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Stereoselective Inactivation of *Torpedo californica* Acetylcholinesterase by Isomalathion: Inhibitory Reactions with (1*R*)- and (1*S*)-Isomers Proceed by Different Mechanisms

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The present study was undertaken to test the hypothesis that acetylcholinesterase (AChE) inhibition by isomalathion stereoisomers proceeds with different primary leaving groups for (1*R*)- and (1*S*)-isomers. Consistent with results obtained with enzyme from other species, AChE from *Torpedo californica* (TcAChE) was stereoselectively inhibited by isomalathion isomers with the (1*R*,3*R*)-isomer exhibiting greater potency than (1*S*,3*S*)-isomalathion. TcAChE modified by (1*R*)-isomers readily reactivated in the presence of 2-pralidoxime methiodide (2-PAM), whereas enzyme inhibited by (1*S*)-isomalathions was intractable toward reactivation. Computer-based molecular modeling showed that the ligand positioned as the primary leaving group was diethyl thiosuccinyl for (1*R*)-isomers and thiomethyl for (1*S*)-isomalathions. Mass spectral analysis revealed that inhibition of TcAChE by (1*R*)-isomers resulted in an *O,S*-dimethyl phosphate adduct, as expected from expulsion of the diethyl thiosuccinyl ligand. In contrast, inactivation of the enzyme by (1*S*)-isomalathions yielded an *O*-methyl phosphate adduct, consistent with initial loss of thiomethyl followed by displacement of the diethyl thiosuccinyl group. The findings demonstrate that the inhibitory reactions of TcAChE with (1*R*)- and (1*S*)-isomalathions proceed by different mechanisms involving distinct primary leaving groups.

Introduction

Isomalathion, *S*-[1,2-bis(ethoxycarbonyl)ethyl]-*O,S*-dimethyl phosphorodithiolate, is an OP[1] compound formed from thermal or photochemical isomerization of malathion (*1*, *2*). This isomerization results in a 1000-fold increase in potency for inhibition of AChE (E.C. 3.1.1.7) (*3*, *4*). Isomalathion has four stereoisomers, owing to two asymmetric centers, one at phosphorus and the other at a carbon atom (Figure 1). Chirality at phosphorus but not carbon was rendered from isomerization.

AChE from mammalian and avian species was shown to be stereoselectively inhibited by the isomers of isomalathion with the following order of potency: (1*R*,3*R*) > (1*R*,3*S*) > (1*S*,3*R*) > (1*S*,3*S*) (*5*−*7*). For rat brain AChE, the (1*R*,3*R*)-stereoisomer was found to be 29-fold more potent than (1*S*,3*S*)-isomalathion (*5*).

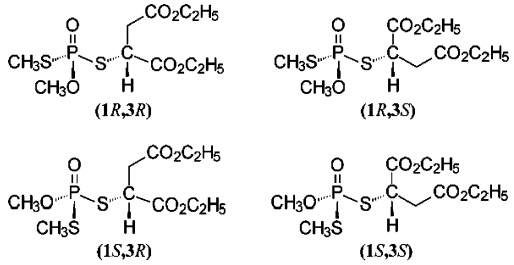

Figure 1. Four stereoisomers of isomalathion with asymmetric phosphorus and carbon atoms designated as positions 1 and 3, respectively.

Rat brain AChE inactivated by (1*R*)-isomalathion readily reactivated, but enzyme modified by the (1*S*)-isomers was refractory toward reactivation even in the presence of an oxime nucleophile (*5*, *6*). These same results were obtained for bovine erythrocyte and hen brain AChE (*8*). To account for the difference in post-inhibitory kinetics observed between enzyme inhibited by (1*R*)- and (1*S*)-isomers, it was proposed that (1*R*)- and (1*S*)-isomalathions inhibit AChE by different mechanisms (*5*, *6*). It was postulated that inactivation of AChE by (1*R*)-isomers proceeds with loss of diethyl thiosuccinate (*9*) but that inhibition of enzyme by (1*S*)-isomalathions occurs with expulsion of thiomethyl followed

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[1] Abbreviations: AChE, acetylcholinesterase; ATCh, acetylthiocholine; BSA, bovine serum albumin; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); EeAChE, electric eel AChE; MH^+, protonated molecule; OP, organophosphorus; 2-PAM, pyridine-2-aldoxime methiodide; PI-PLC, phosphatidylinositol specific phospholipase C; SELDI-TOF-MS, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry; TcAChE, *Torpedo californica* acetylcholinesterase.

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by a rapid postinhibitory reaction to yield an aged[2] AChE protein.

To test the hypothesis of a change in primary leaving group preference, the configurationally equivalent enantiomers of isoparathion methyl, *O*-[4-nitrophenoxy]-*O*,*S*-dimethyl phosphorothiolate, were used as standards of comparison in previous studies, because 4-nitrophenol is expelled as the primary leaving group to yield an *O*,*S*-dimethyl phosphate adduct. It was found that enzyme modified by (1*R*)-isomalathions had rate constants of reactivation (k_3) comparable to that for AChE inhibited by the configurationally equivalent (*S*)-isoparathion methyl (*5*, *6*, *8*). This evidence supports the hypothesis that diethyl thiosuccinate is the primary leaving group for the (1*R*)-isomers and that the inhibitory adduct is an *O*,*S*-dimethyl phosphate (*9*).

In contrast to the result obtained with the (1*R*)-isomers, AChE inactivated by (1*S*)-isomalathions did not reactivate at rates comparable to enzyme modified by the configurationally equivalent (*R*)-isoparathion methyl (*5*, *6*, *8*). It was also discovered that AChE modified by the (1*S*,3*S*)-isomer aged at a rate too rapid for measurement with the techniques employed ($t_{1/2}$ < 1 min) (*8*). These data suggest that diethyl thiosuccinate is not the primary leaving group for (1*S*)-isomalathions and that an *O*,*S*-dimethyl phosphate adduct is not generated upon reaction of AChE with (1*S*)-isomers. Instead, the results indicate that the inhibitory adduct is *O*-methyl-*S*-diethylsuccinyl phosphorothiolate, which rapidly undergoes a postinhibitory reaction to yield an aged enzyme.

It was recently demonstrated using kinetics and MS that inactivation of electric eel AChE by (1*S*,3*S*)-isomalathion proceeds with loss of thiomethyl as the primary leaving group and that aging of the inhibitory adduct yields an *O*-methyl phosphate moiety covalently bound to the enzyme (*10*). These findings further support the hypothesis that a difference in inhibitory mechanism occurs between AChE inactivated by (1*R*)- vs (1*S*)-isomers. Even with these results, however, it was still not possible to conclude unequivocally that the mechanism of inactivation of AChE was different between the (1*R*)- and the (1*S*)-isomers, because the adducts resulting from inhibition with (1*R*)-isomalathions reactivated too quickly ($t_{1/2}$ < 5 min) to permit their characterization.

The present study was undertaken to test the hypothesis that inhibition of AChE by (1*R*)- vs (1*S*)-isomalathions proceeds with distinct primary leaving groups: diethyl thiosuccinate and thiomethyl for the (1*R*)- and (1*S*)-isomers, respectively. *Torpedo californica* AChE (TcAChE) was chosen for this study because the crystal structure for this species of enzyme has been determined (*11*), enabling rigorous molecular modeling experiments to be carried out. Enzyme was treated with each isomer of isomalathion, and bimolecular rate constants of inhibition (k_i) were determined to ascertain whether this species of AChE was stereoselectively inhibited as previously seen with enzyme from other species. TcAChE inactivated by each isomalathion isomer was treated with an excess of oxime reactivator, and rate constants of reactivation (k_3) were measured to verify that *Torpedo* enzyme inhibited by (1*S*)- but not (1*R*)-isomers exhibited intractability toward reactivation as observed for other species of AChE. The orientation of each isomalathion stereoisomer in the active site of TcAChE was deduced using molecular modeling, including both mechanics and dynamics simulations, enabling the identity of the primary leaving group to be predicted. Furthermore, enzyme modified by each stereoisomer of isomalathion and digested with trypsin was analyzed using MS to identify the inhibitory OP adduct, permitting the unequivocal elucidation of the mechanism of inactivation by each isomer.

[2] Aging refers to a time-dependent process whereby a serine hydrolase previously inhibited by an OP compound becomes intractable toward reactivation upon treatment with nucleophilic reactivators, e.g., oximes or fluoride ion. This definition of aging is an operational one that does not specify the reaction mechanism.

Materials and Methods

Chemicals. The four resolved stereoisomers of isomalathion [(1*S*,3*S*), (1*R*,3*S*), (1*S*,3*R*), and (1*R*,3*R*)] were prepared and characterized as described previously (*12*). Chemical purity of these inhibitors was >99% as assessed by ^{1}H and ^{31}P NMR, GC, HPLC, and combustion analysis. Stereoisomer configurations were assigned based on single-crystal X-ray analysis of a strychnine salt precursor. Optical rotations and stereoisomeric purities determined by polarimetry and chiral HPLC, respectively, were as follows: (1*S*,3*S*) $[\alpha]^{25}_D = -44.8°$ (c = 0.580, $CHCl_3$), 95%; (1*R*,3*S*) $[\alpha]^{25}_D = -57.6°$ (c = 0.500, $CHCl_3$), 98%; (1*S*,3*R*) $[\alpha]^{25}_D = +58.6°$ (c = 0.635, $CHCl_3$), 93%; (1*R*,3*R*) $[\alpha]^{25}_D = +42.3°$ (c = 0.620, $CHCl_3$), 94%. ATCh iodide, DTNB, BSA, 2-PAM, and PI-PLC were purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals were reagent grade. Aqueous solutions were prepared in doubly deionized water.

Preparation of TcAChE. Tissue from the electroplax organ of *T. californica* was obtained from Aquatic Research Consultants (San Pedro, CA). After it was rinsed with phosphate buffer (0.1 M sodium phosphate buffer, pH 7.6), the tissue was homogenized in phosphate buffer at room temperature using a glass homogenizer until a fine suspension was generated (~20 strokes). The fine suspension was diluted with phosphate buffer to a final concentration of 0.3% (w/w) before use in kinetics assays. TcAChE was purified and solubilized for SELDI-TOF-MS analysis according to the following procedure of Futerman et al. (*13*). *Torpedo* electroplax tissue (~100 g) was homogenized in a Waring blender. Water (40 mL) was added, and the homogenate was centrifuged at 5000*g* for 30 min at 10 °C. The supernatant was removed (~100 mL), and the pellet was resuspended in water. This was repeated for a total of three washes at room temperature. After the last wash, the pellet was suspended in 10 mM Tris buffer (pH 7.5 at 25 °C) to a final volume of 36 mL. PI-PLC was added to the suspension with a final concentration of 1 U/mL, and the mixture was incubated at 30 °C for 19 h with frequent vortexing. The suspension was centrifuged at 5000*g* for 30 min at 10 °C, and the supernatant was removed and centrifuged at 100 000*g* for 1 h at 4 °C. After it was diluted 1:10 with water, the supernatant solution was aliquoted into a Biomax-50 centrifugal concentrating device containing a 50 kDa filter (Millipore, Bedford, MA) and centrifuged at 5000*g* for 10 min. The resulting supernatant solution was concentrated by a factor of 100. AChE activity was determined to be ~950 U/mL using ATCh as substrate at 25 °C.

TcAChE Inhibition. Kinetics experiments involving TcAChE were performed in phosphate buffer at 25 °C using a modification of the method of Ellman et al. (*14*). Inhibitor was dissolved in acetone and diluted in phosphate buffer. The final acetone concentration was ≤1% (w/w) and did not affect enzyme activity. Enzyme and inhibitor were preincubated for various measured periods of time, and at the end of each period, substrate solution was added containing ATCh and DTNB (final concentrations: ATCh, 1.0 mM; DTNB, 0.32 mM). Residual activity was determined by measuring the change in absorbance at 412 nm over a 1−2 min period using a SPECTRAmax 340 microplate reader at 25 °C (Molecular Devices Corporation, Sunnydale, CA).

Determination of k_i Values. The apparent bimolecular rate constant of inhibition (k_i) was determined as previously described (*15*, *16*). The reaction with inhibitor (I) proceeded with [I] > 10 [enzyme] and [I] ≪ K_D, so that the k_i could be measured using pseudo-first-order kinetics. Linear regression was used to calculate the slopes of primary kinetic plots of ln(% activity remaining) vs time (t) to obtain apparent first-order rate constants of inhibition (k') at each [I] and to determine the k_i from the slopes of secondary plots of $-k'$ vs [I].

TcAChE Reactivation. TcAChE was incubated with each of the four stereoisomers of isomalathion at concentrations required to yield 90% inhibition in 10 min. Reactions were performed at 25 °C in phosphate buffer. Concentrations of each isomer used for inhibition of TcAChE were as follows: (1*R*,3*R*), 2.6 μM; (1*R*,3*S*), 9.5 μM; (1*S*,3*R*), 9.8 μM; and (1*S*,3*S*), 50 μM. An aliquot of the enzyme solution was then diluted 1:100 (v/v) in phosphate buffer (25 °C) containing 200 μM 2-PAM. Aliquots were withdrawn at timed intervals and assayed for activity as described above for TcAChE inhibition.

Determination of k_3. The apparent first-order rate constant of reactivation (k_3) was determined according to the method of Clothier et al. (*9*). The following equation was used to calculate k_3, where A is the activity of the control, A_t is the activity of inhibited enzyme at time = t, and A_0 is the activity of inhibited enzyme at 0 min: ln(100/% inhibition) = $k_3 \cdot t$; % inhibition = $[(A - A_t)/(A - A_0)] \times 100$. Residual activities were determined as described above for AChE inhibition and reactivation. The slope of the least-squares best-fit line was determined using linear regression, and only the linear portion of the graph was used in the calculation (0−10 min).

Statistical Analysis. All values are reported as mean ± SE from at least three separate experiments. All statistics were calculated using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA). The significance of differences between pairs of means was determined by two-tailed unpaired t-tests ($p < 0.05$). An F-test was performed to determine whether slopes of regression lines of reactivation plots were significantly nonzero ($p < 0.05$).

Molecular Modeling. Molecular modeling experiments were performed on an SGI Octane computer (Mountain View, CA) using InsightII 2000 software with Affinity, Biopolymer, and Discover modules (Molecular Simulations, Inc., San Diego, CA). The crystal structure of TcAChE (2ACE) (*11*) was downloaded from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (http://www.rcsb.org/pdb/). Missing residues and hydrogen atoms were added (pH 7.6) using the Biopolymer module. Isomalathion isomer structures were initially positioned in the active site of TcAChE with the phosphorus atom adjacent to the catalytic Ser 200 and the phosphoryl oxygen within a hydrogen-bonding distance of the oxyanion hole. The inhibitor and a subset of TcAChE atoms located within a 10 Å radius of the inhibitor were designated as flexible, while the rest of the enzyme was held rigid. Tethering was applied from the inhibitor phosphoryl oxygen to the oxyanion hole so that they were kept within hydrogen-bonding distance (*17*). The ligand was confined to a 2 Å radius to prevent it from drifting away from the active site during the initial minimization that was performed in vacuo. Docking was accomplished using a minimization process involving two phases followed by a molecular dynamics simulation (*18*). The first round of minimization (100 steps) involved a Monte Carlo approach that took into consideration only van der Waals interactions. Coulombic terms were turned off, and the constraint factors were set to a minimal value. During the second round of minimization (100 steps), Coulombic terms were turned back on (distance-dependent dielectric constant ϵ = 78.0) and constraint factors were set back to full values. Simulated annealing was performed with a total of 50 dynamics stages with each lasting 100 fs. Starting and ending temperatures were set at 500 and 300 K, respectively. Structures generated from this procedure were then subjected to a final round of minimization (1000 steps) similar to the second phase initially used.

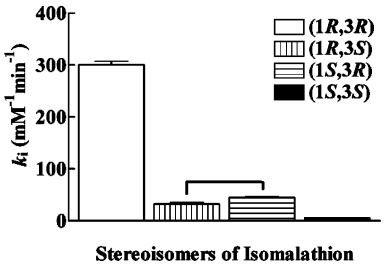

Figure 2. k_i values for inhibition of TcAChE by the four stereoisomers of isomalathion. All values are significantly different unless connected by a bar. Significant differences were identified by one way ANOVA ($\alpha = 0.05$) using the Newman−Keuls post hoc test for multiple comparisons ($n = 3$ or 4).

Preparation of TcAChE for SELDI-TOF-MS Analysis. PI-PLC-solubilized TcAChE in 50 mM ammonium bicarbonate buffer, pH 7.8, was incubated with ∼40 μM each stereoisomer of isomalathion for 20 min at 25 °C, resulting in >90% loss of control activity. To verify that PI-PLC-solubilized enzyme modified by each of the four isomalathion isomers exhibited postinhibitory kinetics similar to that measured for tissue-bound TcAChE, an aliquot was removed and diluted 1:3000 (v/v) with 0.1 M sodium phosphate buffer (pH 7.6) containing 200 μM 2-PAM and 1 mg/mL BSA at 25 °C. The activity in the aliquot was measured at 0 and 20 min as described above for TcAChE inhibition and reactivation. Acetonitrile was added to both control and isomalathion-treated samples to a final concentration of 10% (v/v), and trypsin was subsequently added to a final concentration of 40 μg/mL. Samples were incubated for 1 h at 25 °C. The sequence of TcAChE was obtained from Schumacher et al. (*19*) using the ESTHER database (http://www.ensam.inra.fr), and the MS-Digest feature of ProteinProspector version 3.2.1 (http://prospector.ucsf.edu) was used to predict the average and monoisotopic masses of resulting peptides. According to the previously mentioned reference and resources, the active site peptide containing the catalytic Ser 200 (shown in boldface) has the following sequence: TVTIFGE**S**AGGASVGMHILSPGSR (theoretical MH^+ at m/z 2332.7).

SELDI-TOF-MS Analysis. A 1:10 dilution of the peptide fraction in water (2 μL) was mixed on an H4 Protein Chip (Ciphergen Biosystems, Inc., Fremont, CA) with 1 μL of a 50% (v/v) acetonitrile/50% (v/v) 1% trifluoroacetic acid (w/v) solution saturated with α-cyano-4-hydroxycinnamic acid. Samples were allowed to air-dry and crystallize before loading the plate into the mass spectrometer. A PBS-II SELDI-TOF mass spectrometer (Ciphergen Biosystems, Inc.) equipped with a nitrogen laser (337 nm, 4 ns pulse width) was used to obtain mass spectra. Analysis was carried out with an acceleration voltage of 20 kV, and 50−100 shots were averaged for each spectrum. The instrument was externally calibrated with human angiotensin-I, fibrinopeptide B, and dynorphin A (Ciphergen Biosystems, Inc.).

Results

Inhibition of TcAChE by Isomalathion Isomers. At the concentrations of inhibitors used, pseudo-first-order kinetics was observed, allowing the determination of k_i for each stereoisomer. Values of k_i (mM^{-1} min^{-1}) are shown in Figure 2 for the reaction of isomalathion with TcAChE. The order of potency for each isomer was found to be as follows: (1*R*,3*R*) > (1*R*,3*S*) = (1*S*,3*R*) > (1*S*,3*S*).

Table 1 lists the ratios of k_i values for isomalathion isomers with AChE from various species, enabling a species comparison for AChE stereoselectivity. The ratio of rate constants for the most to least potent isomalathion stereoisomer was 53.8, one of the highest reported to date (Table 1). As shown in Table 1, the stereoselectivity of

Table 1. Ratios of k_i Values for the Inhibition of AChE from Various Species by the Four Stereoisomers of Isomalathion[a]

	ratios of k_i values[b]					
enzyme	(1*R*,3*R*)/(1*S*,3*S*)	(1*R*,3*R*)/(1*R*,3*S*)	(1*R*,3*R*)/(1*S*,3*R*)	(1*R*,3*S*)/(1*S*,3*S*)	(1*R*,3*S*)/(1*S*,3*R*)	(1*S*,3*R*)/(1*S*,3*S*)
TcAChE[c]	53.8 ± 2.9	9.32 ± 0.75	6.74 ± 0.42	5.78 ± 0.36	0.722 ± 0.03	7.99 ± 0.30
EeAChE[d]	60.6 ± 7.3	4.56 ± 0.77	3.64 ± 0.53	13.3 ± 1.78	0.801 ± 0.08	16.6 ± 1.66
HBAChE[e]	15.2 ± 1.82	2.10 ± 0.36	5.44 ± 0.78	7.23 ± 0.97	2.59 ± 0.26	2.79 ± 0.28
HEAChE[f]	12.4 ± 0.3	2.79 ± 0.07	6.74 ± 0.48	4.44 ± 0.48	2.42 ± 0.31	1.83 ± 0.23
BEAChE[g]	21.4 ± 0.2	2.97 ± 0.07	9.18 ± 0.69	7.21 ± 0.52	3.09 ± 0.31	2.33 ± 0.24
RBAChE[h]	28.9 ± 1.2	3.30 ± 0.38	4.28 ± 0.26	8.75 ± 1.18	1.30 ± 0.12	6.75 ± 0.63

[a] The asymmetric phosphorus and carbon atoms are designated as positions 1 and 3, respectively. [b] Values shown are mean ± SE (n = 3 or 4). SE calculated using standard rules of error propagation for quotients (*20*). [c] Present study. [d] Electric eel AChE (*24*). [e] Hen brain AChE (*7*). [f] Human erythrocyte AChE (*21*). [g] Bovine erythrocyte AChE (*21*). [h] Rat brain AChE (*5*, *6*).

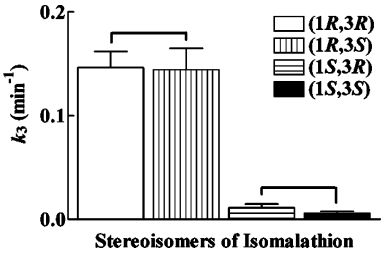

Figure 3. Kinetic constants for reactivation of TcAChE modified by isomalathion stereoisomers. Values of k_3 for oxime-mediated reactivation of TcAChE inhibited by the four isomers of isomalathion (n = 3 or 4). Values of k_3 for diastereomers in each set of isomalathions, i.e., (1*R*)- and (1*S*)-isomers, were found not to be significantly different as identified by two-tailed unpaired *t*-tests ($p > 0.05$) and are connected by a bar.

TcAChE is similar to that of EeAChE but strikingly different from that of mammalian and/or avian AChE for the enantiomeric ratios of (1*R*,3*R*)/(1*S*,3*S*) and (1*R*,3*S*)/(1*S*,3*R*).

Reactivation of TcAChE Modified by Isomalathion Isomers. Enzyme inactivated by (1*R*)-isomers readily reactivated in the presence of 2-PAM with a measurable k_3 of >0.140 min^{-1} as shown in Figure 3. Substantial activity (70−80%) could be restored to TcAChE modified by (1*R*)-isomalathions after a 20 min treatment with 2-PAM. Values of k_3 for enzyme inhibited by (1*R*,3*R*)- and (1*R*,3*S*)-diastereomers were not significantly different ($p > 0.05$). TcAChE modified by (1*S*,3*R*)- or (1*S*,3*S*)-isomalathion was refractory toward oxime-mediated reactivation with k_3 values of 0.0111 and 0.00608 min^{-1}, respectively (Figure 3). Values of k_3 for enzymes inhibited by (1*S*,3*R*)- and (1*S*,3*S*)-isomalathion were not significantly different ($p > 0.05$). These data are consistent with results from previous studies involving avian and mammalian AChE (*5*, *6*, *8*) and indicate that the difference in inhibitory mechanism between (1*R*)- and (1*S*)-isomalathions is conserved for TcAChE.

PI-PLC-solubilized TcAChE modified by (1*R*)-isomalathions and treated with 200 μM 2-PAM regained 70−80% of its former catalytic activity, but oxime treatment of enzyme inhibited by (1*S*)-isomers restored only 5−10% of its former catalytic activity. This result is identical to that observed for tissue-bound TcAChE and confirms that both preparations of the enzyme react with isomalathion stereoisomers in the same manner.

Docking of Isomalathion in the Active Site of TcAChE. Results of the molecular modeling experiments are shown in Figures 4 and 5. (1*R*,3*R*)- and (1*R*,3*S*)-Isomalathion were found to be oriented in the active site of TcAChE with the diethyl thiosuccinyl group pointing toward the gorge entrance and positioned apically to the catalytic Ser 200 hydroxyl group. In contrast, the calculated configurations of the (1*S*,3*R*)- and (1*S*,3*S*)-isomers

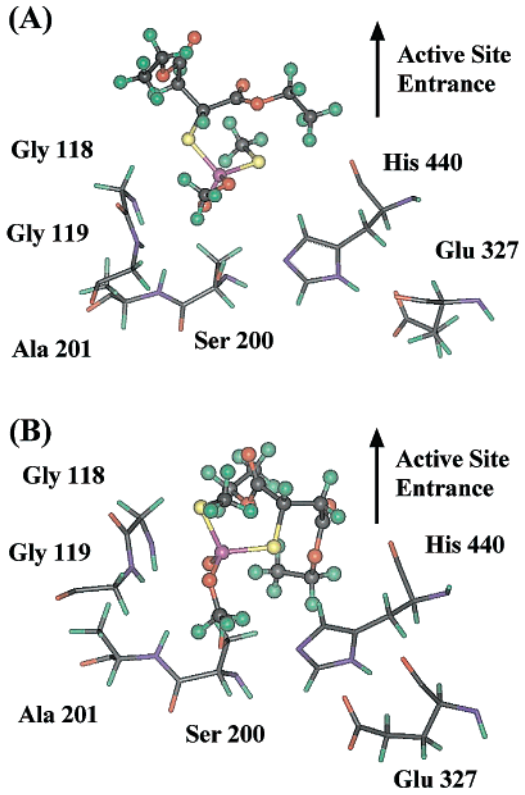

Figure 4. Results of computer-based docking experiments for isomalathion enantiomers and TcAChE. (A) (1*R*,3*R*)- and (B) (1*S*,3*S*)-isomalathion in the active site of TcAChE. The isomalathion enantiomers are in ball-and-stick format, whereas TcAChE residues are displayed as sticks. Colors were assigned to atoms as follows: C, gray; H, green; N, blue; O, red; P, purple; and S, yellow. Only the catalytic triad and active site residues in close proximity to isomalathion are shown. The catalytic Ser 200 is located directly beneath the isomalathion phosphorus atom. Diethyl thiosuccinyl is predicted to be positioned apically to the active site Ser 200 for (1*R*,3*R*)-isomalathion. In contrast, thiomethyl is predicted to be positioned apically to Ser 200 for the (1*S*,3*S*)-enantiomer. The active site entrance is located directly above the isomalathion molecule in each panel as indicated by the arrow.

of isomalathion predicted the thiomethyl group to be pointed toward the gorge entrance and oriented apically to the active site Ser 200. For (1*S*)-isomalathions, the diethyl thiosuccinyl ligand was predicted to be oriented toward the anionic site, Trp 84. For both the (1*R*)- and the (1*S*)-isomers of isomalathion, the methoxy ligand was adjacent to the acyl binding pocket (Phe 288 and Phe 290) (*22*), and the phosphoryl oxygen was in close proximity to the members of the oxyanion hole (Gly 118 and Gly 119).

One of the predicted structures for the (1*S*,3*R*)-isomer was oriented in the active site of TcAChE with the diethyl thiosuccinyl moiety positioned as the primary leaving

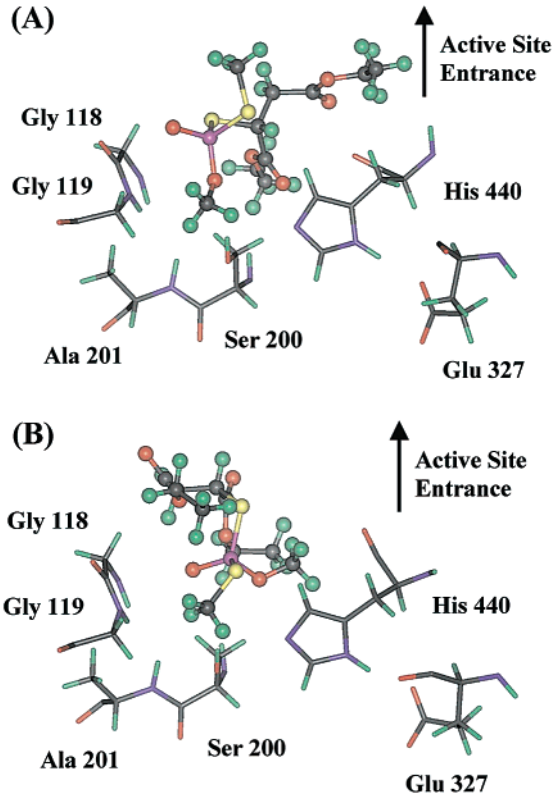

Figure 5. Predicted models of (1*S*,3*R*)-isomalathion in the active site of TcAChE. (A) Thiomethyl positioned as primary leaving group. (B) Diethyl thiosuccinyl positioned as the primary leaving moiety. The energy difference between these two models is 21 kcal/mol, with A being lower in energy. The isomalathion molecules are in ball-and-stick format, whereas TcAChE residues are displayed as sticks. Colors were assigned to atoms as follows: C, gray; H, green; N, blue; O, red; P, purple; and S, yellow. Only the catalytic triad and active site residues in close proximity to isomalathion are shown. The active site entrance is located directly above the isomalathion molecule in each panel as indicated by the arrow.

group (Figure 5B). This result was not observed for (1*S*,3*S*)-isomalathion (Figure 4B). In the model shown in Figure 5B, the thiomethyl is proximal to the acyl binding pocket, Phe 288 and Phe 290. The calculated energy difference between the models showing thiomethyl (lower energy) vs diethyl thiosuccinyl (higher energy) was found to be 21 kcal/mol. Thus, thiomethyl was predicted to be the more favorable leaving group for both (1*S*)-isomers of isomalathion.

SELDI-TOF-MS Analysis of TcAChE Modified by Isomalathion. Representative spectra for TcAChE treated with isomalathion isomers are shown in Figure 6. In the spectrum of control TcAChE, a peak was observed at *m*/*z* 2333.0 (± 0.8) with average mass corresponding to that of the unmodified active site peptide (calculated MH^+ at *m*/*z* 2332.7; Table 2, Figure 6). Peaks with *m*/*z* at 2455.1 (± 0.9) and 2454.3 (± 0.9) were found in the TcAChE samples treated with (1*R*,3*R*)- and (1*R*,3*S*)-isomalathion and subsequently digested with trypsin, respectively (Table 2). The average mass of these peaks corresponds to the active site peptide with an *O*,*S*-dimethyl phosphate adduct. Peaks with *m*/*z* at 2425.8 (± 0.9) and 2425.7 (± 0.6) were found in the samples treated with (1*S*,3*R*)- and (1*S*,3*S*)-isomalathion and subsequently digested with trypsin, respectively (Table 2). The average mass of these peaks corresponds to the active site peptide with an *O*-methyl phosphate adduct.

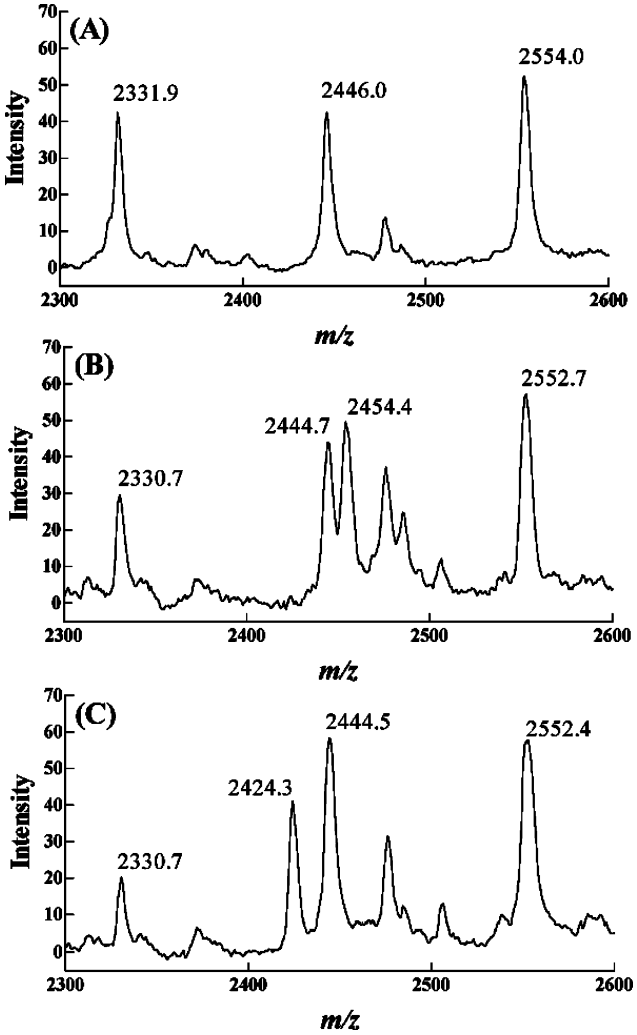

Figure 6. Representative SELDI-TOF-MS spectra of tryptic digest peptides from TcAChE inhibited by isomalathion isomers. (A) Control TcAChE peptides. Peak with *m*/*z* at 2331.9 represents the unmodified active site peptide containing the catalytic Ser 200. (B) Peptides of enzyme modified by (1*R*,3*R*)-isomalathion. Peak with *m*/*z* at 2454.4 corresponds to the active site peptide with a covalently bound *O*,*S*-dimethyl phosphate adduct. (C) Peptides of TcAChE inhibited by the (1*S*,3*S*)-stereoisomer. Peak with *m*/*z* at 2424.3 represents the active site peptide with a covalently bound *O*-methyl phosphate adduct. The peaks at *m*/*z* 2446 and 2554 represent AChE peptides corresponding to residues 270−289 and 150−174, respectively.

Discussion

It was previously found that mammalian and avian AChE are stereoselectively inactivated by isomalathion showing differences for k_i values among all stereoisomers with the order of potency being (1*R*,3*R*) > (1*R*,3*S*) > (1*S*,3*R*) > (1*S*,3*S*) (*5*−*7*). Stereoselective inactivation of AChE has been demonstrated for other chiral OP compounds, such as soman, which also contains an asymmetric phosphorus and an asymmetric carbon in one of its ligands (*22*, *23*).

As shown in Figure 2, the stereoselectivity of TcAChE for isomalathion isomers is different from that of enzyme from mammalian and avian species, with k_i values for (1*R*,3*S*)- and (1*S*,3*R*)-enantiomers being the same. Such a result is similar to that obtained for the inhibition of EeAChE by isomalathion isomers (*24*) and suggests that these two enzymes share similar active site topology that differentiates them from mammalian and avian AChE.

Table 2. Observed MH^+ for Unmodified and Modified Active Site Peptides of TcAChE[a]

isomalathion isomer	MH^+ (average mass)	
	active site peptide[b]	Δm[d]
control (none)	2333.0 ± 0.8[c]	
(1*R*,3*R*)	2455.1 ± 1.0	123.9 ± 0.1[e]
(1*R*,3*S*)	2454.3 ± 0.9	123.8 ± 0.4[e]
(1*S*,3*R*)	2425.8 ± 0.9	94.0 ± 0.6[f]
(1*S*,3*S*)	2425.7 ± 0.6	93.8 ± 0.2[f]

[a] Conditions used are described in Materials and Methods. [b] Active site peptide containing the catalytic Ser 200 in the control (unmodified) and isomalathion-treated (modified) samples. Values shown are mean ± SE for $n = 3$ experiments, except the control, for which $n = 6$ experiments. [c] Theoretical MH^+ at m/z 2332.7. [d] Difference in mass between the modified and the unmodified active site peptides identified in spectra of TcAChE modified by isomalathion isomers and digested with trypsin that takes into account the Ser proton lost in the organophosphorylation reaction. Values shown are mean ± SE for $n = 3$ experiments. [e] Mass corresponds to an *O*,*S*-dimethyl phosphate adduct ($\Delta m = 124.1$, theoretical). [f] Mass corresponds to an *O*-methyl phosphate adduct ($\Delta m = 94.0$, theoretical).

The most profound difference between the TcAChE and the enzyme from mammalian or avian sources revealed in the present study was the ratios for k_i values of enantiomeric pairs as shown in Table 1. A change in stereochemistry from the (1*R*,3*R*)- to the (1*S*,3*S*)-enantiomer results in a ratio of 53.8 for TcAChE, which is much higher than values for mammalian or avian enzymes but comparable to that for EeAChE. Furthermore, the (1*R*,3*S*)-/(1*S*,3*R*)-enantiomer ratio for the *Torpedo* enzyme is only 0.722, comparable to that for EeAChE but strikingly different from values for mammalian or avian AChE. These data reveal that TcAChE and EeAChE have comparable stereoselectivity for isomalathion isomers that is different from other AChE species studied and further indicate structural similarity in the catalytic centers of these picine enzymes.

TcAChE inhibited by (1*R*)-isomers readily reactivated in the presence of 200 μM 2-PAM with $k_3 > 0.140$ min^{-1}, corresponding to a $t_{1/2} < 5$ min, but enzyme modified by (1*S*)-isomalathions was refractory toward reactivation with $k_3 < 0.012$ min^{-1}, corresponding to $t_{1/2} > 60$ min. Such a finding is comparable to that previously reported for AChE from various species and has been demonstrated using kinetics to be the result of loss of different primary leaving groups for (1*R*)- vs (1*S*)-isomers (*5*, *6*, *8*, *10*). Results of reactivation experiments reported in the present paper suggest that the difference in inhibitory mechanism postulated to occur between (1*R*)- and (1*S*)-isomalathions for mammalian and avian AChE is conserved for TcAChE.

Although the ratio of k_3 values for enzyme inactivated by (1*S*,3*R*)- vs (1*S*,3*S*)-diastereomers was found to be 1.8, the individual k_3 values were small as compared to those for the (1*R*)-isomers and not significantly different from each other ($p > 0.05$). For other species of AChE, however, a difference in k_3 values between the two (1*S*)-isomers was observed and determined to be statistically significant (*8*, *10*). Taken together, these findings suggest that the difference in the mechanism of inhibition of AChE between (1*R*)- and (1*S*)-isomalathions may be occurring to a greater degree for the (1*S*,3*S*)-isomer than for the (1*S*,3*R*)-isomer.

Molecular modeling was employed to determine the orientation of each of the isomalathion stereoisomers in the active site of TcAChE, and results of these virtual experiments lend support to the hypothesis of a mechanistic shift between the (1*R*)- and the (1*S*)-isomers. Such an approach has been used recently to study the interaction of enantiomeric OP compounds with the active site of AChE (*25*−*27*). In the present study, both (1*R*,3*R*)- and (1*R*,3*S*)-isomalathion were predicted to be aligned in the catalytic center with the diethyl thiosuccinyl moiety pointing toward the gorge entrance and therefore positioned as the primary leaving group (Figure 4A; data not shown for (1*R*,3*S*)-isomalathion). Clothier et al. (*9*) predicted that diethyl thiosuccinyl is the primary leaving moiety for the isomalathions, and data presented in this paper support this hypothesis for the (1*R*)-isomers.

In contrast, docking experiments of (1*S*,3*R*)- and (1*S*,3*S*)-isomalathion in the active site of TcAChE predicted thiomethyl to be apical to the catalytic Ser 200 and therefore positioned as the primary leaving group (Figures 4B and 5A). Thiomethyl has also been demonstrated to be the primary leaving moiety for the inhibitory reaction of methamidophos with AChE (*28*, *29*). These results notwithstanding, molecular modeling experiments of (1*S*,3*R*)-isomalathion in the present study also predicted that diethyl thiosuccinyl may be oriented as the primary leaving group for this isomer, even though this solution was energetically less favorable than the one with thiomethyl aligned for ejection from the active site (Figure 5B). Such a result was not observed for (1*S*,3*S*)-isomalathion, for which thiomethyl emerged as the only candidate for displacement. The finding that inhibition of AChE by the (1*S*,3*R*)-isomer may proceed with expulsion of either thiomethyl (as the preferred leaving group) or diethyl thiosuccinyl resulting in formation of two distinct inhibitory adducts may yield an explanation for results obtained in previous studies that reported a difference in rates of reactivation for enzyme modified by (1*S*,3*R*)- vs (1*S*,3*S*)-isomalathion (*5*, *6*, *8*, *10*). At this point, however, the molar ratio for production of the two possible adducts resulting from the loss of either choice of ligand from (1*S*,3*R*)-isomalathion during the inactivation of AChE is unknown, although thiomethyl is the preferred primary leaving group based on the calculated difference in energy between the two models.

The basis for the difference in primary leaving group preference between (1*R*)- and (1*S*)-isomalathions may arise from the specificity of the acyl binding site (Phe 288 and Phe 290), which might preclude efficient accommodation of the diethyl thiosuccinyl and thiomethyl ligands. Using molecular modeling and site-directed mutagenesis, previous work has demonstrated that bulky ligands of OP compounds cannot fit into this specificity pocket, because of steric interference with the two Phe side chains (*30*−*32*).

While the thiomethyl group can be accommodated by the acyl binding pocket, methoxy is the preferred ligand as demonstrated by the molecular modeling experiments in the present study. Evidence to support this conclusion comes from previous work involving inhibition of AChE by enantiomers of isoparathion methyl. Both enantiomers of this compound are thought to organophosphorylate the catalytic serine with loss of 4-nitrophenoxy as the primary leaving group (*33*). If the 4-nitrophenoxy ligand is aligned as the primary leaving group, the methoxy and thiomethyl ligands should be accommodated in the acyl binding pocket of AChE for the (*S*)- and (*R*)-isomers, respectively, upon reaction with AChE (*22*). This contention is supported by the results of two previous studies.

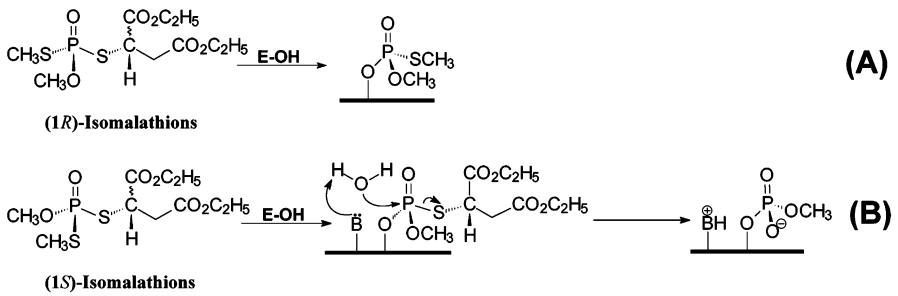

Figure 7. Inactivation of AChE (denoted as E-OH) by isomalathion isomers. (A) Inhibition of AChE by (1*R*)-isomalathions proceeds with loss of diethyl thiosuccinyl to yield an *O*,*S*-dimethyl phosphate adduct. (B) Inactivation of AChE by (1*S*)-isomalathions occurs with loss of thiomethyl to yield an *O*-methyl-*S*-diethylsuccinyl phosphorothiolate adduct that undergoes rapid aging via an S_N2 reaction to yield an *O*-methyl phosphate adduct.

First, (*S*)-isoparathion methyl has been shown to be a 3−15-fold more potent inhibitor of the enzyme from rat brain than the (*R*)-isomer (*33*). Second, Jianmongkol et al. (*8*) found that the (*S*)-enantiomer was >20-fold more potent than (*R*)-isoparathion methyl as an inhibitor of bovine erythrocyte and hen brain AChE.

The difference in primary leaving group preference between (1*R*)- and (1*S*)-isomalathions may be modulated by the stereochemistry of the diethyl thiosuccinyl ligand. For (1*S*)-isomalathions, the diastereomer with the *R*-configuration at carbon may be oriented with the thiomethyl or diethylthiosuccinyl ligands as primary leaving groups. However, changing the carbon chirality to the *S*-configuration prevents the isomalathion molecule from positioning the diethyl thiosuccinyl as the primary leaving moiety, according to results of molecular modeling experiments detailed in this paper. Steric constraints arising from chirality at both the phosphorus and the carbon atoms may force the (1*S*,3*S*)-isomer to orient itself such that the thiomethyl ligand must be the primary leaving group for the inhibitory reaction of TcAChE with isomalathion.

Direct chemical characterization of the adducts resulting from inactivation of TcAChE by each of the four isomers of isomalathion revealed a difference in inhibitory mechanism between (1*R*)- and (1*S*)-stereoisomers (Figure 7). TcAChE treated with (1*R*)-isomalathions resulted in an *O*,*S*-dimethyl phosphate adduct, indicating that diethyl thiosuccinyl was expelled as the primary leaving group. In contrast, inhibition of the enzyme by (1*S*)-isomers resulted in an *O*-methyl phosphate adduct. These findings are in accord with results of a previous study (*10*) and demonstrate that thiomethyl is lost as the primary leaving moiety followed by a rapid postinhibitory reaction to yield an aged enzyme.

The aging reaction for AChE modified by OP compounds is thought to involve dealkylation of the OP adduct via an S_N1 process with scission of a C−O bond and formation of a carbocation on the leaving alkyl moiety (*34*, *35*). This reaction yields an oxyanion in the active site of AChE (*36*), causing the enzyme to be intractable toward reactivation. Results of the present study indicate that aging of AChE inhibited by (1*S*)-isomalathions proceeds via an S_N2 reaction with P−S bond cleavage (Figure 7B). A recent study found that aging of AChE modified by racemic tabun may involve an S_N2 process with scission of a P−N bond (*37*).

In summary, data presented in this paper obtained from experiments involving kinetics, molecular modeling, and MS indicate that the inhibitory reactions of TcAChE with (1*R*)- and (1*S*)-isomers of isomalathion proceed by different mechanisms (Figure 7). TcAChE displayed a differential preference for the four stereoisomers of isomalathion similar to that previously seen for EeAChE (*24*) but distinguishable from that observed for mammalian and avian AChE (*5*, *6*, *8*). TcAChE inhibited by (1*R*)-isomalathions rapidly reactivated with $t_{1/2} < 5$ min in the presence of 200 μM 2-PAM, but enzyme modified by (1*S*)-isomers was refractory toward reactivation. Results of oxime-mediated reactivation experiments for this enzyme were similar to those previously observed for mammalian and avian AChE, indicating that the shift in inhibitory mechanism between (1*R*)- and (1*S*)-isomalathions previously demonstrated for these enzymes using kinetics likewise occurs for TcAChE (*5*, *6*, *8*). Molecular modeling experiments involving the four isomers of isomalathion in the active site of TcAChE also yielded evidence to support the hypothesis of a shift in inhibitory mechanism between (1*R*)- and (1*S*)-isomalathions. While the (1*R*)-isomers were predicted to be oriented with diethyl thiosuccinyl as the primary leaving group, (1*S*)-isomalathions were positioned with thiomethyl as the primary leaving moiety. The (1*S*,3*R*)-isomer was found to be oriented with either thiomethyl or diethyl thiosuccinyl as the ligand to be expelled, although the alignment with thiomethyl as the primary leaving group was energetically more favorable. Mass spectral analysis of TcAChE inhibited by each isomalathion isomer revealed that inactivation by (1*R*)-isomers yields an *O*,*S*-dimethyl phosphate adduct, but inhibition of the enzyme by (1*S*)-isomers results in an *O*-methyl phosphate adduct. The results of experiments involving MS, therefore, provided unequivocal evidence supporting the stereochemically distinct pathways of AChE inhibition predicted by kinetics and molecular modeling (Figure 7). Given that AChE is a member of a superfamily of proteins termed the α/β-hydrolase fold (*38*, *39*), it would be of interest to discover if the mechanistic shift demonstrated to occur for inactivation of this enzyme by isomalathion stereoisomers is conserved for structurally related serine hydrolases.

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References

(1) Metcalf, R. L., and March, R. B. (1953) The isomerization of organic thionophosphate insecticides. *J. Econ. Entomol.* **46**, 288−294.

(2) Chukwudebe, A., March, R. B., Othman, M., and Fukuto, T. R. (1989) Formation of trialkyl phosphorothioate esters from organophosphorus insecticides after exposure to either ultraviolet-light or sunlight. *J. Agric. Food Chem.* **37**, 539−545.

(3) Thompson, C. M., Frick, J. A., Natke, B. C., and Hansen, L. K. (1989) Preparation, analysis, and anticholinesterase properties of *O*,*O*-dimethylphosphorothioate isomerides. *Chem. Res. Toxicol.* **2**, 386−391.

(4) Aldridge, W. N., Miles, J. W., Mount, D. L., and Verschoyle, R. D. (1979) The toxicological properties of impurities in malathion. *Arch. Toxicol.* **42**, 95−106.

(5) Berkman, C. E., Quinn, D. A., and Thompson, C. M. (1993) Interaction of acetylcholinesterase with the enantiomers of malaoxon and isomalathion. *Chem. Res. Toxicol.* **6**, 724−730.

(6) Berkman, C. E., Ryu, S., Quinn, D. A., and Thompson, C. M. (1993) Kinetics of the postinhibitory reactions of acetylcholinesterase poisoned by chiral isomalathion: A surprising nonreactivation induced by the R_P stereoisomers. *Chem. Res. Toxicol.* **6**, 28−32.

(7) Jianmongkol, S., Berkman, C. E., Thompson, C. M., and Richardson, R. J. (1996) Relative potencies of the four stereoisomers of isomalathion for inhibition of hen brain acetylcholinesterase and neurotoxic esterase in vitro. *Toxicol. Appl. Pharmacol.* **139**, 342−348.

(8) Jianmongkol, S., Marable, B. R., Berkman, C. E., Talley, 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.

(9) 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.

(10) 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.

(11) Sussman, J. L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L., and Silman, I. (1991) Atomic structure of acetylcholinesterase from *Torpedo californica*: A prototypic acetylcholine-binding protein. *Science* **253**, 872−879.

(12) Berkman, C. E., Thompson, C. M., and Perrin, S. R. (1993) Synthesis, absolute configuration, and analysis of malathion, malaoxon, and isomalathion enantiomers. *Chem. Res. Toxicol.* **6**, 718−723.

(13) Futerman, A. H., Low, M. G., and Silman, I. (1983) A hydrophobic dimer of acetylcholinesterase from *Torpedo californica* electric organ is solubilized by phosphatidylinositol-specific phospholipase C. *Neurosci. Lett.* **40**, 85−89.

(14) 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.

(15) Aldridge, W. N., and Reiner, E. (1972) *Enzyme Inhibitors as Substrates*, North-Holland Publishing Co., Amsterdam.

(16) Richardson, R. J. (1992) Interactions of organophosphorus compounds with neurotoxic esterase. In *Organophosphates: Chemistry, Fate, and Effects* (Chambers, J. E., and Levi, P. E., Eds.) pp 299−323, Academic Press, San Diego.

(17) Ordentlich, A., Barak, D., Kronman, C., Ariel, N., Segall, Y., Velan., B., and Shafferman, A. (1996) The architecture of human acetylcholinesterase active center probed by interactions with selected organophosphate inhibitors. *J. Biol. Chem.* **271**, 11953−11962.

(18) MSI technical literature for Affinity module.

(19) Schumacher, M., Camp, S., Maulet, Y., Newton, M., MacPhee-Quigley, K., Taylor, S. S., Friedmann, T., and Taylor, P. (1986) Primary structure of *Torpedo californica* acetylcholinesterase deduced from its cDNA sequence. *Nature* **319**, 407−409.

(20) Harris, D. C. (1991) *Quantitative Chemical Analysis*, 3rd ed., pp 41−45, W. H. Freeman and Company, New York.

(21) Jianmongkol, S. (1998) *Stereoselective Influence of Isomalathion on Neurotoxicity: Inhibition of Acetylcholinesterase and Neurotoxic Esterase.* Ph.D. Dissertation, The University of Michigan, Ann Arbor.

(22) Taylor, P., Radic, Z., Hosea, N. A., Camp, S., Marchot, P., and Berman, H. A. (1995) Structural basis for the specificity of cholinesterase catalysis and inhibition. *Toxicol. Lett.* **82**−**83**, 453−458.

(23) Benschop, H. P., Konings, C. A., Van Genderen, J., and De Jong, L. P. (1984) Isolation, anticholinesterase properties, and acute toxicity in mice of the four stereoisomers of the nerve agent soman. *Toxicol. Appl. Pharmacol.* **72**, 61−74.

(24) Richardson, R. J., Doorn, J. A., Talley, T. T., and Thompson, C. M. (2000) Inhibitory and postinhibitory kinetics of inhibition of electric eel acetylcholinesterase (AChE) after inhibition with the chiral probe isomalathion. *Toxicol. Sci.* **54**, 339 (abstract 1591).

(25) Hirashima, A., Kuwano, E., and Eto, M. (2000) Docking study of enantiomeric fonofos oxon bound to the active site of *Torpedo californica* acetylcholinesterase. *Bioorg. Med. Chem.* **8**, 653−656.

(26) Bernard, P. P., Kireev, D. B., Chrétien, J. R., Fortier, P. L., and Coppet, L. (1998) 3D model of the acetylcholinesterase catalytic cavity probed by stereospecific organophosphorous inhibitors. *J. Mol. Model.* **4**, 323−334.

(27) Bernard, P. P., Kireev, D. B., Pintore, M., Chrétien, J. R., Fortier, P. L., and Froment, D. (2000) A CoMFA study of enantiomeric organophosphorus inhibitors of acetylcholinesterase. *J. Mol. Model.* **6**, 618−629.

(28) Thompson, C. M., and Fukuto, T. R. (1982) Mechanism of cholinesterase inhibition by methamidophos. *J. Agric. Food. Chem.* **30**, 282−284.

(29) 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.

(30) Ordentlich, A., Barak, D., Kronman, C., Benschop, H. P., De Jong, L. P., Ariel, N., Barak, R., Segall, Y., Velan, B., and Shafferman, A. (1999) Exploring the active center of human acetylcholinesterase with stereomers of an organophosphorus inhibitor with two chiral centers. *Biochemistry* **38**, 3055−3066.

(31) Taylor, P., Hosea, N. A., Tsigelny, I., Radic, Z., and Berman, H. A. (1997) Determining ligand orientation and transphosphorylation mechanisms on acetylcholinesterase by Rp, Sp enantiomer selectivity and site-directed mutagenesis. *Enantiomer* **2**, 249−260.

(32) Taylor, P., Wong, L., Radic, Z., Tsigelny, I., Bruggerman, R., Hosea, N. A., and Berman, H. A. (1999) Analysis of cholinesterase inactivation and reactivation by systematic structural modification and enantiomeric selectivity. *Chem.-Biol. Interact.* **119**−**120**, 3−15.

(33) Ryu, S., Lin, J., and Thompson, C. M. (1991). Comparative anticholinesterase potency of isoparathion methyl. *Chem. Res. Toxicol.* **4**, 517−520.

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

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

(36) Segall, Y., Waysbort, D., Barak, D., Ariel, N., Doctor, B. P., Grunwald, J., and Ashani, Y. (1993) Direct observation and elucidation of the structures of aged and nonaged phosphorylated cholinesterases by ^{31}P NMR spectroscopy. *Biochemistry* **32**, 13441−13450.

(37) Barak, D., Ordentlich, A., Kaplan, D., Barak, R., Mizrahi, D., Kronman, C., Segall, Y., Velan, B., and Shafferman, A. (2000) Evidence for P−N bond scission in phosphoroamidate nerve agent adducts of human acetylcholinesterase. *Biochemistry* **39**, 1156−1161.

(38) Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., Harel, M., Remington, S. J., Silman, I., Schrag, J. D., Sussman, J. L., Verschueren, K. H. G., and Goldman, A. (1992) The α/β-hydrolase fold. *Protein Eng.* **5**, 197−211.

(39) Cygler, M., Schrag, J. D., Sussman, J. L., Harel, M., Silman, I., Gentry, M. K., and Doctor, B. P. (1993) Relationship between sequence conservation and three-dimensional structure in a large family of esterases, lipases, and related proteins. *Protein Sci.* **2**, 366−382.

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