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Probing the Active Sites of Butyrylcholinesterase and Cholesterol Esterase with Isomalathion: Conserved Stereoselective Inactivation of Serine Hydrolases Structurally Related to Acetylcholinesterase

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Previous work has shown that acetylcholinesterase (AChE), a member of the α/β-hydrolase superfamily, is stereoselectively inhibited by the four stereoisomers of isomalathion. Recent kinetic and mass spectral data demonstrated that a difference in mechanism of inactivation exists for AChE treated with (1*R*)- versus (1*S*,3*S*)-stereoisomers. This study sought to determine whether other α/β-hydrolases are stereoselectively inhibited by isomalathion and if the difference in mechanism of AChE inactivation between (1*R*)- and (1*S*,3*S*)-isomers is conserved for other α/β-hydrolases. Bimolecular rate constants of inhibition (k_i) were measured for human and equine butyrylcholinesterase (HBChE and EBChE, respectively) and bovine cholesterol esterase (BCholE) with all four isomers. Isomalathion isomers inhibited these enzymes with the following order of potency: (1*R*,3*R*) > (1*R*,3*S*) > (1*S*,3*R*) ≥ (1*S*,3*S*). Ratios of k_i values for the most potent to the least potent isomer were 10.5 (HBChE), 11.9 (EBChE), and 68.6 (BCholE). Rate constants of reactivation (k_3) were measured for enzyme inhibited by isomalathion isomers. HBChE, EBChE, and BCholE inactivated by the (1*R*)-isomers readily reactivated. However, enzymes modified by (1*S*)-isomalathions were refractory toward reactivation, and k_3 values were not significantly different from zero for HBChE and BCholE treated with the (1*S*,3*S*)-isomer. Computer-based docking experiments were performed for BCholE with (1*R*,3*R*)- and (1*S*,3*S*)-enantiomers. Calculated structures predicted a difference in primary leaving group: diethyl thiosuccinate for (1*R*,3*R*)-isomalathion and thiomethyl for the (1*S*,3*S*)-isomer. The data demonstrate that the α/β-hydrolases used in this study are stereoselectively inhibited by isomalathion. Furthermore, the results suggest that the mechanistic shift demonstrated to occur for inhibition of AChE by (1*R*)- versus (1*S*,3*S*)-isomers is conserved for butyrylcholinesterase and cholesterol esterase.

Introduction

Butyrylcholinesterase (BChE, EC 3.1.1.8)[1] and cholesterol esterase (CholE, EC 3.1.1.13) belong to a large family of proteins called the α/β-hydrolase fold, which includes other esterases such as acetylcholinesterase (AChE, EC 3.1.1.7; refs *1* and *2*). Certain hydrolases belonging to this family have a catalytic triad containing Ser-His-Asp/Glu that is essentially the mirror image of that found in serine proteases, such as trypsin and chymotrypsin (*2*, *3*). As with serine proteases, esterases of the α/β-hydrolase fold are susceptible to inhibition by organophosphorus (OP) compounds, with the most notable example being AChE.

AChE is potently inhibited by OP nerve agents and the oxon form of OP pesticides, exhibiting remarkable stereoselectivity for particular enantiomers/isomers. Asymmetric OP inhibitors have been used to probe the active site of this enzyme. It was found that (*S*)-somans[2] inactivated AChE from various species with bimolecular rate constants (k_i) >1000-fold larger than those for (*R*)-somans (*4*, *5*). Previous work has demonstrated that this enzyme is stereoselectively inhibited by the four isomers of isomalathion (*6*, *7*; see Figure 1 for structures), an impurity formed from thermal or photochemical isomerization of the widely used pesticide malathion (*8*, *9*). For both soman and isomalathion, asymmetry at the phosphorus and carbon atoms has a significant effect on k_i values.

A difference in postinhibitory kinetics has been observed for AChE inactivated by the stereoisomers of

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[1] Abbreviations: AChE, acetylcholinesterase; BCholE, bovine pancreatic cholesterol esterase; BSA, bovine serum albumin; BChE, butyrylcholinesterase; BTCh, butyrylthiocholine; CholE, cholesterol esterase; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); EBChE, equine serum butyrylcholinesterase; HBChE, human serum butyrylcholinesterase; OP, organophosphorus; pNPB, *p*-nitrophenyl butyrate; 2-PAM, pyridine-2-aldoxime methiodide.

[2] Asymmetry is designated for the phosphorus atom. The alternative nomenclature $P_{R/S}$ or $(R/S)_P$ was not used, because it could be confused with planar chirality.

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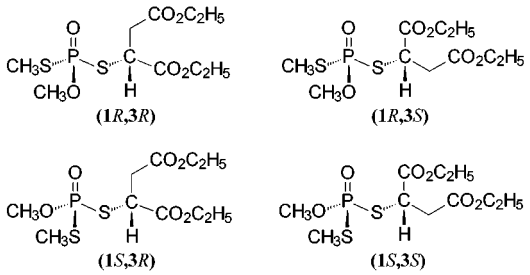


Figure 1. The four stereoisomers of isomalathion with asymmetric phosphorus and carbon atoms designated at positions 1 and 3, respectively.

isomalathion. While enzyme inhibited with (1*R*)-isomalathions readily reactivates both spontaneously and in the presence of pyridine-2-aldoxime methiodide (2-PAM), AChE inactivated with the (1*S*)-isomers is intractable toward reactivation (*10*, *11*). Moreover, a difference in the rate of aging[3] was found to exist between enzyme inhibited by (1*R*)- versus (1*S*)-isomers and even between (1*S*,3*R*)- versus (1*S*,3*S*)-diastereomers (*11*). AChE inhibited with (1*S*,3*S*)-isomalathion aged at a rate too rapid for measurement using conventional techniques.

BChE is potently inhibited by certain OP compounds (*12*, *13*). The enzyme displays stereoselectivity for methyl phosphonates, but to a much lesser degree than AChE (*14*, *15*). For some isomers, the chiral preference is slightly different from that of AChE (*14*). Likewise, soman has been used to probe the active site of BChE; the stereoselectivity of the enzyme for the four isomers of this compound is similar to that of AChE, but not as pronounced. A notable difference is that the (*R*)-isomers of soman, which are essentially inactive for AChE, are potent inhibitors of BChE (*16*, *17*). Aging of BChE inhibited by soman was found to be influenced by the stereochemistry of the OP adduct with rates similar to OP compound-inhibited AChE (*16*).

Few studies have been done to determine the specificity of CholE for OP compounds. Also, postinhibitory kinetics have not been determined for the enzyme inactivated by these agents. Alkyl phosphates were found to be effective inhibitors of CholE and were presumed to phosphorylate the active site Ser (*18*−*20*). To date, no asymmetric OP compounds have been used to probe the active site of CholE; however, Lin et al. demonstrated that the enzyme is inactivated with enantiomeric preference by atropisomers of biarylcarbamates (*21*, *22*).

This study was undertaken to probe the active sites of BChE and CholE with isomalathion and to test the hypothesis that these members of the α/*β*-hydrolase superfamily in addition to AChE exhibit stereoselective inhibition by isomalathion with a difference in primary leaving group preference between (1*R*)- versus (1*S*,3*S*)-isomers. Kinetic constants for inactivation as well as spontaneous and oxime-mediated reactivation were measured for enzyme inhibited by the four stereoisomers of isomalathion. Molecular modeling was used to determine the orientation of the (1*R*,3*R*)- and (1*S*,3*S*)-enantiomers in the active site of bovine pancreatic CholE and predict the primary leaving group for the inhibitory reaction.

[3] Aging refers to a time-dependent process whereby the enzyme becomes refractory 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 mechanism of the reaction.

Material and Methods

Caution: *The OP chemicals synthesized and used in this study are hazardous and should be handled by trained personal in a well-ventilated hood. The OP chemicals described are hydrolyzed by 3M NaOH and rendered inactive as AChE inhibitors.*

Chemicals. The four resolved stereoisomers of isomalathion were prepared and characterized as described previously (*23*). Chemical purity of these inhibitors was >99% as assessed by ^{1}H and ^{31}P NMR, GC, HPLC, and combustion analysis. Isomalathion stereoisomer configurations were assigned based on single-crystal X-ray analysis of a strychnine salt precursor. Optical rotations and stereoisomeric purities (all >90%) were determined by polarimetry and chiral HPLC, respectively. Equine serum butyrylcholinesterase (EBChE), human serum butyrylcholinesterase (HBChE), bovine pancreatic cholesterol esterase (BCholE), butyrylthiocholine iodide (BTCh), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), bovine serum albumin (BSA), *p*-nitrophenyl butyrate (pNPB), and pyridine-2-aldoxime methiodide (2-PAM) were purchased from Sigma Chemical Company (St. Louis, MO). The following proteases and protease substrates, respectively, were purchased from Sigma: α-chymotrypsin (bovine pancreatic, EC 3.4.21.1), succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide; trypsin (bovine pancreatic, 3.4.21.4), benzoyl-Arg-*p*-nitroanilide; and elastase (porcine pancreatic, EC 3.4.21.11), succinyl-Ala-Ala-Ala-*p*-nitroanilide. All other chemicals were reagent grade. Aqueous solutions were prepared in doubly deionized water.

Esterase Inhibition. Kinetics involving BChE were performed in 0.1 M sodium phosphate buffer, pH 7.6, at 37 °C using a modification of the method of Ellman et al. (*24*). CholE kinetics were performed in 0.1 M sodium phosphate buffer containing 0.1 M NaCl and 0.5% (w/w) Triton X-100, pH 7.0, at 37 °C using a modification of the method of Hosie et al. (*25*). Inhibitor was dissolved in acetone and diluted in phosphate buffer. 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 either BTCh and DTNB (BChE assay, final concentrations, BTCh 1.0 mM, DTNB 0.32 mM) or pNPB (CholE assay, final concentration, 400 μM). pNPB was initially solubilized in acetonitrile and subsequently dissolved in buffer with resulting acetonitrile concentration <1% (w/w). Residual activity was determined by measuring the change in absorbance at 412 nm (BChE assay) or 405 nm (CholE assay) over a 1−2 min period using a SPECTRAmax 340 microplate reader at 37 °C (Molecular Devices Corporation, Sunnydale, CA).

Protease Inhibition. Experiments involving α-chymotrypsin, elastase, and trypsin were performed in 0.1 M sodium phosphate buffer, pH 7.6, at 37 °C. Enzymes were incubated with the four stereoisomers of isomalathion for 15 min using the following concentrations: 115 μM (1*R*,3*R*), 148 μM (1*R*,3*S*), 122 μM (1*S*,3*R*), and 98.1 μM (1*S*,3*S*). At the end of the incubation period, the appropriate substrate solution was added at a final concentration of 1 mM. The following substrates were used for assaying protease activity: succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (α-chymotrypsin); succinyl-Ala-Ala-Ala-*p*-nitroanilide (elastase); and benzoyl-Arg-*p*-nitroanilide (trypsin). Residual activity was determined colorimetrically as described above for esterases except using a wavelength of 410 nm.

Determination of k_i Values. The bimolecular rate constant of inhibition (k_i) was determined according to the method of Aldridge and Reiner (*26*) as reviewed in Richardson (*27*). The inhibitory reaction proceeded with [I] > 10 [enzyme] and [I] < K_m so that the k_i could be approximated using pseudo-first-order kinetics. Linear regression was used to calculate the slopes of ln(% activity remaining) versus time (which yields k') and $-k'$ versus [I] to determine k_i.

Enzyme Reactivation. BChE in 0.1 M sodium phosphate buffer, pH 7.6, was incubated at 30 °C (EBChE) and 37 °C (HBChE) with each of the four stereoisomers of isomalathion

using concentrations to yield ~90% inhibition in 15 min. Concentrations of each isomer used for inhibition of EBChE and HBChE, respectively, were as follows: (1*R*,3*R*), 3.1 and 1.6 μM; (1*R*,3*S*), 4.5 and 4.6 μM; (1*S*,3*R*), 30 and 12 μM; and (1*S*,3*S*), 38 and 19 μM. An aliquot of the enzyme solution was then diluted 1:40 (v/v) with or without 2-PAM (500 μM). Reactivation was performed in 0.1 M sodium phosphate buffer (pH 7.6) at 30 °C (EBChE) and 37 °C (HBChE). Aliquots were withdrawn at timed intervals and assayed for activity as described above using a plate reader. A separate control was run to determine the effect of 2-PAM on BTCh hydrolysis. BCholE in 0.1 M sodium phosphate buffer, pH 7.6, was incubated with isomalathion with concentrations to yield 80% [(1*R*)-isomers] and 90% [(1*S*)-isomers] inhibition in 10 min [(1*R*)- and (1*S*,3*R*)-isomers] and 20 min [(1*S*,3*S*)-isomer)] at 37 °C. A longer time period was needed to achieve 90% inactivation for (1*S*,3*S*)-isomalathion with BCholE, because of the low inhibitory potency of this isomer. Concentrations of each isomer used for inhibition of BCholE were as follows: (1*R*,3*R*) 13 μM; (1*R*,3*S*), 13 μM; (1*S*,3*R*), 120 μM; and (1*S*,3*S*), 190 μM. An aliquot of enzyme solution was then diluted 1:200 (v/v) with 0.1 M sodium phosphate buffer (pH 7.0) at 37 °C, containing 0.1 M NaCl and 0.5% w/w Triton X-100. Aliquots were withdrawn at timed intervals and assayed for activity as described above using a plate reader.

Determination of k_3 Values. The apparent first-order rate constant of reactivation (k_3) was determined according to the method of Clothier et al. (*28*). The following equation was used to calculate k_3: ln(100/% inhibition) = k_3t; % inhibition = $[(A - A_t)/(A - A_0)] \times 100$, 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. 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 (0−12 min, spontaneous; 0−8 min, oxime-mediated).

Statistical Analysis. All values are reported as mean ± SE from at least three separate experiments. All statistics and regressions were calculated using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA). Significance of differences between means was determined by one-way ANOVA ($\alpha = 0.05$) using the Newman-Keuls post hoc test for multiple comparisons or 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. All 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 BCholE (*29*; 1AKN.pdb) was downloaded from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (http://www.rcsb.org/pdb/). Hydrogen atoms were added (pH 7.0) using the Biopolymer module. (1*R*,3*R*)- and (1*S*,3*S*)-isomalathion structures were initially positioned in the active site of BCholE. The inhibitor and a subset of BCholE 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 presumptive oxyanion hole so that they were kept within hydrogen-bonding distance (*30*). The ligand was confined to a 2 Å radius to prevent it from drifting away from the active site during the initial minimization that is performed in vacuo. Docking was accomplished using a minimization process involving two phases followed by a molecular dynamics simulation (*31*). The first round of minimization (100 step) 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 step), Coulombic terms were turned back on (distance dependent dielectric constant $\epsilon = 78.0$) and constraint factors 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 step) similar to the second phase initially used.

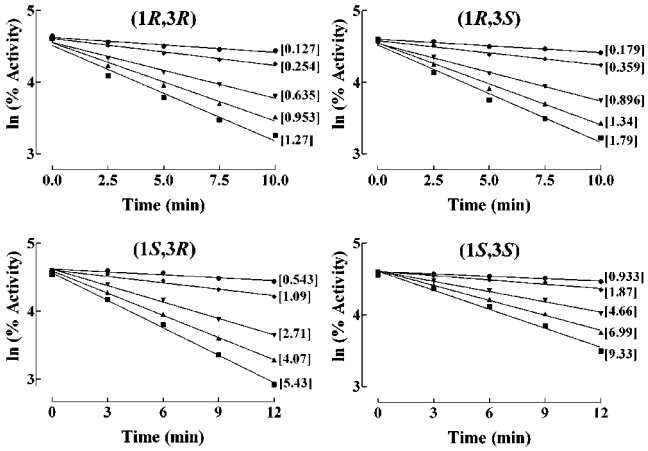


Figure 2. Representative primary plots for the time course of inhibition of HBChE by the four stereoisomers of isomalathion. As described in the Materials and Methods, the slope of each line represents k' (min^{-1}). The concentration of each isomer (μM) is indicated in brackets next to each curve.

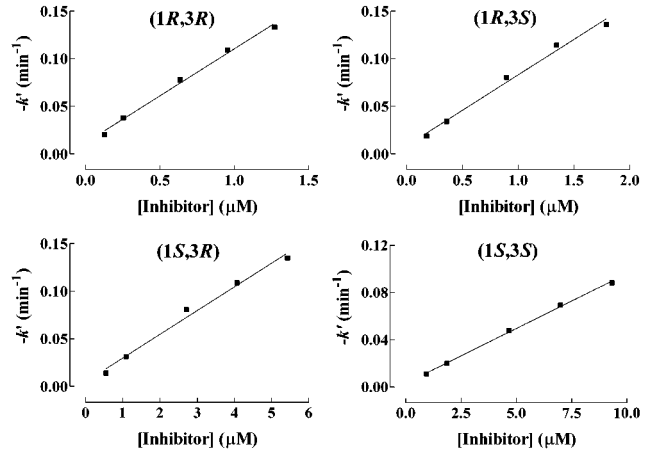


Figure 3. Representative secondary plots of $-k'$ (min^{-1}) versus [I] (μM) for isomers of isomalathion with HBChE. The slopes of these plots yield the k_i for each stereoisomer.

Results

Inhibition of BChE and CholE by Isomalathion. Representative primary plots are shown in Figure 2 for the time course of inactivation of HBChE by the four isomers of isomalathion. At the concentrations of inhibitors used, pseudo-first-order kinetics were observed. Slopes of primary plots were linear with respect to inhibitor concentration (Figure 3), permitting the determination of k_i for each stereoisomer. Values of k_i (mM^{-1} min^{-1}) are shown in Figure 4 for the enzymes used in this study. Ratios of k_i values for the most potent to the least potent stereoisomer were 10.5 (HBChE), 11.9 (EBChE), and 68.6 (BCholE). For all enzymes, the order of potency was found to be (1*R*,3*R*) > (1*R*,3*S*) > (1*S*,3*R*) ≥ (1*S*,3*S*).

Inhibition of Proteases by Isomalathion. An attempt was made to determine inhibitory potency of isomalathion stereoisomers with the serine proteases

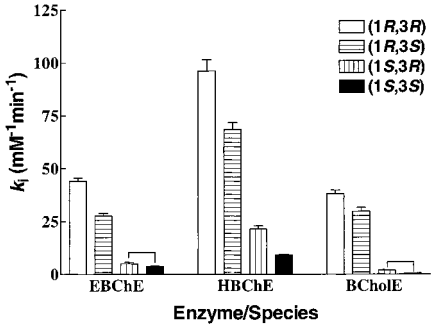


Figure 4. The k_i values for the inhibitory reaction of isomalathion with BCholE, EBChE, and HBChE. For each species, the k_i values are significantly different unless connected with a bar. Significant differences were identified by one-way ANOVA ($\alpha = 0.05$) using Newman-Keuls *post hoc* test for multiple comparisons.

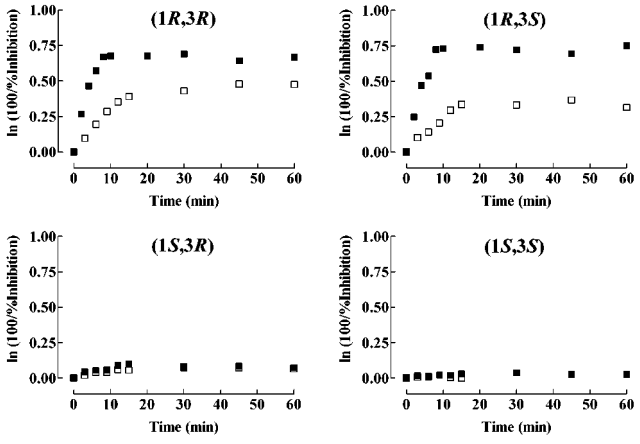


Figure 5. Representative plots of spontaneous (□) and oxime-mediated (■) reactivation for HBChE inhibited by isomalathion stereoisomers. The slopes of the initial linear portion of each graph represent k_3 (min^{-1}).

α-chymotrypsin, elastase, and trypsin. At concentrations of ∼100 μM for each of the four isomers, no inhibition was observed for any of the proteases examined.

Reactivation of BChE and CholE Inhibited by Isomalathion. Representative reactivation plots are shown for HBChE inhibited by isomalathion isomers in Figure 5. EBChE, HBChE, and BCholE inactivated by (1*R*)-isomers reactivated spontaneously with a measurable k_3 (min^{-1}) as shown in Figure 6A. The k_3 values for enzymes inhibited by (1*R*,3*R*)- and (1*R*,3*S*)-isomalathion were not significantly different. BChE and CholE modified by (1*S*)-isomers were refractory toward spontaneous reactivation, and a k_3 value could not be measured for EBChE, HBChE, or BCholE inactivated by (1*S*,3*S*)-isomalathion, because the slopes of the reactivation plots were not significantly different from zero. The presence of 500 μM 2-PAM increased the rate of reactivation for BChE as shown in Figure 6B. EBChE inhibited by (1*S*,3*R*)- and (1*S*,3*S*)-isomalathion had oxime-mediated k_3 values that were significantly different, and a k_3 could not be measured for HBChE modified by the (1*S*,3*S*)-isomer. No functional equivalent to 2-PAM was found for BCholE. Although uncharged oximes are effective reactivators of carboxylesterase (*32*), these compounds were ineffective for reactivating isomalathion-inhibited CholE. The presence of 1 mM 2,3-butanedione monoxime, diisonitrosoacetophenone, or NaF did not increase the rate of reactivation for this enzyme (results not shown).

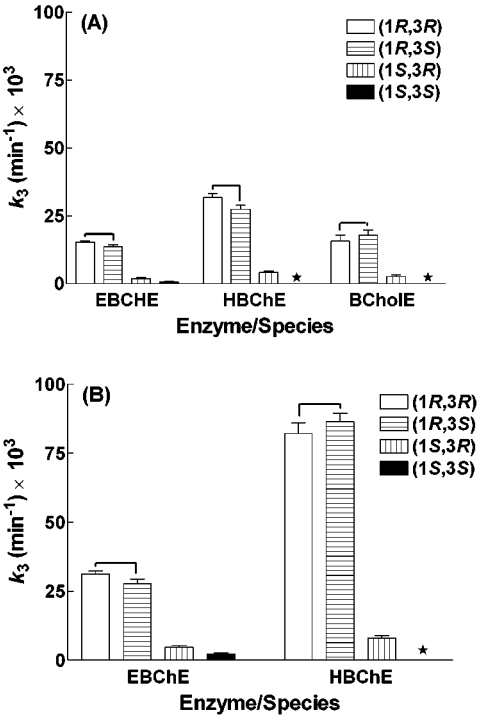


Figure 6. The k_3 values for (A) spontaneous reactivation of BCholE, EBChE, and HBChE inhibited by isomalathion stereoisomers and (B) oxime-mediated reactivation of EBChE and HBChE inactivated by isomalathion stereoisomers. For each enzyme/species, k_3 values for (1*R*)- or (1*S*)-pairs are significantly different unless connected by a bar. Significant differences were identified by two-tailed unpaired *t*-tests ($p < 0.05$). (★) Slopes of reactivation plots were determined to be not significantly different from zero using an *F*-test ($p > 0.05$).

Molecular Modeling of (1*R*,3*R*)- and (1*S*,3*S*)-Isomalathion with BCholE. Results of the molecular modeling experiments are shown in Figure 7. (1*R*,3*R*)-Isomalathion was found to be oriented in the active site of BCholE with the diethyl thiosuccinate group pointing toward the gorge-type opening and positioned apically to the active site Ser 194 hydroxyl group (Figure 7A). In contrast, the calculated configuration of the (1*S*,3*S*)-enantiomer was with the thiomethyl group pointed toward the gorge-type opening and oriented apically to the active site Ser 194 (Figure 7B). In both cases, the methoxy ligand was proximal to Trp 227, Phe 324, and Phe 393, and the phosphoryl oxygen was in close proximity to the putative oxyanion hole: Gly 107, Ala 108, and Ala 195 (*29*).

Discussion

Previous studies found that AChE from various species is stereoselectively inactivated by isomalathion with differences for k_i values between all stereoisomers with the order of potency being (1*R*,3*R*) > (1*R*,3*S*) > (1*S*,3*R*) > (1*S*,3*S*) (*6*, *7*). The same result was discovered for the mean values of k_i for BChE and CholE as shown in Figure 4, although the values for the (1*S*,3*R*)- and (1*S*,3*S*)-isomers were not statistically different for EBChE and BCholE. Chirality at both the phosphorus and carbon atoms influenced the rate of the inhibitory reaction of isomalathion with the enzymes used in this study. These observations confirm the expectation that steric interactions between the enzyme and these inhibitors are similar for AChE, BChE, and CholE. Surprisingly, CholE exhibited the greatest discrimination between (1*R*,3*R*)-

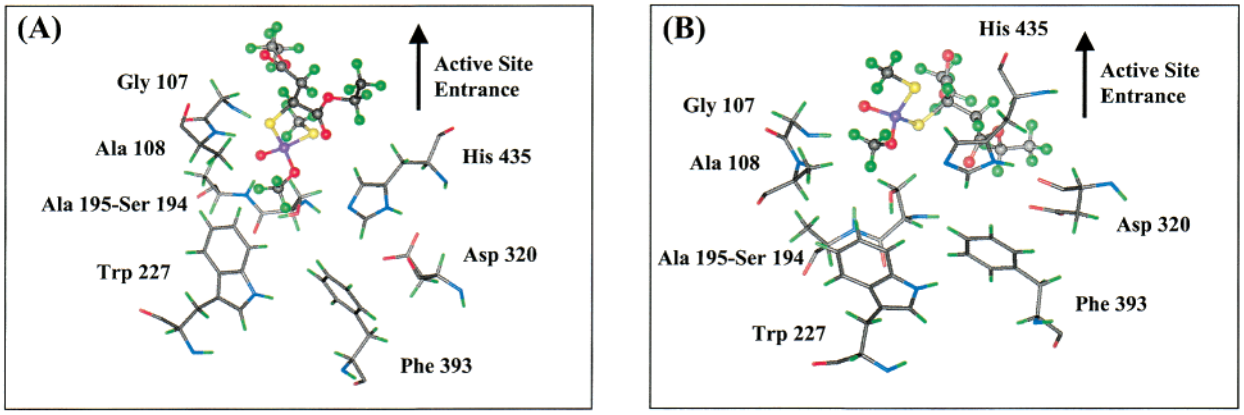


Figure 7. Results of docking experiments for (A) (1*R*,3*R*)- and (B) (1*S*,3*S*)-isomalathion in the active site of BCholE. Experimental procedures used to determine structures are described in Materials and Methods. Both enzyme and inhibitor were kept flexible during the minimization process and dynamics simulation. The isomalathion enantiomers are in ball-and-stick format while the BCholE residues are displayed as sticks. Colors were assigned to atoms as follows: C = black, H = green, N = blue, O = red, P = purple, S = yellow. Only the catalytic triad and active site residues in close proximity to isomalathion are shown. The catalytic Ser 194 is located directly below the isomalathion phosphorus atom. Diethyl thiosuccinate is predicted to be positioned apically to the active site Ser 194 for (1*R*,3*R*)-isomalathion. In contrast, thiomethyl is predicted to be positioned apically to Ser 194 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.

Table 1. Ratios of k_i Values for the Inhibition of AChE, BChE, and CholE 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*)
EBChE	11.9 (± 0.4)	1.60 (± 0.09)	8.65 (± 1.21)	7.46 (± 0.51)	5.41 (± 0.78)	1.38 (± 0.20)
HBChE	10.5 (± 0.6)	1.40 (± 0.10)	4.50 (± 0.40)	4.47 (± 0.43)	3.20 (± 0.25)	2.33 (± 0.18)
BCholE	68.6 (± 3.1)	1.28 (± 0.10)	19.3 (± 2.0)	53.6 (± 5.3)	15.1 (± 1.8)	3.55 (± 0.43)
HEAChE[c]	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[c]	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[d]	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 at positions 1 and 3, respectively. [b] Values shown are mean ± SE. SE calculated using standard rules of error propagation for quotients (*36*). [c] Human and bovine erythrocyte AChE (*37*). [d] Rat brain AChE (*6*).

versus (1*S*,3*S*)-enantiomers with a ratio of 68.6 for k_i values, which is the largest number reported to date for α/β-hydrolases. Although, it allows accommodation of bulky lipid and cholesterol esters, the active site of CholE is highly stereoselective, as revealed by the isomalathion probes.

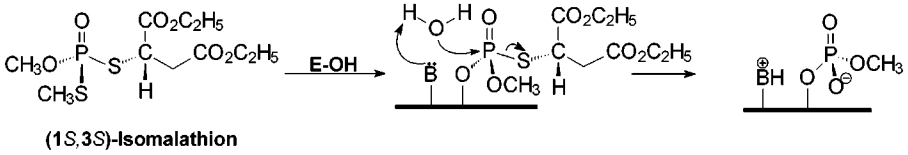
The catalytic triad in the active site of serine proteases bears a mirror-image relationship to that of *Torpedo* AChE (*2*, *3*). Accordingly, it has been found that α-chymotrypsin has reversed enantioselective preference for chiral phosphonates and phosphonothiolates compared to two fungal lipases (*33*, *34*). Unfortunately, none of the stereoisomers of isomalathion was a potent inhibitor of the serine proteases used in this study. Because of this noninhibition, a comparison of stereoselectivity between serine esterases and proteases could not be made. However, it is noteworthy that there is such a pronounced difference in sensitivity to inhibition by isomalathions between these two categories of serine hydrolases.

Although BChE and CholE were inactivated by the isomers of isomalathion in the same order of potency as observed for AChE, these enzymes displayed less preference for the (1*R*,3*R*)- versus (1*R*,3*S*)-diastereomer compared to AChE (Table 1). Apparently for (1*R*)-isomers, the asymmetry at the carbon atom does not affect the accommodation of isomalathion in the active site of BChE and CholE as much as it does for AChE. This suggests that the regions of the catalytic pockets of these enzymes that interact with the diethyl thiosuccinate moiety of (1*R*)-isomalathion are less discriminatory than the corresponding components of the active site of AChE.

With the exception of the (1*R*,3*R*)/(1*R*,3*S*) ratios, the k_i ratios for BChE are similar to those for AChE (Table 1). For both of these enzymes, altering isomalathion stereochemistry resulted in comparable changes with respect to inhibitory potency. This suggests that these two enzymes have similar active site topology. In contrast, k_i ratios for BCholE were profoundly different from those for AChE and BChE with the exception of the (1*R*,3*R*)/(1*R*,3*S*) and (1*S*,3*R*)/(1*S*,3*S*) ratios. This indicates that the active site of CholE may contain distinct structural features distinguishing it from the catalytic pockets of cholinesterases. Changing the stereochemistry at the phosphorus atom while holding asymmetry constant at the carbon produced ratios of 19.3 and 53.6 for the *R* and *S* configurations at carbon, respectively, thus demonstrating the remarkable stereoselectivity of this enzyme.

As shown in Table 1, the largest k_i ratios for BChE and CholE resulted from a change in stereochemistry at the phosphorus atom, i.e., (1*R*)- to (1*S*)-isomalathions, for diastereomeric and enantiomeric pairs. It is important to note that the k_i is a function of the affinity of the inhibitor for the enzyme and the rate of phosphorylation (*26*, *35*). Such a large contrast in the k_i values between the (1*R*)- and (1*S*)-isomalathions may simply indicate that the enzymes have a greater affinity for one set of isomers. Another possibility is that the inhibitory reaction of these enzymes with (1*R*)- and (1*S*)-isomalathions involves sufficiently different orientations of each set of isomers in the respective active sites to favor loss of different primary leaving groups, which would be re-

Scheme 1. Inhibition of AChE by (1*S*,3*S*)-isomalathion with subsequent aging (*38*)



(1*S*,3*S*)-Isomalathion

flected in different initial affinities and subsequent phosphorylation rates.

The measured k_3 values indicate a choice between the alternate hypotheses listed above, namely, that inactivation of BChE and CholE by (1*R*)- versus (1*S*)-stereoisomers proceeds with different primary leaving groups, yielding distinct inhibitory adducts. As for AChE (*10*, *11*), BChE and CholE inhibited by (1*R*)-isomalathion readily reactivated, but either enzyme inactivated by the (1*S*)-isomers was refractory toward reactivation. A >5-fold difference was observed in k_3 values for BChE and CholE modified by (1*R*)- versus (1*S*)-isomalathions, suggesting that each set of isomers produces a different inhibitory adduct. BChE and CholE modified by (1*R*,3*R*)- and (1*R*,3*S*)-diastereomers had k_3 values that were not significantly different, implying that these two isomers generate the same organophosphorylated protein with identical stereochemistry in the OP moiety that is covalently bonded to the active site serine. If diethyl thiosuccinate were the primary leaving group, then an *O*,*S*-dimethyl phosphate adduct with the same stereochemistry should be produced in each case. It is possible that the (1*R*)- and (1*S*)-stereoisomers produce different enantiomers of the *O*,*S*-dimethyl phosphate adduct, and this accounts for the discrepancy in measured rates of reactivation. However, studies using the configurationally equivalent enantiomers of isoparathion methyl (*10*, *11*) and peptide mass mapping with mass spectrometry (*38*) have demonstrated that this is not the case for AChE, and that a shift in primary leaving group preference occurs for enzyme inactivated by (1*R*)- versus (1*S*,3*S*)-isomers.

Interestingly, a fundamental difference in reactivation appears to exist for enzyme inactivated by (1*S*,3*R*)- versus (1*S*,3*S*)-isomalathion, indicating an influence of the stereochemistry at the chiral carbon on the reactivation. Whereas a finite k_3 for spontaneous reactivation could be measured for HBChE and BCholE inhibited by (1*S*,3*R*)-isomer, reactivation could not be detected for these enzymes modified by (1*S*,3*S*)-isomalathion. Furthermore, HBChE modified by this stereoisomer could not be reactivated even in the presence of 500 μM 2-PAM. This result indicates that the shift in primary leaving group preference between (1*R*)- and (1*S*)-isomers may be occurring to a greater degree for the (1*S*,3*S*)-compound than for its (1*S*,3*R*)-diastereomer. Moreover, AChE modified by (1*S*,3*S*)- but not (1*S*,3*R*)-isomalathion aged at a rate too rapid for measurement by conventional techniques (*11*).

Results of the molecular modeling experiment for (1*R*,3*R*)- and (1*S*,3*S*)-isomalathion in the active site of CholE lend support to the hypothesis of a mechanistic shift between the (1*R*,3*R*)- and (1*S*,3*S*)-isomers. Each enantiomer adopted a different orientation in the catalytic pocket, suggesting a difference in primary leaving groups. For the (1*R*,3*R*)-isomer, the diethyl thiosuccinate moiety was positioned as the primary leaving group. On the other hand, docking experiments of (1*S*,3*S*)-isomalathion in the active site of CholE predicted thiomethyl to be the primary leaving group. The primary leaving group for the reaction of AChE with (1*S*,3*S*)-isomalathion has been demonstrated to be the thiomethyl (*10*, *11*, *38*), in accord with results described in this paper.

For both (1*R*,3*R*)- and (1*S*,3*S*)-enantiomers, the methoxy group is proximal to Trp 227, Phe 324, and Phe 393. These aromatic residues might constitute an acyl binding pocket that precludes efficient accommodation of the thiomethyl or diethyl thiosuccinate groups.

The aging reaction of AChE modified by OP compounds is thought to proceed via dealkylation of the OP adduct in an S_N1 manner with formation of a carbocation on the leaving alkyl moiety (*39*, *40*). Studies have shown that this appears to be the case for organophosphorylated BChE as well (*41*, *42*). In contrast, recently generated kinetic and mass spectral evidence demonstrate that AChE inhibited by (1*S*,3*S*)-isomalathion undergoes aging via an S_N2 mechanism as shown in Scheme 1 with loss of diethyl thiosuccinate as the secondary leaving group (*38*). Analysis of k_i and k_3 values associated with BChE and CholE inhibited by isomalathion isomers taken together with the results of molecular modeling experiments reported in this paper suggest that this unique mechanism of nonreactivation following inhibition by (1*S*,3*S*)-isomalathion may be conserved for these α/β-hydrolases as well.

In summary, the four stereoisomers of isomalathion were used as chiral probes to study the active sites of BChE and CholE. The hypothesis was tested that these α/β-hydrolases in addition to AChE are stereoselectively inhibited by isomalathion with a shift in primary leaving group preference between (1*R*)- and (1*S*,3*S*)-isomers. EBChE, HBChE, and BCholE were inactivated by the stereoisomers with an order of potency previously seen for mammalian AChE (*6*, *7*) but with less preference for (1*R*,3*R*)- versus (1*R*,3*S*)-diastereomers. For the first time, it was demonstrated that CholE has a stereochemical preference for an OP inhibitor, and chirality at both phosphorus and carbon atoms influenced k_i values. Both BChE and CholE modified by isomalathion reactivated with a pattern similar to that of AChE inactivated by the four isomers (*10*, *11*). Molecular modeling experiments predicted that the primary leaving group for the inhibitory reaction of BCholE with (1*R*,3*R*)-isomalathion is diethyl thiosuccinate, but for the reaction of the enzyme with the (1*S*,3*S*)-enantiomer, it is thiomethyl. Interestingly, the kinetic data and results of the molecular modeling experiments suggest that the mechanism of inhibition and aging for AChE with (1*S*,3*S*)-isomalathion as shown in Scheme 1 (*38*) appears to be conserved for BChE and CholE. Work is in progress to identify the adduct(s) formed by each stereoisomer in the inhibitory reaction of isomalathion with EBChE using peptide mass mapping with mass spectrometry. When the crystal structure of BChE is available, molecular modeling experiments of isomalathion with BChE would lend further insight into the proposed stereochemically de-

termined shift in primary leaving group that appears thus far to be conserved among the α/β-hydrolases similar to AChE.

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