OMPA-treated BChE by diafiltration in a Centricon 100,000 molecular weight cutoff centrifugal filter (Amicon, Beverly, MA). The BChE tetramer has a molecular weight of 340,000, which allowed the BChE protein to be concentrated in the upper compartment, whereas iso-OMPA with a molecular weight of 342.3 grams/mole passed through the filter. The concentrated BChE was diluted with 2 ml of 10mM  $\text{NH}_4\text{HCO}_3$  0.01% azide pH 8.1 and reconcentrated. Iso-OMPA-treated BChE (16  $\mu\text{g}$ ) and control BChE were denatured in a boiling water bath for 10 min and cooled to room temperature before digestion with 0.8  $\mu\text{g}$  trypsin at pH 8.1, overnight at  $37^{\circ}\text{C}$ . The mass of the modified BChE peptide was determined from mass spectra acquired in the MALDI-TOF/TOF mass spectrometer.

**HPLC purification.** Pepsin-digested BChE samples were purified by HPLC for the purpose of obtaining a peptide sample relatively free of contaminating peptides. Purification decreased ion suppression effects and therefore gave an improved MS/MS spectrum in the MALDI-TOF/TOF mass spectrometer. Samples were injected onto a Phenomenex C18 column,  $100\times 4.6\text{mm}$  using a Waters 625 LC system. Peptides were eluted with a 60-min gradient starting with 100% buffer A (0.1% trifluoroacetic acid in water) and ending with 60% buffer B (acetonitrile containing 0.09% trifluoroacetic acid) at a flow rate of 1 ml/min. One-ml fractions were collected. The volume of each fraction was decreased to about 50  $\mu\text{l}$  by vacuum centrifugation before 1- $\mu\text{l}$  aliquots were analyzed with a MALDI-TOF/TOF mass spectrometer.

**Enrichment of iso-OMPA-modified peptide by binding to titanium oxide.** The active site BChE peptic peptide FGES<sub>198</sub>AGAAS modified on serine 198 by iso-OMPA was enriched on titanium oxide beads (Liyasova *et al.*, 2011) using the solvents reported by Kweon and Håkansson (2006). Titansphere beads (5  $\mu$  bulk media, part number 5020–7500; GL Sciences Inc., Torrance, CA) were manually packed into a micro spin column (TopTip 1–10  $\mu$ l, part number TT1EMT; Glygen Corp., Columbus, MD) to a bed height of 3 mm (2 mg TiO<sub>2</sub>). Titansphere tips were washed with 3.3% formic acid before samples were loaded on the tips. Digests were centrifuged through the TiO<sub>2</sub> micro column at a speed of 2000rpm in a Sorvall MC12V microfuge (1500  $\times$  g). After the sample had passed through the tip, the tip was washed with 75  $\mu$ l water. Bound peptides were released with 20  $\mu$ l of 0.5% piperidine (pH 11). Samples were dried by vacuum centrifugation and dissolved in 5  $\mu$ l of 5% acetonitrile, 0.1% trifluoroacetic acid. One- $\mu$ l aliquots were spotted onto a 384-well target plate (cat. no. 1016629; Applied Biosystems, Foster City, CA), dried, and overlaid with DHB matrix in preparation for analysis with the MALDI-TOF/TOF mass spectrometer.

**Mass spectrometry.** MALDI mass spectra were acquired on a MALDI-TOF/TOF 4800 mass spectrometer (Applied Biosystems, Framingham, MA). Mass spectra were taken in negative reflector mode with DHB matrix for peptides from pepsin digestion and in positive reflector mode with CHCA matrix for peptides from digestion with trypsin. Each spectrum was the average of 3000 laser shots taken with the laser energy adjusted to 6000V for negative mode and 5000V for positive mode. MS/MS fragmentation spectra were taken using a 1kV positive ion method, with the timed ion selector enabled, the metastable ion suppression on, and the precursor ion mass window set to  $\pm$  1 Da. Laser intensity was adjusted to obtain maximum signal intensity without exceeding the saturation limit. Spectra were analyzed with Data Explorer Software. The amino acid sequences of the peptides were determined by manual inspection of the MS/MS fragmentation spectra, with the aid of the MS-Product algorithm from Protein Prospector (<http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msproduct>) and the Proteomics Toolkit (<http://db.systemsbiology.net:8080/proteomicsToolkit/FragIonServlet.html>) from DB Systems Biology.

**Molecular modeling.** The topological description of tabun thiocholine was built using acpype and the general amber force field (Wang *et al.*, 2004). The structure of hAChE was prepared from chain A of PDB entry 3lii. The structure of hBChE was prepared from PDB entry 1p0i. The missing surface loops were rebuilt using MODELLER 9v8 (Eswar *et al.*, 2007). The networks of crystallographic water molecules of human AChE and BChE were conserved in the initial models. Molecular dynamics simulations were carried out using GROMACS 4.5.4 (Hess *et al.*, 2008) and the Amber99sb forcefield (Hornak *et al.*, 2006). The Lennard-Jones interactions were cut off at 1 nm. The long-range electrostatic interactions were handled using the particle-mesh Ewald method for determining long-range electrostatics (10 Å cutoff). Temperature was set to 310K and was kept constant using a Berendsen thermostat (with a coupling time constant of 0.1 ps) (Berendsen *et al.*, 1984). Pressure with a reference value of 1 bar was controlled by a Berendsen barostat (with a coupling time constant of 0.5 ps). To build the initial structure of the hAChE/tabun thiocholine, tabun thiocholine was manually docked into hAChE and hBChE. The complexes were immersed in a periodic water box of cubic shape with a minimal distance of 10 Å to any edge and periodic boundary conditions. The box was solvated using the TIP3P solvation model. Chloride and sodium counter ions at a concentration of 50mM were added to neutralize the simulation system. After energy minimization using a 500-step steepest descent method, the tabun thiocholine complex was subjected to equilibration at 310K and 1 bar for 50 ps under the conditions of position restraints for heavy atoms of the protein. All bond lengths were constrained using the LINCS algorithm (Hess *et al.*, 1997). The resulting conformation was optimized by a final 500-step steepest descent minimization.

**Safety considerations.** Tabun is highly toxic and is classified as a schedule 1 chemical as defined in the Chemical Weapons Convention. Tabun was used in a military laboratory certified for work with nerve agents. Nerve agent model

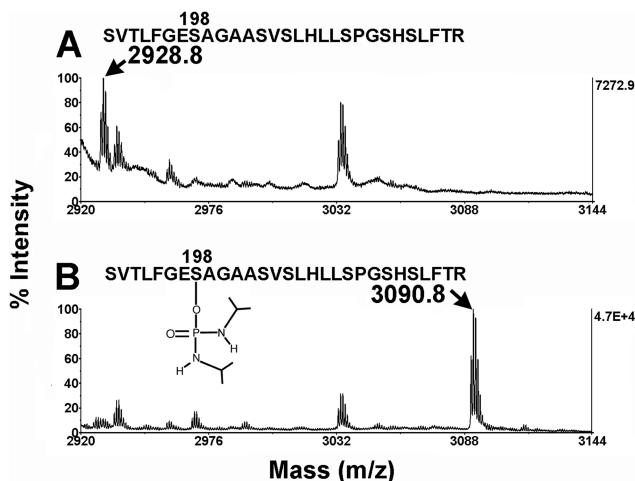
compounds are less toxic than authentic nerve agents (Kalisiak *et al.*, 2011). The amounts available were lower than the amount that would intoxicate a human. Used vials and pipette tips were detoxified in 0.1M sodium hydroxide. Personal protective equipment was worn.

## RESULTS

### Iso-OMPA Adds 162 Da to Serine 198 of Human BChE

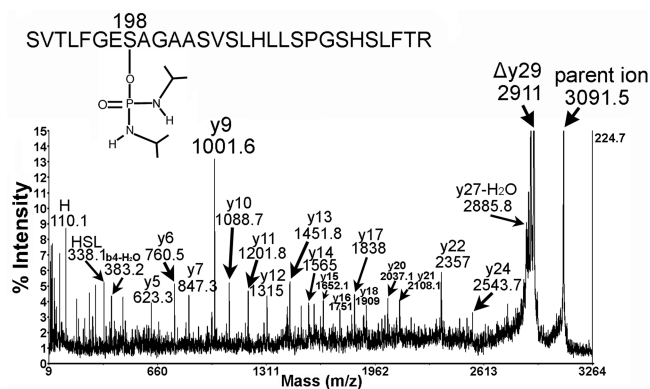
BChE activity was completely inhibited after incubation with 0.25mM iso-OMPA. Mass spectrometric analysis of the trypsin-digested BChE showed that the inhibited BChE was covalently modified on serine 198, the active site serine of BChE. In Figure 2A, the unmodified active site peptide of human BChE, SVTLFGES<sub>198</sub>AGAASVSLHLLSPGSHSLFTR, is at 2928.8 m/z. In the iso-OMPA-inhibited BChE sample, Figure 2B, the mass of the 2928.8 peptide disappeared and a new peptide at 3090.8 m/z (162 Da heavier) appeared. The mass of the adduct is consistent with the structure indicated in Figure 2B, wherein one half of the original iso-OMPA is covalently bound to serine 198, and the other half is displaced by reaction with serine 198. Absence of a peak at 3048 m/z indicated that the iso-OMPA adduct on BChE did not lose an alkyl group during sample preparation and 3-day incubation at 37°C. That is, there was no evidence for aging of the iso-OMPA adduct. Furthermore, there was no evidence for hydrolysis of both P-N bonds because there was no peak at 3008 m/z (Fig. 2B).

Confirmation of the identity of the 3091 Da mass was obtained with a MALDI-TOF/TOF mass spectrometer. The MS/MS spectrum (Fig. 3) supports the peptide sequence



**FIG. 2.** Mass spectra of tryptic digests of BChE with and without iso-OMPA. MS spectra were acquired with CHCA matrix on a MALDI-TOF/TOF mass spectrometer. (A) Highly purified human BChE digested with trypsin. The active site peptide has the sequence SVTLFGES<sub>198</sub>AGAASVSLHLLSPGSHSLFTR and a mass of 2928.8 Da in positive mode. (B) The same BChE preparation treated with 0.25mM iso-OMPA, which inhibited 100% of the BChE activity, was digested with trypsin. The active site peptide from this preparation had a mass of 3090.8 Da in positive mode. The additional mass of 162 Da was on active site serine 198 (accession no. gi34810860 for human BChE protein).



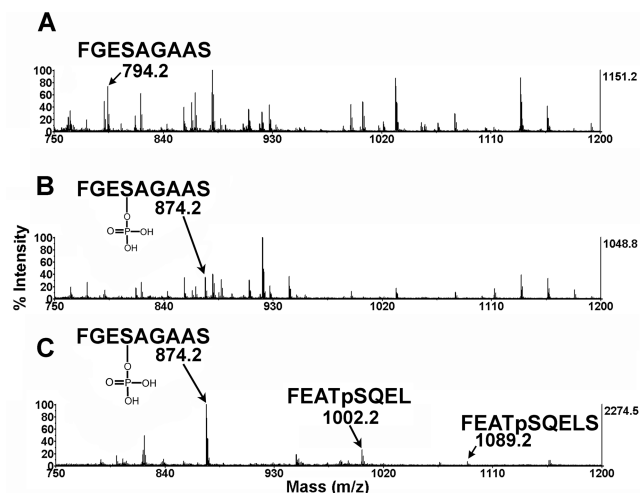


**FIG. 3.** MS/MS spectrum confirming the identity of the iso-OMPA-modified residue in human BChE as serine 198. The spectrum was acquired in positive mode with a MALDI-TOF/TOF 4800 mass spectrometer. The symbol  $\Delta$  represents elimination of 180 Da from serine 198 due to loss of the OP and a molecule of water. The y-axis has been magnified to enhance the low-intensity peptide fragments, thus truncating the high-intensity mass at 2911 Da and the parent ion mass at 3091.5 Da.

SVTLFGES<sub>198</sub>AGAASVSLHLLSPGSHSLFTR, where serine 198 is the modified amino acid. Masses of the y-ions and internal fragment (HSL at 338.1) support the sequence. The modified residue is identified as serine 198 from the mass of the y22 ion, which is 162 Da heavier than the y22 ion from control BChE (Gilley *et al.*, 2009). Additional evidence that serine 198 is the modified residue comes from the mass interval between y21 and y22 (249 Da), which is equal to the dehydro mass of serine (87 Da) plus the mass of the adduct (162 Da). The y24 and y27-H<sub>2</sub>O ions also carry an additional mass of 162 Da, consistent with modification of serine 198. The most intense peak (Fig. 3) has a mass of 2911 Da due to neutral loss of 180 Da from the parent ion. The iso-OMPA-serine bond underwent  $\beta$ -elimination during collision-induced dissociation and resulted in release of the organophosphorus molecule (162 Da) plus a molecule of water (18 Da). Elimination of the organophosphorus (OP) molecule plus a molecule of water from OP serine is a characteristic feature of all OP-serine adducts (Fidder *et al.*, 2002; Gilley *et al.*, 2009) examined to date.

#### Recognition of an Artifact: Acid Hydrolysis of the P-N Bond

Digestion of human BChE with pepsin at pH 2 yielded the nine-residue active site peptide FGES<sub>198</sub>AGAAS at 794.2 m/z (Fig. 4A). Digestion of iso-OMPA-inhibited BChE with pepsin at pH 2 yielded a new peptide at 874.2 m/z (Figs. 4B and C) and resulted in disappearance of the unlabeled active site peptide at 794.2 m/z. Thus, the mass of the active site peptide increased by 80 Da rather than the expected 162 Da. An added mass of 80 Da corresponds to addition of phosphate (HPO<sub>3</sub>). This result was taken as evidence that both P-N bonds were hydrolyzed from the iso-OMPA adduct under the acid conditions of pepsin digestion (pH 2) and incubation with 1% trifluoroacetic acid in the DHB matrix. The CHCA matrix used for MALDI-TOF analysis of tryptic peptides (Fig. 2B) contained only

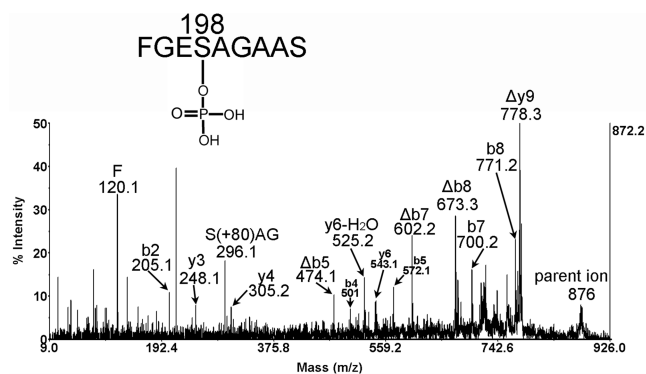


**FIG. 4.** MALDI-TOF mass spectra of control unmodified BChE and iso-OMPA-inhibited BChE digested with pepsin. Spectra were acquired in negative mode with DHB matrix at a laser voltage of 6000 V. (A) Highly purified human BChE digested with pepsin. The active site peptide, FGES<sub>198</sub>AGAAS, has a mass of 794.2 m/z in negative mode. (B) BChE treated with 0.25 mM iso-OMPA, which inhibited 100% of the BChE activity, digested with pepsin. The active site peptide, FGEpSAGAAS, has a mass of 874.2 m/z in negative mode. The added mass of 80 Da is from phosphate. The acidic conditions of pepsin digestion cleaved both P-N bonds. (C) MS spectrum of the digest in (B) after enrichment of phosphopeptides on titanium oxide. The peaks at 1002.2 and 1089.2 Da are phosphopeptides of porcine pepsin.

0.1% trifluoroacetic acid. There was no evidence of P-N bond hydrolysis in CHCA matrix under those conditions.

The peak at 874.2 Da was stronger after enrichment of phosphopeptides on titanium oxide (Fig. 4C). Porcine pepsin (accession no. P00791) is phosphorylated on Ser68 by endogenous kinases (Sielecki *et al.*, 1990). The mass at 1002.2 m/z is the phosphorylated peptide FEATpSQEL from porcine pepsin. A missed cleavage yielded a second phosphorylated porcine pepsin peptide with the sequence FEATpSQELS and a mass of 1089.2 m/z.

Confirmation of the identity of the peptide at 874.2 m/z (negative mode) was obtained with a MALDI-TOF/TOF mass spectrometer. The peptide was prepared for MS/MS analysis by offline HPLC purification. MS/MS spectra were acquired in positive mode because signal intensity was good in positive mode but weak in negative mode. The parent ion mass is 876.2 Da in positive mode. The MS/MS spectrum of the 876.2 Da parent ion is shown in Figure 5. The masses of the b- and y-ions support the sequence FGEpS<sub>198</sub>AGAAS where the phosphate is on serine 198. The most intense peak (Fig. 5) has a mass of 778.3 Da, which represents neutral loss of 98 Da from the parent ion. The 98 Da loss corresponds to loss of phosphate (minus 80 Da) plus loss of a molecule of water (minus 18 Da) and resulted in transformation of serine 198 to dehydroalanine. Loss of 98 Da during fragmentation in the mass spectrometer is characteristic of peptides containing phosphoserine. The b4, b5, b7, and b8 ions and the internal fragment at 296.1 m/z carry phosphate on serine 198, whereas  $\Delta$ b5,  $\Delta$ b7,  $\Delta$ b8, and  $\Delta$ y9 ions represent fragments that lost 98 Da from serine 198.

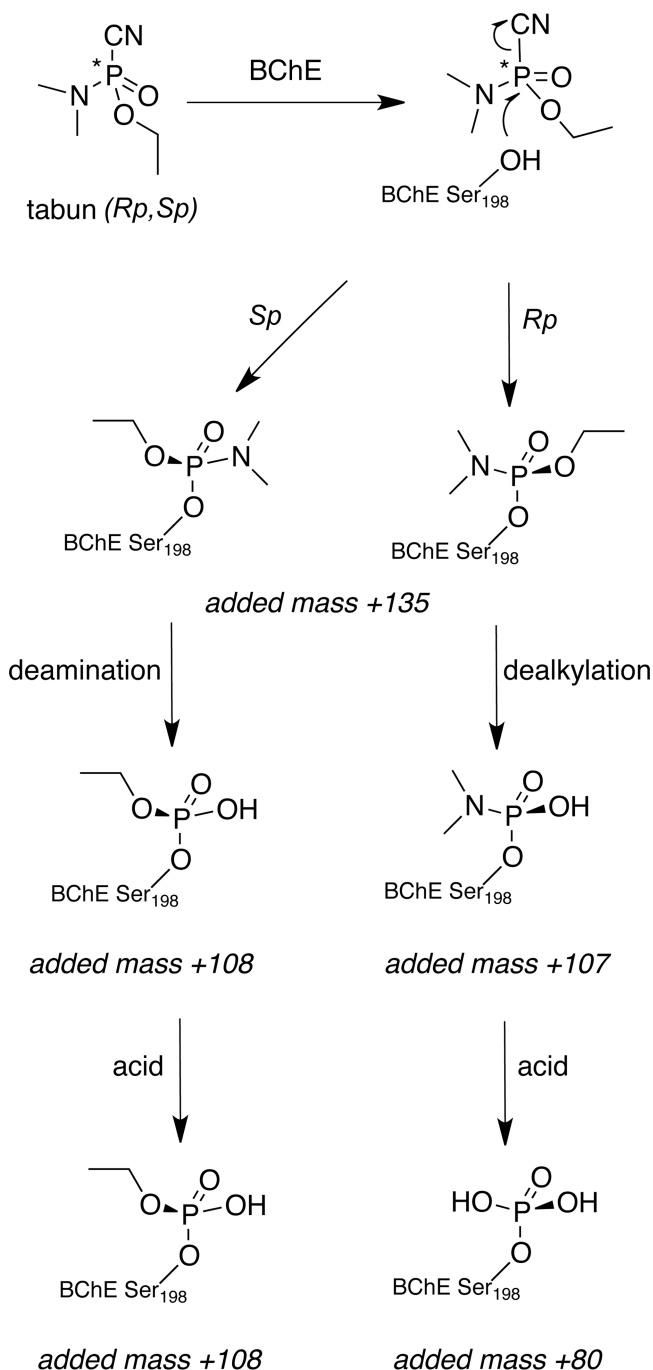


**FIG. 5.** Further evidence of artifactual deamination of P-N bonds during digestion with pepsin at pH 2. Parent ion 876.3 m/z is the active site peptide from iso-OMPA-inhibited human BChE digested with pepsin. The MS/MS spectrum of parent ion 876.3 Da, in positive mode, shows major y- and b-ions of peptide FGEpSAGAAS. The fragment ion masses support phosphorylation of serine 198, the active site serine. The symbol  $\Delta$  represents fragments which underwent loss of phosphate and a molecule of water, thus converting phosphoserine into dehydroalanine. The y-axis has been magnified to enhance the low-intensity peptide fragments, thus truncating the high-intensity mass at 778.3 Da.

#### A Method to Distinguish Aging of Rp-Tabun From Sp-Tabun Adducts on BChE

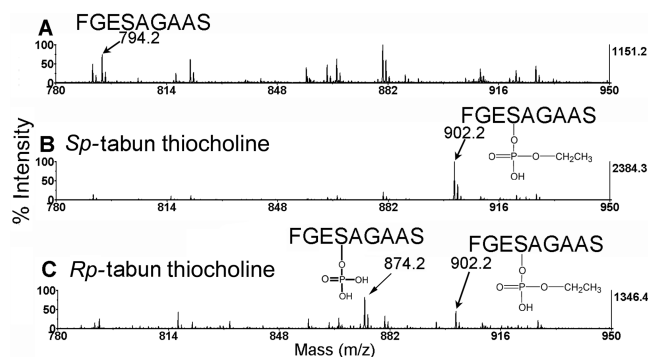
Tabun has a center of chirality about phosphorus and can exist as two stereoisomers (*Sp* and *Rp* forms; Fig. 1). The two enantiomers react with BChE to form adducts with different stereochemistry, assuming that both isomers undergo *in-line* nucleophilic substitution, i.e., the cyano leaving group and the catalytic serine are on opposite sides of the phosphorus atom (Fig. 6). The initial BChE adducts are indistinguishable by mass spectrometry because both stereoisomers lose cyanide to yield an adduct with an additional mass of 135 Da (Gilley *et al.*, 2009). However, the two stereoisomers of tabun age differently (Carletti *et al.*, 2008). It was proposed that the *Sp*-tabun adduct is oriented in the BChE active site to promote aging through deamination. By contrast, the *Rp*-tabun adduct is oriented to promote aging through O-dealkylation. Figure 6 shows that the added mass from aged *Sp*-tabun is 108 Da (corresponding to loss of the N-dimethyl group), whereas that from *Rp*-tabun is 107 Da (corresponding to loss of the O-ethyl group). This small difference in mass can be difficult to distinguish by mass spectrometry (Gilley *et al.*, 2009). By taking advantage of the acid labile nature of the P-N bond, we have devised a mass spectrometry method to conclusively prove the aging pathway for each stereoisomer. Figure 6 shows that the adduct that has aged by deamination is unaffected by acid and has an additional mass of 108 Da even after pepsin digestion at pH 2. By contrast, the adduct that aged by dealkylation has lost dimethylamine at pH 2 to yield a peptide with an added mass of 80 Da.

The proposed sequence of events (Fig. 6) was tested on human BChE that was inhibited by *Sp*- and *Rp*-tabun thiocholine model compounds (Fig. 1). It must be noted that because the priority of the substituent is different in tabun and tabun thiocholine, *Sp*-tabun thiocholine forms the same adduct as *Rp*-tabun assuming *in-line* nucleophilic substitution. Conversely,



**FIG. 6.** Aging pathways of tabun adducts can be identified by taking advantage of the acid labile P-N bond. Both stereoisomers of tabun (*Rp*, *Sp*) make an initial adduct with human BChE that adds a mass of 135 Da to the active site serine. The adduct formed by *Sp*-tabun ages through deamination to form ethoxyphosphate, which is stable in acid, yielding a peptide with an added mass of 108 Da when BChE is digested with pepsin. In contrast, the adduct formed by *Rp*-tabun ages through O-dealkylation to form dimethylaminephosphate, which is unstable in acid, yielding a peptide with an added mass of 80 Da when the BChE adduct is digested with pepsin.

*Rp*-tabun thiocholine forms the same adduct as *Sp*-tabun. Each conjugate was allowed to age for 4 days before it was digested with pepsin at pH 2. The digests were analyzed with

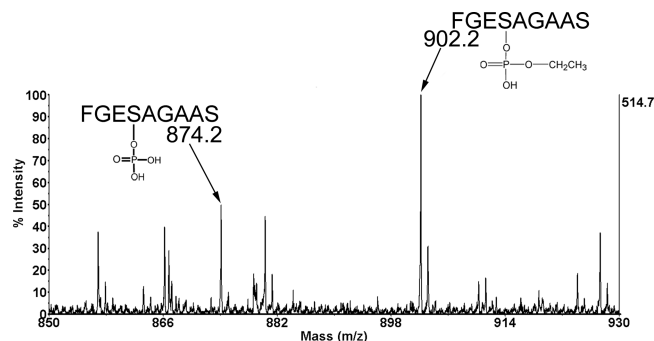


**FIG. 7.** The *Sp*-tabun thiocholine adduct on BChE ages by deamination, whereas the *Rp*-tabun thiocholine adduct ages by O-dealkylation. The MALDI-TOF mass spectra show masses of the human BChE active site peptide produced by digestion with pepsin at pH 2. (A) Highly purified control human BChE digested with pepsin. The active site peptide FGES<sub>198</sub>AGAAS has a mass of 794.2 m/z in negative mode. (B) BChE treated with *Sp*-tabun thiocholine and digested with pepsin yields peptide FGES<sub>198</sub>AGAAS with a mass of 902.2 m/z in negative mode. (C) BChE treated with *Rp*-tabun thiocholine and digested with pepsin yields a mass of 874.2 m/z in negative mode and a less intense peak at 902.2 m/z that is assumed to arise from contamination by *Sp*-tabun thiocholine.

MALDI-TOF mass spectrometry. Figure 7A shows peptide FGES<sub>198</sub>AGAAS at 794.2 m/z from control BChE digested with pepsin. Figure 7B shows that BChE inhibited with *Sp*-tabun thiocholine and digested with pepsin has a new peak at 902.2 m/z and has no peak at 794.2 m/z. The difference in mass between 902.2 and 794.2 Da is 108 Da, consistent with aging through deamination for the *Sp*-tabun thiocholine adduct. There is no peak at 874.2 m/z (Fig. 7B), which confirms that ethoxyphosphate is stable in acid. Figure 7C shows that BChE inhibited with *Rp*-tabun thiocholine and digested with pepsin has new peaks at 874.2 and 902.2 m/z. The difference in mass between 874.2 and 794.2 is 80 Da, which is consistent with aging through O-dealkylation for the *Rp*-tabun thiocholine adduct followed by hydrolysis of the dimethylamine adduct at pH 2. The peak at 902.2 m/z (Fig. 7C) reflects contamination of *Rp*-tabun thiocholine with a small amount of *Sp*-tabun thiocholine.

#### Aging of Authentic Tabun-BChE Adduct

The authentic nerve agent tabun is a racemic mixture of both *Sp* and *Rp* enantiomers. Human BChE treated with authentic tabun, and allowed to age before digestion with pepsin, yielded the two adducts as shown in Figure 8. The peak at 902.2 m/z in negative mode represents aging through deamination, whereas the peak at 874.2 m/z represents aging by dealkylation. This result shows that both stereoisomers of tabun inhibit human BChE (Tenberken *et al.*, 2010b) and that adducts with racemic tabun age by deamination and by dealkylation. The relative concentrations of adducts (Fig. 8) cannot be estimated because peak height depends not only on concentration but also on the ease with which a peptide ionizes.



**FIG. 8.** Mechanism of aging of human BChE inhibited by authentic racemic tabun. Digestion of tabun-inhibited BChE with pepsin at pH 2 yielded peptides that represent aging by two mechanisms: dealkylation (874.2 m/z) of the *Rp*-tabun adduct and deamination (902.2 m/z) of the *Sp*-tabun adduct.

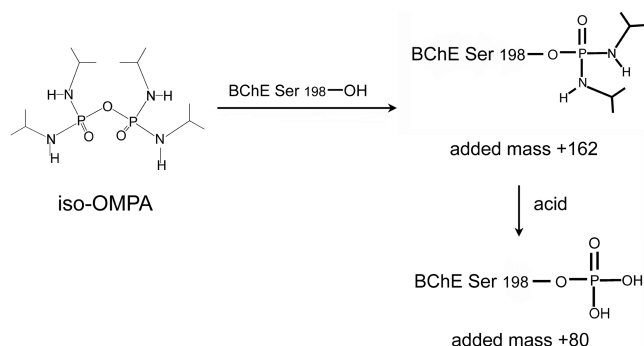
## DISCUSSION

### Summary of Mass Spectrometry Results for *iso*-OMPA-Inhibited Human BChE

Our studies lead to the conclusion that at neutral pH, *iso*-OMPA covalently modifies human BChE on the active site serine 198. The modification adds a mass of 162 Da to serine 198 to make the structure shown in Figure 9. An artifact is introduced when the BChE protein is acidified to pH 2 during pepsin digestion. At this pH, acid hydrolyzes the P-N bonds to yield phosphorylated serine having an added mass of 80 Da.

### Acid Labile P-N Bond in Tabun Thiocholine Identifies the Aging Pathway

The information gained from our study of *iso*-OMPA-inhibited BChE was used as a prelude to analyze the aging pathways of the stereoisomers of nerve agent model compounds of tabun. The nerve agent model compounds afford adducts identical to that of authentic tabun in that they add a mass of 135 Da to the active site serine (Gilley *et al.*, 2009). We devised a scheme in which aging through deamination was clearly distinguishable from aging through O-dealkylation (see Fig. 6). Our mass spectrometry results conclusively prove that *Sp*-tabun thiocholine-inhibited BChE ages by deamination, whereas *Rp*-tabun thiocholine-inhibited BChE ages by O-dealkylation. Because *Sp*-tabun thiocholine forms the same adduct as authentic *Rp*-tabun assuming *in-line* nucleophilic substitution in both cases and because the *Rp*-tabun adduct on BChE and AChE ages by O-dealkylation (Carletti *et al.*, 2008), it follows that the *in-line* nucleophilic substitution assumption for the model compounds must be wrong. The tabun thiocholine model compounds do not appear to react via *in-line* substitution. The alternative mechanism places the leaving group in the choline-binding pocket of BChE rather than pointing toward the mouth of the gorge (Nachon *et al.*, 2010). This situation seems favorable for tabun thiocholine, whose choline head can interact with Trp82 of the



**FIG. 9.** Reaction of iso-OMPA with human BChE to yield an adduct on the active site serine 198. The diisopropylphosphoramidate adduct with an added mass of 162 Da is identified by digesting BChE with trypsin at pH 8. In contrast, an artifact with an added mass of 80 Da is produced by digesting iso-OMPA-inhibited BChE with pepsin at pH 2.

choline-binding pocket. Conclusive proof of this scenario could be achieved by solving the x-ray structure of BChE inhibited by enantiomers of tabun thiocholine.

#### Enantioselectivity of the Stereoisomers of Tabun for BChE and AChE

Kinetic and crystal structure analysis of human BChE and human AChE inhibition by racemic tabun has shown that both enzymes react preferably with *Rp*-tabun although the exact ratio of inhibition constants of the *Sp* and *Rp* enantiomers has not been reported (Carletti *et al.*, 2008, 2010). The present mass spectrometry study qualitatively shows that both enantiomers of tabun inhibit human BChE, and that after aging, the tabun-BChE adduct contains two chemically distinct modifications. The results show that the enantioselectivity of BChE for *Rp*-tabun is sufficiently weak to allow the detection of the *Sp*-tabun adduct. Determination of the exact enantioselectivity requires pure enantiomers of tabun, which were not available to us but have been prepared in limited quantity by Tenberken *et al.* (2010a). However, as discussed below, to address this question, *Sp* and *Rp* enantiomers of a tabun nerve agent model compound were studied.

The crystal structures of human AChE and BChE inhibited by racemic tabun showed that aging proceeds through O-dealkylation. Before aging, the dimethylamino group of tabun was located in the acyl-binding pocket, whereas the ethoxy group was located in the choline-binding pocket, near the catalytic histidine known to catalyze the dealkylation. In aged crystals, the ethoxy group was absent. It was further shown that tabun analogs, differing by the nature of the alkyl group on the nitrogen atom, form adducts with BChE with the amino group in the acyl-binding pocket (Carletti *et al.*, 2009). Similar to authentic tabun, their ethoxy group is also in the choline-binding pocket and also ages by dealkylation. One notable exception was the N-methyl analog (instead of N-dimethyl for tabun) whose methylamino group is located in the choline-binding pocket and ages, presumably by deamination, whereas

the ethoxy group remains intact in the acyl-binding pocket (Nachon *et al.*, 2010). The rule seems to be that the aging substituent is located in the choline-binding pocket near the catalytic histidine. The aging substituent can be either an amino or an ethoxy group; the mechanism of aging is defined by orientation in the active site rather than by identity as an amino or an ethoxy group.

The orientation of the substituents on the chiral center of *Sp*-tabun thiocholine is equivalent to that of authentic *Rp*-tabun. It was therefore expected that *Sp*-tabun thiocholine would age by dealkylation. The result was opposite to expectation. We found that *Sp*-tabun thiocholine aged by deamination. An explanation for this observation takes into account rules developed from crystal structure analysis (Carletti *et al.*, 2008, 2009, 2010; Nachon *et al.*, 2010): (1) Positively charged groups face the choline-binding pocket, (2) the aging substituent is located in the choline-binding pocket, and (3) the leaving group must be located in an apical position of the trigonal-bipyramidal transition state. These rules pose a dilemma because the leaving group and the aging substituent cannot occupy the same site at the same time. Furthermore, when the leaving group faces the choline-binding pocket, the leaving group occupies an equatorial position in the trigonal-bipyramidal transition state.

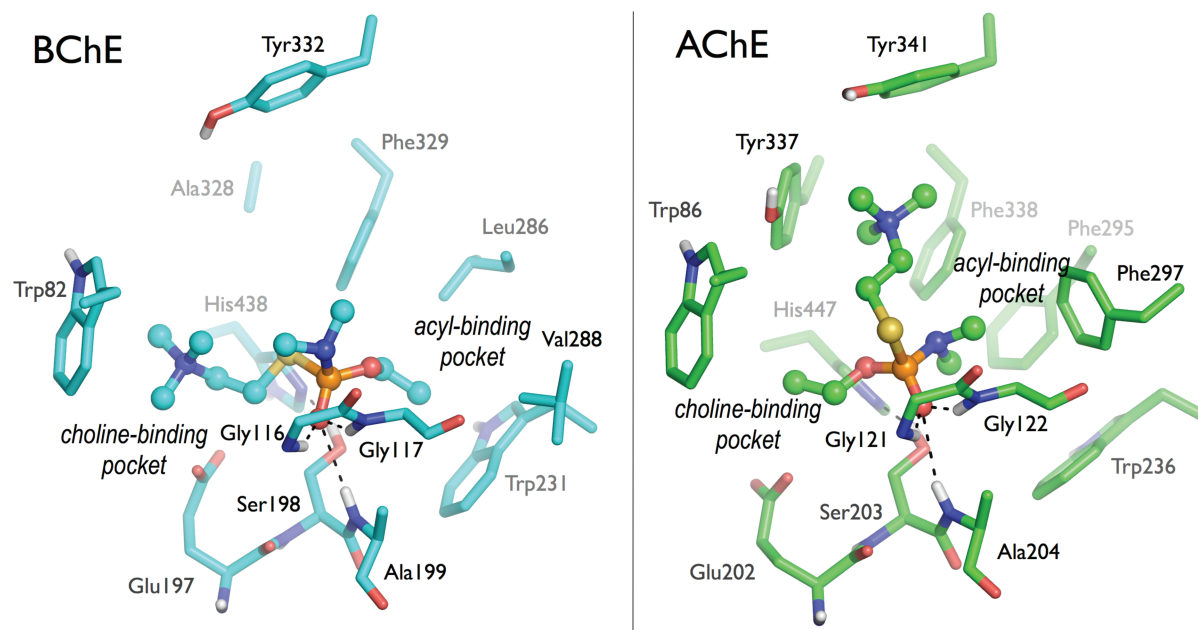
A similar dilemma has been solved previously for an N-methyl tabun analog (Nachon *et al.*, 2010). It was proposed that initially the leaving group faces the choline-binding pocket so that the leaving group occupies an equatorial position in the trigonal-bipyramidal transition state. The transition intermediate rearranges, placing the leaving group in an apical position. After the bond between the phosphorus atom and the leaving group breaks, the aging substituent occupies the choline-binding site. This mechanism of Berry pseudorotation is proposed to explain aging by deamination of the *Sp*-tabun thiocholine model compound.

Figure 10 illustrates binding of *Sp*-tabun thiocholine to the active site of BChE, with the thiocholine leaving group facing the choline-binding pocket. After bond cleavage, the ethoxy group will be in the acyl-binding pocket and the dimethylamino group in the choline-binding pocket, consistent with aging by deamination. Conclusive proof of this scenario could be obtained by solving the x-ray structure of BChE inhibited by enantiomers of tabun thiocholine.

The second panel (Fig. 10) suggests that binding of *Sp*-tabun thiocholine to AChE is restricted by the small size of the acyl-binding pocket. The acyl-binding pocket can accommodate the dimethylamino group but not the ethoxy group. The model predicts that *Sp*-tabun thiocholine adducts of AChE will age by O-dealkylation.

It is important to know how tabun analogs fit into the AChE active site because model compounds are used to make conjugates of human AChE aimed at testing new reactivators of tabun-inhibited AChE (Kalisiaik *et al.*, 2011, 2012). A specific enantiomer of a tabun model compound might under-represent the adducts formed by authentic racemic tabun. The





**FIG. 10.** Molecular modeling of *Sp*-tabun thiocholine in the active site of human BChE (left) and AChE (right). Residues are represented by sticks, tabun thiocholine by sticks and balls, and hydrogen bonds by dashes. In human BChE, the acyl-binding pocket is sufficiently large to accommodate the ethoxy group, so that the thiocholine leaving group lies in the choline-binding pocket. The P=O oxygen is H-bonded to oxyanion hole residues Gly116/Gly117/Ala199. The dimethylamino group is on the opposite side of the entering catalytic serine (Ser198). In human AChE, the size of the acyl-binding pocket is limited by bulky aromatic residues (Phe295 and Phe297). The dimethylamino is the only substituent able to fit in this pocket. It follows that the ethoxy group lies in the choline-binding pocket. The P=O oxygen is H-bonded to oxyanion hole residues Gly121/Gly122/Ala204. The thiocholine leaving group is on the opposite side of the entering serine, ideally for *in-line* substitution, and the choline head makes cation- $\pi$  interactions with Tyr337, Tyr341, and Phe338.

consequence could be that the rate of reactivation for the conjugate formed by *Sp*-tabun thiocholine would not represent the rate of reactivation for the conjugate formed by authentic racemic tabun. Therefore, results of reactivation studies with oximes of AChE inhibited by nerve agent model compounds must be interpreted carefully.

### Significance

In the event that a population is exposed to nerve agents, investigators will need to identify the nerve agent. Identification by mass spectrometry is currently the most sensitive method. The fact that phosphoramidates are acid labile can be used to distinguish between tabun analogs and nerve agents that have no P-N bond.

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