Organophosphorus Acid Anhydride Hydrolase Activity in Human Butyrylcholinesterase: Synergy Results in a Somanase

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ABSTRACT: Organophosphorus acid anhydride (OP) "nerve agents" are rapid, stoichiometric, and essentially irreversible inhibitors of serine hydrolases. By placing a His near the oxyanion hole of human butyrylcholinesterase (BChE), we made an esterase (G117H) that catalyzed the hydrolysis of several OP, including sarin and VX [Millard et al. (1995) *Biochemistry 34*, 15925−15930]. G117H was limited, however, because it was irreversibly inhibited by pinacolyl methylphosphonofluoridate (soman); soman is among the most toxic synthetic poisons known. This limitation of G117H has been overcome by a new BChE (G117H/E197Q) that combines two engineered features: spontaneous dephosphonylation and slow aging (dealkylation). G117H/E197Q was compared with the single mutants BChE G117H and E197Q. Each retained cholinesterase activity with butyrylthiocholine as substrate, although k_{cat}/K_m decreased 11-, 11- or 110-fold for purified G117H, E197Q, or G117H/E197Q, respectively, as compared with wild-type BChE. Only G117H/E197Q catalyzed soman hydrolysis; all four soman stereoisomers as well as sarin and VX were substrates. Phosphonylation and dephosphonylation reactions were stereospecific. Double mutant thermodynamic cycles suggested that the effects of the His and Gln substitutions on phosphonylation were additive for P_SC_R or P_RC_R soman, but were cooperative for the P_SC_S stereoisomer. Dephosphonylation limited overall OP hydrolysis with apparent rate constants of 0.006, 0.077, and 0.128 min^{-1} for the $P_{R/S}C_R$, P_SC_S, and P_RC_S soman stereoisomers, respectively, at pH 7.5, 25 °C. We conclude that synergistic protein design converted an archetypal "irreversible inhibitor" into a slow substrate for the target enzyme.

Serum butyrylcholinesterase (BChE[1] ; EC 3.1.1.8) parallels synaptic acetylcholinesterase (AChE; EC 3.1.1.7) in primary amino acid sequence, deduced secondary structure, and active site chemistry; the two enzymes also have overlapping specificity for substrates and inhibitors (reviewed in ref 3). The crystalline protein structure and the main purpose of AChE in cholinergic neurotransmission are well resolved, while the tertiary structure and natural function of BChE are unknown. Both AChE and BChE are like "B-type" proteases because they are susceptible to rapid phosphylation[2] of an active site Ser by organophosphorus acid anhydride (OP) inhibitors (reviewed in refs 4 and 5). The OP reaction with serine hydrolases serves as a classic example of an "irreversible inhibitor" (*6*). Our goal is to modify human BChE so that it rapidly and spontaneously dephosphylates to catalyze inhibitor hydrolysis. Engineered OP hydrolase (OPAAH) activity may find future application as a "catalytic scavenger" to protect populations at risk for exposure to OP pesticides or chemical warfare agents, or as an agricultural tool to protect beneficial predators of undesirable insects.

Limited OPAAH activity was developed in human BChE by replacing Gly117(*119*),[3] believed to participate in the "oxyanion hole", with histidine (*7*−*9*). The resultant enzyme, G117H, retained esterase activity but also catalyzed the hydrolysis of sarin and VX (highly toxic nerve agents). Faster catalytic rates were found for hydrolysis of OP with two alkoxy substituents at the phosphorus, for example, diisopropyl phosphorofluoridate (DFP), diethoxy phosphorylthiocholine (Echothiophate), or diethyl *O*-(4-nitrophenyl)-phosphate (paraoxon) (Figure 1). The only OP tested that G117H could not hydrolyze was soman.

Soman is a remarkably potent nerve agent that exploits 60−70% of the natural catalytic efficiency of AChE to

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[1] Abbreviations: 2-PAM, pyridine-2-aldoxime methiodide; AChE, acetylcholinesterase (EC 3.1.1.7); BChE, butyrylcholinesterase (EC 3.1.1.8); BTCh, butyrylthiocholine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EMP-enzyme, *O*-ethyl methylphosphonylated-enzyme; i-PrMP-enzyme, *O*-isopropyl methylphosphonylated-enzyme; OP, organophosphorus acid anhydride inhibitor; OPAAH, organophosphorus acid anhydride hydrolase; sarin, *O*-isopropyl methylphosphonofluoridate; SE, standard error; soman, *O*-pinacolyl methylphosphonofluoridate; PMP-enzyme, *O*-pinacolyl methylphosphonylated-enzyme; VX, *O*-ethyl *S*-(diisopropylaminoethyl) methylphosphonothiolate; WT, wild-type, natural human BChE.

[2] "Phosphylated"denotes phosphorylation and phosphonylation reactions without distinction (*1*).

[3] By convention, the italicized number in parentheses following an amino acid residue refers to the homologous amino acid position in the sequence of *Torpedo californica* AChE. For example, BChE S198-(*200*) corresponds to AChE S200.

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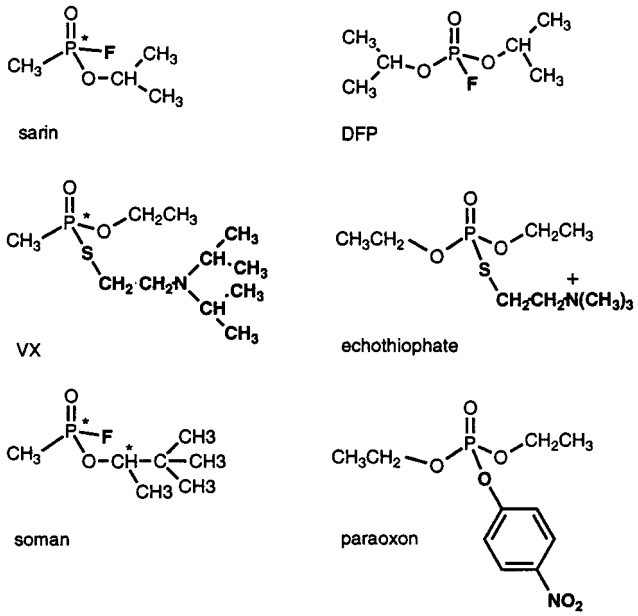


FIGURE 1: OP inhibitors converted to substrates. Each may be considered as an anhydride of two acids: (1) an alkyl alkylphosphonic or dialkyl phosphoric acid, and (2) HX (where X is the leaving group in Scheme 1). The acids are the products of the overall reaction of OPs with the His117 enzymes (Scheme 1); this is the basis for adopting the nomenclature "organophosphorus acid anhydride hydrolase activity". Asterisks denote chiral centers and the leaving groups are shown with bold text.

achieve rapid phosphonylation (*10*); inhibited enzyme then catalyzes dealkylation of the pinacolyl group (*11-13*). Dealkylation is one pathway of "aging" because it results in an irreversibly inhibited enzyme that resists all known forms of chemical reactivation or medical treatment (reviewed in refs 14 and 15). We hypothesized that BChE G117H failed to hydrolyze soman because the engineered dephosphonylation rate remained much slower than the dealkylation reaction of soman aging. For comparison, the aging rate constant for *O*-pinacolyl methylphosphonylated (PMP)-BChE is approximately 0.07 min^{-1} at pH 7.5, 25 °C (*16*), whereas G117H dephosphonylation rate constants are only 0.004−0.007 min^{-1} (*7*). As a test of this hypothesis, a second modification was attempted to slow aging of G117H and thereby to confer somanase activity.

MATERIALS AND METHODS

Construction and Expression of the Mutants. Starting from the solved structure of AChE (*17*), homology-based computer models of human BChE were used to design mutations (*18*) (Figure 2). Three variants of human BChE were made: (1) Gly117(*119*) was changed to His (G117H); (2) Glu197(*199*) was changed to Gln(E197Q); or (3) the two were changed together (G117H/E197Q).

Mutants were made from cDNA for the human wild-type (WT) BChE gene (*19*). Site-directed mutagenesis, cloning, and sequencing were accomplished as described (*9*). Chinese hamster ovary cells (CHO−K1; American Type Culture Collection, No. CCL 61, Rockville, MD) were transfected by calcium phosphate coprecipitation, and the recombinant enzyme was collected into serum-free medium (Ultraculture 12-725B, BioWhittaker, Walkersville, MD). Medium was

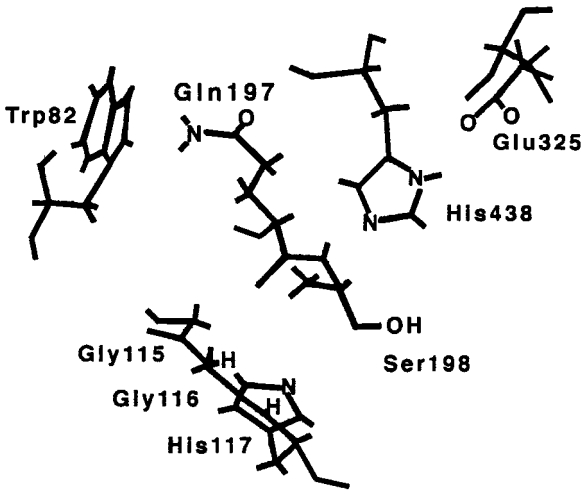


FIGURE 2: Relative positions of amino acids altered in this study. A computer model of the active site of human BChE G117H/E197Q is shown. The active site triad is Ser198, His438, and Glu325. The altered trimethyl subsite includes Gln197 and Trp82; the presumed "oxyanion hole"includes main chain amide hydrogen atoms (depicted as H) of Gly116 and His117 as well as the His117-$N^{\epsilon 2}$. For orientation, the point-to-point distances from the Ser198-O^{γ} to the His117-$N^{\epsilon 2}$ and Gln197-$N^{\epsilon 2}$ are 7 and 9 Å, respectively. The α carbons of His117 and Gln197 are separated by 10 Å in this model.

concentrated by ultrafiltration with Amicon PM10 membranes (Amicon Inc., Beverly, MA), sterile-filtered, and assayed for esterase activity.

Substrate Kinetics. Kinetic parameters were calculated from the change in optical density at 412 nm for solutions of recombinant enzyme and butyrylthiocholine iodide (BTCh; Sigma, St. Louis, MO) in 0.067 M Na/K phosphate buffer containing 2 mM DTNB, pH 7.5, 25 °C (*20*).

Catalyzed hydrolysis of BTCh was fit to a model for "substrate activation" that assumes binary (ES) and ternary (SES) enzyme−substrate complexes form, both capable of yielding products, but the latter at a greater rate than the former (reviewed in ref 21):

$$v = \left(\frac{1 + b[\mathrm{BTCh}]/K_{SS}}{1 + [\mathrm{BTCh}]/K_{SS}}\right)\left(\frac{V_{max}}{1 + K_m/[\mathrm{BTCh}]}\right) \qquad (1)$$

where [BTCh] is the substrate concentration (ranges given in Table 1); K_m is the Michaelis constant; K_{SS} is the substrate activation constant; V_{max} is the maximum velocity for the ES complex; and *b* is the ratio of catalytic rate constants for the SES and ES complexes.

For determination of the molar catalytic activity, k_{cat}, recombinant enzymes were purified by an established three-step procedure: (1) concentration; (2) iterative affinity chromatography using procainamide-Sepharose; and (3) ion-exchange chromatography using DEAE-Sephacel (Pharmacia, Piscataway, NJ) *(9, 22)*. The purified enzyme concentrations were determined from ultraviolet absorptivity; human BChE has a molar absorption coefficient of 1.53×10^5 $M^{-1}cm^{-1}$ at 280 nm (*22*). The k_{cat} values for E197Q and G117H/E197Q were calculated by converting V_{max} (eq 1) to the concentration of substrate hydrolyzed per minute (*20*) and dividing by the enzyme concentration. The k_{cat} for 99% pure G117H with BTCh was reported previously (*9*).

Table 1: Comparison of Cholinesterase Activity[a]

	[BTCh] (mM)	K_m (mM)	K_{SS} (mM)	b	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} min^{-1})
WT	0.007−10	0.025 ± 0.006	0.9 ± 0.1	3.1	24 000[b]	1×10^9
E197Q	0.007−85	0.078 ± 0.005	23 ± 4	1.7	7000 ± 1000	9×10^7
G117H	0.007−30	0.10 ± 0.02	0.8 ± 0.1	2.7	9000[b]	9×10^7
G117H/E197Q	0.015−85	0.28 ± 0.02	120 ± 20	5.1	2600 ± 400	9×10^6

[a] The substrate was BTCh. Measured in 0.067 M phosphate buffer, pH 7.5, 25 °C. Mean ± standard error (SE) were from nonlinear regression curve fits directly to eq 1 (error calculation reviewed in ref 74). [b] Data of Lockridge et al. (*9*).

Table 2: Comparison of Soman Inhibition Constants[a]

soman	BChE	(n)	[soman] (μM)	k_i ± SE (μM^{-1} min^{-1})
P_SC_R	WT	3	0.02−0.2	5 ± 1
	E197Q	6	0.5−3	0.23 ± 0.02
	G117H	9	0.2−3	0.548 ± 0.005
	G117H/E197Q	5	4−9	0.025 ± 0.002 (0.03)[b]
P_RC_R	WT	5	0.04−0.2	6 ± 2
	E197Q	3	1−10	0.35 ± 0.05
	G117H	4	8−80	0.033 ± 0.004
	G117H/E197Q	6	8−200	0.0021 ± 0.0002
P_SC_S	WT	3	0.02−0.06	40 ± 5
	E197Q	9	0.02−0.2	5 ± 2
	G117H	8	3−60	0.019 ± 0.008
	G117H/E197Q	6	3−30	0.036 ± 0.009 (0.05)[b]

[a] Measured in 0.067 M phosphate buffer, pH 7.5, 25 °C using (n) different concentrations of soman in the range shown. [b] k_i values in parentheses were calculated from eq 5, which considers competition from spontaneous reactivation of G117H/E197Q.

Inhibitor Kinetics. Racemic sarin, soman, or VX were obtained from the Edgewood Research, Development, and Engineering Center (Aberdeen Proving Ground, MD) and determined to be >98% pure by gas chromatography. Resolved stereoisomers of soman[4] were obtained from Prins Maurits Laboratory TNO (Rijswijk, The Netherlands). The extent of fluoride-catalyzed racemization at the chiral phosphorus for each soman stereoisomer was measured before use (*23*, *24*). *Sarin, soman, and VX are extremely toxic and were handled according to established safety regulations governing chemical warfare materials.*

Pseudo-first-order rates for progressive inhibition (ρ) were determined at different concentrations of soman (ranges given in Table 2) in 0.067 M Na/K phosphate buffer, pH 7.5, 25.0 °C. Residual cholinesterase activity was measured using a standard assay of 1 mM BTCh for WT, G117H, or E197Q and 7.5 mM BTCh for G117H/E197Q. At least two separate measurements of ρ were made for each soman concentration by direct nonlinear curve fit of residual esterase activity to a single-exponential decay (reviewed in ref 25). Concentrations of the soman stereoisomers were corrected 5−11% for the measured degree of racemization at the chiral phosphorus. To minimize error from spontaneous reactivation, soman concentrations were as high as was practicable (*26*).

Apparent Interaction Energies. For BTCh hydrolysis, k_{cat}/K_m is an apparent second-order rate constant for enzyme *acetylation* that is independent of either enzyme or substrate concentration (reviewed in ref 27). The k_i for soman inhibition is an apparent second-order rate constant for the *phosphonylation* reaction (reviewed in ref 25). From these "specificity constants" for enzyme acetylation and phosphonylation, we calculated relative apparent interaction energies, ΔG_{app} (*28*):

$$\Delta G_{app} = -RT \ln\left(\frac{k'_{II}}{k_{II}}\right) \quad (2)$$

where k_{II} and k_{II}' are the relevant specificity constants for reactions of an enzyme and a modified form of the enzyme (mutation), respectively; T is thermodynamic temperature; and R is the gas constant, 1.987 cal/(mol·K).

Double Mutant Cycles. Interdependence of the altered side chains (His117 and Gln197) also was tested by constructing double-mutant, thermodynamic cycles (*29*, *30*). Four separate cycles connected the relative ΔG_{app} values (eq 2) for the reactions of the enzymes with BTCh or with each of the three soman stereoisomers (Table 3). For each cycle, we calculated a thermodynamic "coupling energy", defined as $|\Delta G1\text{-}\Delta G1'|$ (reviewed in ref 31).

Spontaneous Reactivation. Enzyme was inhibited to 4−14% residual activity, and then excess inhibitor was removed by gel filtration under a centrifugal force of 1000 *g* at 4 °C (*32*). Gel filtration columns (Sephadex G-25, fine; Boehringer Mannheim, Indianapolis, IN) contained 0.067 M Na/K phosphate buffer, pH 7.5. Reactivation was followed with time by diluting less than 10 μL of the reactivating sample (column eluate) into 1 mL of phosphate buffer, pH 7.5, 25 °C, containing BTCh and DTNB.

Spontaneous reactivation of enzyme in aqueous solution was treated as a first-order process:

$$v_t = V_\infty[1 - e^{-k_{obs}t}] \quad (3)$$

where k_{obs} is the observed reactivation rate constant; v_t is initial velocity at time t (zero time was defined as entry of the enzyme−inhibitor mix into the G-25 column); and V_∞ is the maximum velocity achieved after reactivation. The initial velocity of reactivating sample in this assay is directly proportional to the concentration of free enzyme.

For biphasic reactivation, data were fit to an equation that included two rate constants, $k_{obs}(1)$ and $k_{obs}(2)$, and the amplitudes of each exponential phase, A_1 and A_2:

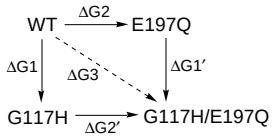
$$v = A_1(1 - e^{-k_{obs}(1)t}) + A_2(1 - e^{-k_{obs}(2)t}) \quad (4)$$

To determine if spontaneous reactivation introduced significant error into the observed inhibition rate constants, we recalculated k_i for some OP−enzyme complexes using a model that includes the reactivation rate constant (*27*):

$$(v_o - v_t) = [(k_i v_o i)/(k_i i + k_{obs})][1 - \exp(-(k_i i + k_{obs})t)] \quad (5)$$

[4] The four soman stereoisomers were resolved optically. Absolute configuration of the chiral carbon was known from the synthetic precursors: C(−) = C_R and C(+) = C_S; provisional assignment for the chiral phosphorus was made by conventional, chemical correlation with solved structures: P(−) = P_S and P(+) = P_R (*2*).

Table 3: Double Mutant Cycles of Apparent Interaction Energies (ΔG_{app})[a]



enzymes compared	BTCh (k_{cat}/K_m) (kcal/mol)	P_SC_R-soman k_i (kcal/mol)	P_RC_R-soman k_i (kcal/mol)	P_SC_S-soman k_i (kcal/mol)
$\Delta G1$ WT → G117H	+1.4 (1.1−1.7)	+1.3 (1.2−1.4)	+3.1 (2.8−3.3)	+4.5 (4.3−4.9)
$\Delta G1'$ E197Q → G117H/E197Q	+1.4 (1.1−1.6)	+1.3 (1.2−1.4)	+3.0 (2.9−3.2)	+2.9 (2.5−3.3)
$\Delta G2$ WT → E197Q	+1.4 (1.2−1.7)	+1.8 (1.7−2.0)	+1.7 (1.4−1.9)	+1.2 (1.0−1.6)
$\Delta G2'$ G117H → G117H/E197Q	+1.4 (1.2−1.6)	+1.8 (1.7−2.0)	+1.6 (1.5−1.8)	−0.4 (−0.8−0)
$\Delta G3$ WT → G117H/E197Q	+2.8 (2.5−3.1)	+3.1 (3.0−3.3)	+4.7 (4.4−4.9)	+4.2 (3.9−4.4)
$\lvert\Delta G1-\Delta G1'\rvert$[b]	0 (0−0.6)	0 (0−0.2)	0.1 (0−0.4)	1.6 (1.2−2.4)

[a] Separate cycles were considered for each ligand; arrows in the diagram connect unmodified to modified enzyme at each stage of a cycle. Error ranges in parentheses were calculated from the SE of k_{cat}/K_m or k_i (Table 1 or 2, respectively). [b] Coupling free energy for each cycle (reviewed in ref 31).

Residual velocity, v_t, at time t, after mixing of enzyme and OP was fit directly to eq 5; k_{obs} is the reactivation rate constant (from eq 3); v_o is the initial velocity for the reaction of the uninhibited enzyme with BTCh; and i is the soman concentration.

Measurement of Soman Hydrolysis During Reactivation. In some experiments, soman hydrolysis was measured indirectly during the reactivation phase: (1) G117H/E197Q was inhibited to less than 14% residual activity with racemic soman; (2) excess inhibitor was removed by gel filtration, and fresh soman (1 μM) was added to the column eluate; and (3) changes in the concentration of soman were followed with time by incubating aliquots for 10 min with eel AChE (Sigma Chemical, St. Louis, MO) and measuring residual AChE activity in a standard acetylthiocholine assay (*33*). The purpose of adding fresh soman to the inhibited enzyme after gel filtration was to determine whether the observed spontaneous reactivation of G117H/E197Q resulted from OP hydrolysis or from the dissociation of noncovalently bound inhibitor. Hydrolysis should have decreased the soman concentration during G117H/E197Q reactivation. Measurement of soman concentration in this assay is based on the fact that soman rapidly and stoichiometrically inactivates eel AChE.

Aging. After inhibition with soman, some samples of G117H were incubated with 10 mM 2-PAM for 24 h in phosphate buffer, pH 7.5, 25 °C. 2-PAM has been shown to reactivate phosphonylated BChE before, but not after, the enzyme undergoes aging by dealkylation.

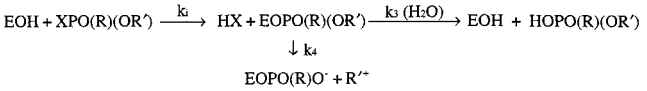
Aging of G117H/E197Q was assessed after completion of the spontaneous reactivation experiments. Final cholinesterase activity achieved was compared among samples incubated either with phosphate buffer (control) or with OP for different lengths of time (2−48 h) at pH 7.5, 25 °C.

RESULTS

Cholinesterase Activity. G117H, E197Q, and G117H/E197Q were secreted as active esterases by the CHO K1 cells. Final elution profiles from DEAE-Sephacel and the gel electrophoresis pattern of purified enzymes showed that E197Q was greater than 95% pure and G117H/E197Q was 80−90% pure.

Both the natural affinity and catalytic power of BChE were weakened by replacement of Glu197. The specificity constant (k_{cat}/K_m) decreased by about 10-fold for G117H or E197Q and about 100-fold for the double mutant (Table 1). Although substrate activation was observed for all three mutants with cationic BTCh ($b > 1$; Table 1), the affinity constant for the ternary complex (K_{ss} in eq 1) increased by 25- or 130-fold for E197Q or G117H/E197Q, respectively, as compared with WT (Figure 3).

Scheme 1[a]



[a] EOH is active enzyme; XPO(R)(OR′) is an OP inhibitor with leaving group X; k_i is the apparent, bimolecular rate constant for overall phosphonylation (neglects reversible complexes); EOPO(R)(OR′) is phosphonylated, inactive enzyme; k_3 is the dephosphonylation rate constant; HOPO(R)(OR′) is hydrolyzed inhibitor; k_4 is the aging rate constant; $EOPO(R)O^-$ is aged enzyme; and R'^+ is the theoretical product of dealkylation (a carbonium that rapidly rearranges to alcohol and alkene products (*52*).

Inhibition Rate Constants. Inhibition by soman was consistent with Scheme 1. Rapid phosphonylation precluded accurate measurement of inhibition constants governing intermediate complexes (reviewed in refs 14 and 25). The high soman concentrations and short incubation times used to determine k_i apparently were adequate to avoid large errors from spontaneous reactivation (results of eq 5 in Table 2).

The k_i values gauged the relative reactivity of each soman stereoisomer with the mutant enzymes. Differences in k_i among the soman isomers showed that G117H, E197Q, and G117H/E197Q were stereoselective. As reported previously for BChE from horse or human serum (*16*, *34*), P_SC_S-soman was the most potent inhibitor of WT or E197Q. The G117H substitution, however, altered BChE stereospecificity; P_SC_R-soman was a better inhibitor of G117H than was P_SC_S-soman, as judged by k_i (Table 2).

Apparent Interaction Energies. Relative to WT, each of the three mutants showed unfavorable ΔG_{app} with BTCh or soman. The E197Q substitution penalized reactions with BTCh or the three soman stereoisomers to approximately the same extent (1−2 kcal/mol; Table 3). The G117H substitution also was unfavorable by 1−2 kcal/mol for reaction with BTCh or P_SC_R soman, but G117H penalized phosphonylation with P_RC_R- or P_SC_S-soman to a greater extent (3−5 kcal/mol; Table 3). Apparently, G117H/E197Q

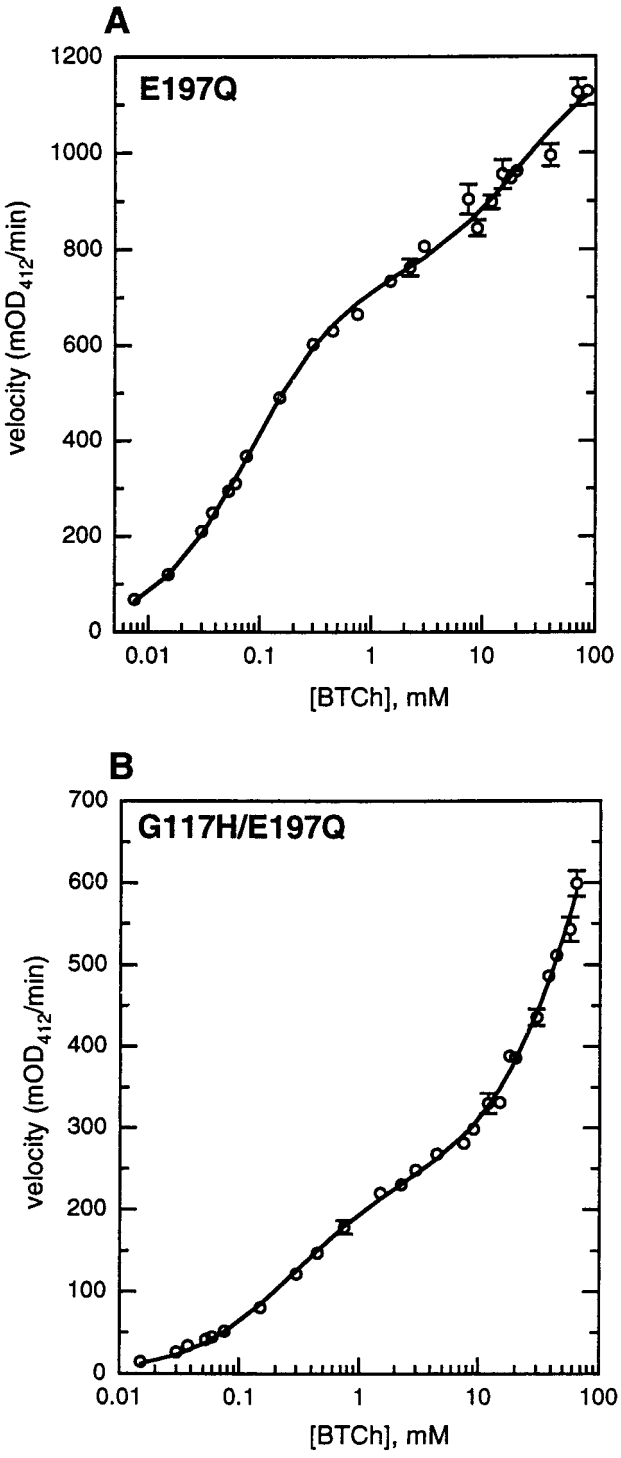


FIGURE 3: Cholinesterase activity of E197Q (panel A) and G117H/E197Q (panel B). The substrate was BTCh. Each point is the mean ± SE of 3−5 measurements. Some SE are smaller than the symbols used for the mean. The lines shown are nonlinear curve fits to eq 1. Comparable data for WT and G117H were published previously (*15*).

was more reactive with P_SC_S soman than was the single mutant G117H, accounting for the only favorable ΔG_{app} (negative sign) in Table 3. The ΔG_{app} values were not interpreted as binding energies, but were taken as an overall measure for the loss of WT specificity caused by each mutation in the reactions with BTCh or soman (*35*).

Double Mutant Cycles. The coupling energy for reaction with BTCh as well as those for phosphonylation with P_SC_R-

Table 4: Spontaneous Reactivation Rate Constants[a] ($\times 10^3$ min^{-1})

	G117H/E197Q	G117H	WT or E197Q
		Sarin	
$k_{obs(1)}$	62 ± 5	5 ± 2[b]	<0.05[c]
$k_{obs(2)}$	0.7 ± 0.2[d]		
		VX	
$k_{obs(1)}$	78 ± 9	7 ± 1[b]	<0.05
$k_{obs(2)}$	0.7 ± 0.2[d]		
		Soman	
P_SC_R	6.0 ± 0.6	<0.05	<0.05
P_RC_R	6.0 ± 0.1		
P_SC_S	77 ± 9	<0.05	<0.05
P_RC_S	128 ± 8		

[a] Measured in 0.067 M phosphate buffer, pH 7.5, 25 °C. Mean ± SE were from 3−7 separate reactivation experiments fit independently to eqs 3 or 4. [b] Data of Millard et al. (*7*). [c] Lower limit for detection of spontaneous reactivation was 0.05×10^{-3} min^{-1}. [d] Reactivation was biphasic under conditions described in the text. Rate constants were obtained using eq 4.

or P_RC_R-soman were not significantly greater than zero (Table 3). Zero coupling energy has been called "additivity" because the cycle balances exactly; the ΔG_{app} for G117H can be added to that for E197Q to arrive at the ΔG_{app} for G117H/E197Q (Table 3). This result meant that the effects of the altered side chains in the double mutant were independent of each other. Reaction with P_SC_S-soman, in contrast, showed a coupling energy of 1.6 kcal/mol (Table 3). Measurable coupling energy meant that the effects of His117 and Gln197 were apparently cooperative (*36*).

Spontaneous Reactivation. After phosphylation, BChE may either regain esterase activity (spontaneous reactivation) or undergo an internal dealkylation (one form of aging). Often these two reactions compete (Scheme 1). Only G117H/E197Q regained esterase activity after removal of excess soman (Figure 4). For pure stereoisomers of soman, spontaneous reactivation followed a single-exponential function (eq 3) for ≥80% of the reaction. Reactivation rate constants for PMP-G117H/E197Q depended upon the stereochemistry of soman (Table 4).

Spontaneous reactivation of racemic PMP-G117H/E197Q, *O*-ethyl methylphosphonylated (EMP)-G117H/E197Q, or *O*-isopropyl methylphosphonylated (i-PrMP)-G117H/E197Q was biphasic (Figures 4A, 5, and 6). The sum of the squares of the residuals for each nonlinear curve fit showed that the two exponential terms of eq 4 described reactivation after inhibition by racemic OP better than did eq 3.

To characterize the biphasic reactivation of G117H/E197Q further, rate constants were measured as a function of incubation time with VX (up to 48 h). VX was used for these experiments because of its prolonged stability in neutral, aqueous solutions (*37*). Short incubations of G117H/E197Q with VX (≤2 h) yielded biphasic reactivation curves; each rate constant, $k_{obs}(1)$ and $k_{obs}(2)$, accounted for an amplitude of approximately 50% of the total enzyme activity recovered. At 24 h incubation with VX, only about 25% reactivated at the faster rate; after 48 h in VX, essentially all of the EMP-G117H/E197Q reactivated at the slower rate (Figure 6).

Aging. Spontaneous reactivation of PMP-G117H was below detection after a 30-min incubation with soman (Figure 4B,C). The loss of esterase activity was attributed to aging because inhibited enzyme could not be reactivated by 20 h

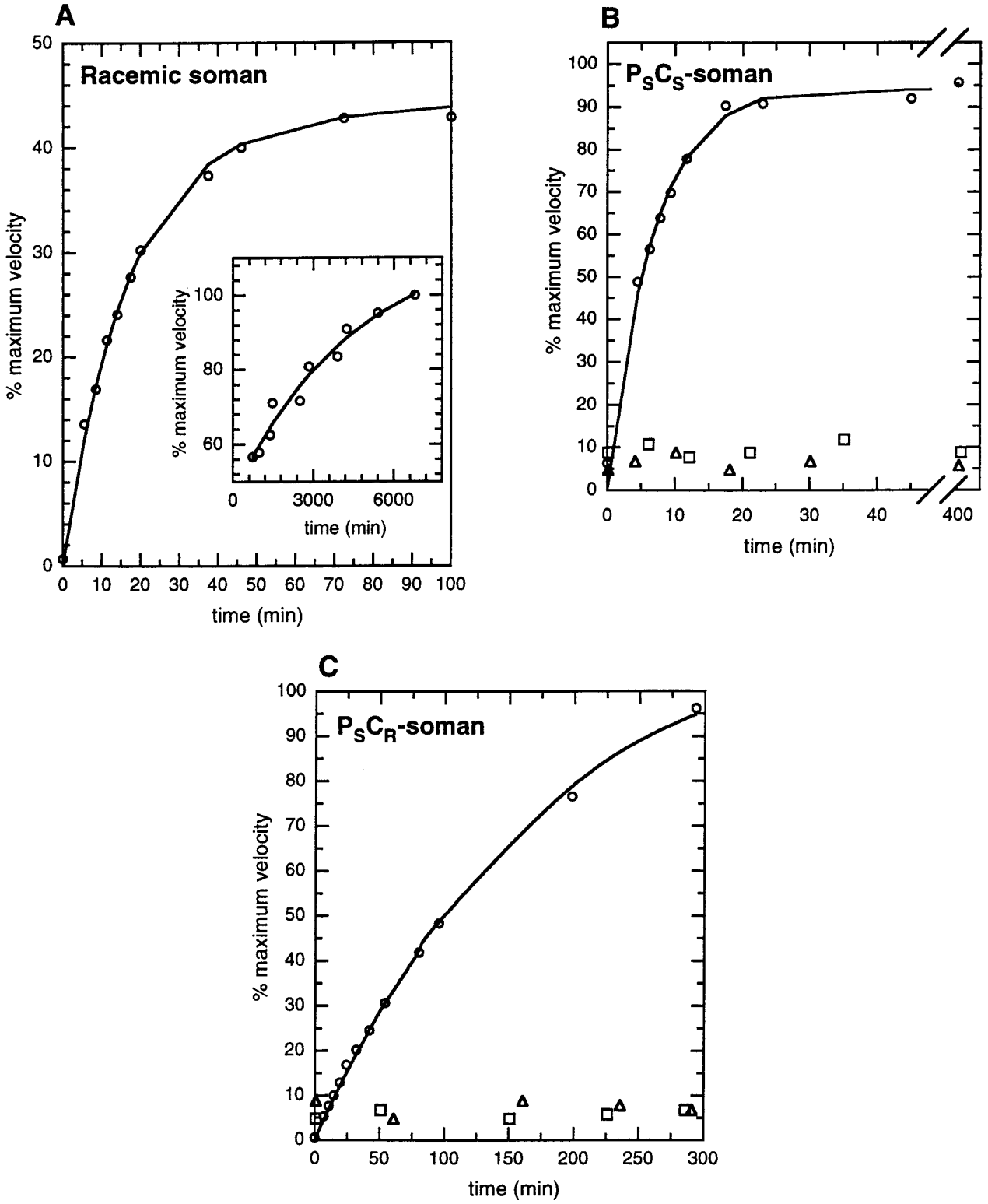


FIGURE 4: G117H/E197Q reactivates spontaneously after soman inhibition. Spontaneous reactivation of racemic PMP-G117H/E197Q was biphasic (panel A; the inset shows slower phase). Spontaneous reactivation of P_SC_S-PMP-G117H/E197Q (panel B; circles) or P_SC_R-PMP-G117H/E197Q (panel C; circles) each followed a single exponential. Neither G117H (triangles; panels B and C) nor E197Q (squares; panels B and C) showed measurable spontaneous reactivation. The "% maximum velocity" was based upon esterase activity of control enzyme incubated with buffer instead of OP, but otherwise treated identically. The lines for exponential reactivation are nonlinear curve fits to eqs 3 or 4. Representative experiments shown were in phosphate buffer, pH 7.5, 25 °C.

incubation with a large excess (10 mM) of 2-PAM in phosphate buffer, pH 7.5.

After removal of unbound soman by gel filtration, G117H/E197Q reactivated to >90% of the esterase activity of control enzyme incubated with buffer instead of OP (Figure 4). The total recovered esterase activity was not reduced by increasing the incubation time with OP (up to 24 h in racemic soman, or 48 h in racemic VX, at pH 7.5, 25 °C). Enzyme recovery after gel filtration was not quantitative, however, and the uncertainty among comparing eluates was as high

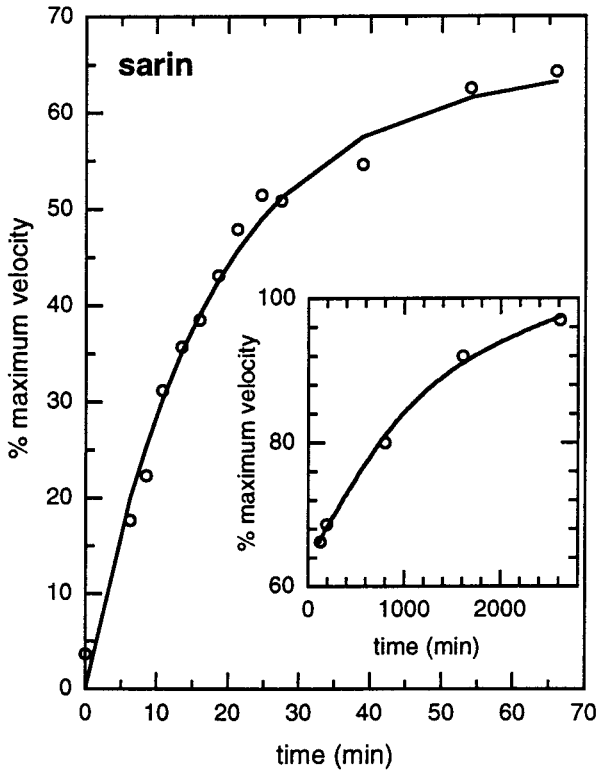


FIGURE 5: G117H/E197Q reactivates spontaneously after sarin inhibition. Spontaneous reactivation of racemic, i-PrMP-G117H/E197Q was biphasic (the inset shows slower phase). The "% maximum velocity"was based upon esterase activity of control enzyme incubated with buffer instead of OP, but otherwise treated identically. The lines for exponential reactivation are nonlinear curve fits to eqs 3 or 4. Representative experiment shown was in phosphate buffer, pH 7.5, 25 °C.

as 20%. Consequently, aging or other processes of "non-reactivatibility" (*38*) were not rigorously excluded. We conclude, however, that loss of G117H/E197Q activity from aging was insignificant in comparison with spontaneous reactivation under the conditions of the experiments (i.e., $k_3 > k_4$ in Scheme 1).

OPAAH Activity. Soman was hydrolyzed during reactivation of G117H/E197Q, as judged by the indirect eel AChE assay. Spontaneous reactivation, therefore, was not due to simple dissociation of a noncovalent enzyme-OP complex (*7*). Catalyzed hydrolysis of C_S-soman by G117H/E197Q was rapid enough to observe OPAAH activity on a minute time scale without removal of excess inhibitor. Incubation of G117H/E197Q with a 10-fold excess of soman resulted in an inhibited steady state that reversed with a rate constant of approximately 0.06 min^{-1} (Figure 7). Inhibited G117H/E197Q regained >90% of its original esterase activity. Spontaneous reversal of inhibition from steady state in the presence of excess inhibitor substantiated OPAAH activity. From the steady-state inhibition experiments, we estimated the ratio of k_3/k_2 (*27*):

$$v_{ss}/(v_o - v_{ss}) = k_3/(ik_i) + k_3/k_2 \qquad (6)$$

where v_o and v_{ss} are the uninhibited and steady-state BTCh velocities, respectively; i is soman concentration; k_i and k_3 are from Scheme 1; and k_2 is the phosphonylation rate constant. The ratio of k_3/k_2 for P_SC_S-soman was approximately 0.1. The rate-limiting step for overall OPAAH activity, therefore, was dephosphonylation, k_3.

Taking the observed reactivation rate constant as an upper limit for overall OPAAH activity of G117H/E197Q, the enzyme effected modest rate enhancements of ≤200-, 1400-, and 4700-fold above the reported aqueous solvolysis rate constants for the optimal stereoisomers of soman, sarin, and VX, respectively, at pH 7.5 (*37*, *39*, *40*).

Purification of OPAAH Activity. Only medium from cells transfected with the gene for G117H/E197Q contained measurable somanase activity (Figure 4). Furthermore, partial purification of G117H/E197Q did not remove the OPAAH activity. These results were consistent with the earlier conclusion that CHO−K cell-conditioned medium does not contain significant amounts of contaminating OPAAH activity (*9*).

DISCUSSION

The alkyl group(s) of OP inhibitors can interact with at least two active site domains in cholinesterase: (1) the trimethyl subsite (also called the "anionic" or "cation-π" site) and (2) the acyl pocket (reviewed in refs 41 and 42). The trimethyl subsite uses Coulombic binding forces, short-range dispersion forces, and the hydrophobic effect to complement the quaternary ammonium leaving group of natural substrates (*43*−*48*). Amino acid side chains of Glu199 and Trp84 contribute to the structure of the trimethyl subsite in AChE (*49*−*51*).

Aging of soman-inhibited cholinesterase results from rapid dealkylation of the pinacolyl group by a carbonium ion mechanism (*12*, *52*). The cationic leaving group during soman aging, therefore, is a choline analogue. Several independent approaches corroborate that the trimethyl subsite of the enzyme participates in rapid aging: (1) competitive, reversible inhibitors with quaternary nitrogen atoms slow aging of AChE or BChE (*11*, *53*, *54*); (2) a soman analogue with both a choline and a pinacolyl group ages 500-fold more slowly than does soman (*55*); (3) computer simulations predict that the soman pinacolyl group interacts with Glu199 (*56*, *57*); and (4) disruption of the trimethyl subsite by replacement of Glu199 or Trp84 reduces aging rates of PMP-AChE by 100−1000-fold (*58*−*60*). On the basis of a presumed role for the trimethyl subsite in the aging reaction, we replaced Glu197(*199*) in BChE G117H.

Mutants Are Resistant to Soman Inhibition. Overall soman phosphonylation rate constants were decreased for each of the three mutants (Table 2). The decrease in k_i for BChE E197Q was comparable with the 25-fold reduction in k_i reported for soman inhibition of the corresponding human AChE mutant, E202Q (*59*). The k_i for G117H inhibition by P_SC_S-soman decreased 2000-fold as compared with WT, yet neither enzyme-catalyzed measurable soman hydrolysis (Tables 2 and 4). This reinforces our earlier conclusion that placement of bulky side chains in the oxyanion hole of serine hydrolases generally confers "OP resistance," probably by sterically impeding phosphylation, but does not ensure OPAAH activity (*7*).

Somanase Activity. Addition of His117 and replacement of Glu197(*199*) worked synergistically to achieve soman hydrolysis. His117 alone was insufficient to prevent enzyme aging, as shown by a progressive inhibition with soman that was not reversed by oximes. Aging of G117H also was reported after inhibition with echothiophate (*9*). Replacement of Glu197(*199*) in human BChE or Glu202(*199*) in

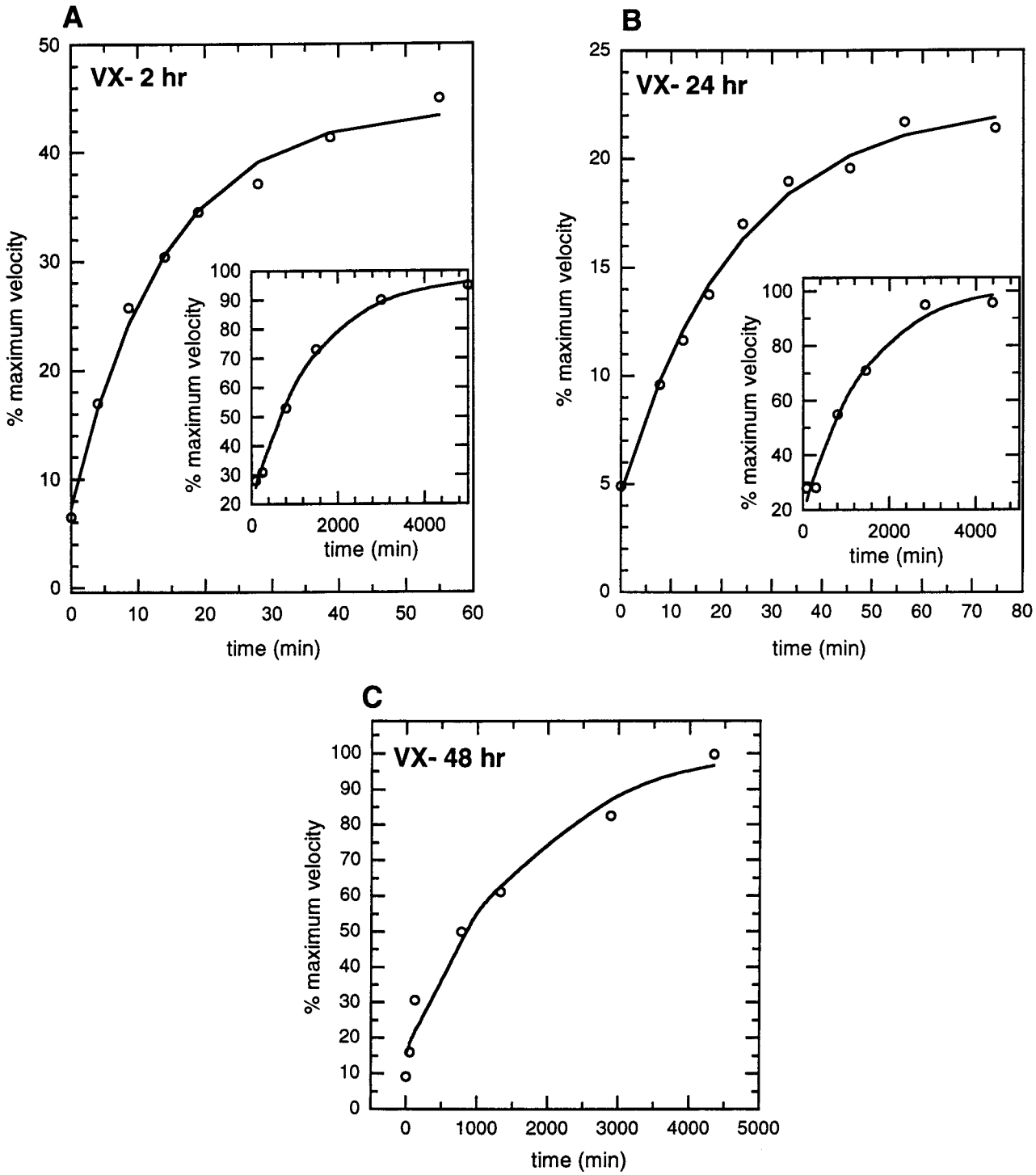


FIGURE 6: Time-dependent change in the spontaneous reactivation of EMP-G117H/E197Q. Spontaneous reactivation of racemic EMP-G117H/E197Q was biphasic after 2 h (panel A) or 24 h (panel B) incubation with VX. After 48 h incubation with VX (panel C) however, the EMP-G117H/E197Q reactivated at the rate constant observed for the "slow"phases of 2 or 24 h (inset of panels A and B). The "% maximum velocity" was based upon esterase activity of control enzyme incubated with buffer instead of OP, but otherwise treated identically. The lines for exponential reactivation are nonlinear curve fits to eqs 3 or 4. Representative experiments shown were in phosphate buffer, pH 7.5, 25 °C.

human AChE (*59*) slowed the aging reaction but did not result in OPAAH activity (Figure 4). Only G117H/E197Q catalyzed soman hydrolysis (Table 4) because it combined a very slow aging rate (E197Q) with enhanced dephosphonylation (G117H).

Previous studies of Glu199 or Trp84 mutants had to measure soman aging rates indirectly with oximes (*58*, *59*). Spontaneous reactivation of soman-inhibited G117H/E197Q, without the use of oximes, shows that disruption of the enzyme's trimethyl subsite directly affects the soman aging reaction. It follows that other alterations in this subsite of BChE G117H, for example, replacement of W82(*84*), also may result in somanase activity.

Stereospecific Phosphonylation. Sarin, soman, and VX each contain a chiral phosphorus that results in stereoisomers; soman is a mixture of four stereoisomers because it also

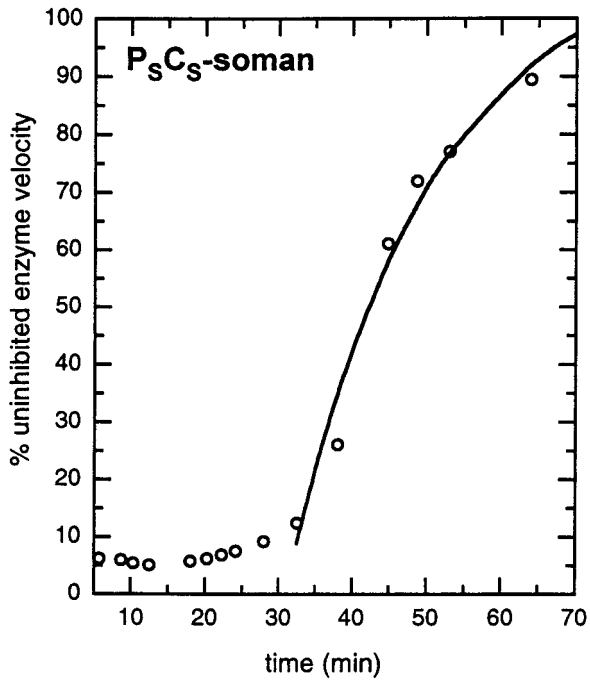


FIGURE 7: Somanase activity of G117H/E197Q. Representative plot showing spontaneous reversal of inhibition from steady-state after inhibition with ≥10-fold molar excess P_SC_S-soman (phosphate buffer, pH 7.5, 22 °C). Active enzyme was measured as a function of time after mixing soman and G117H/E197Q using the standard BTCh assay. The line shown was obtained by a direct fit of velocity to eq 3. At 70 min, less than 10% of the starting soman concentration remained (verified with the eel AChE assay described in text). For comparison with aqueous solvolysis, in the absence of enzyme, >90% of the starting soman concentration remained unhydrolyzed at 60 min in phosphate buffer, pH 7.4, 27 °C (*39*).

contains a chiral carbon (Figure 1). Configuration at the OP chiral centers modulates phosphonylation and aging rate constants of cholinesterase. The stereochemical effect on phosphonylation generally is greater for AChE than for BChE (reviewed in ref 2). Relatively poor accommodation of an OP alkoxyl group in the constrained acyl pocket of AChE may contribute to higher stereoselectivity (*61*, *62*). Despite differences in the phosphonylation and aging reactions for the two enzymes, P_SC_S-soman is the most potent stereoisomer for inhibition of AChE or BChE (*16*).

Double mutant cycles uncovered a qualitative difference for reaction of the BChE mutants with P_SC_S-soman. The effects of His117 and Gln197 were not independent of each other for the phosphonylation reaction with P_SC_S-soman. Independence or thermodynamic additivity was observed, however, for reactions with the other two soman stereoisomers tested as well as for acetylation with BTCh (Table 3). We inferred from this difference that P_SC_S-soman induced a cooperative effect. Several explanations for thermodynamic cooperativity have been proposed that may apply to P_SC_S-soman: (1) conformational change of the enzyme; (2) distortion of the transition state; or (3) reorientation of the ligand (soman) in the active site (*30*).

Stereospecific Dephosphonylation. Spontaneous reactivation was 10−20-fold faster for C_S-PMP-G117H/E197Q than for the corresponding C_R-conjugates (Table 4). Earlier studies found that oxime-induced reactivation was approximately 3-fold faster for P_SC_S-PMP-AChE than for P_SC_R-PMP-AChE (*63*−*65*). Qian and Kovach (*56*) explained the slower oxime-induced reactivation of the C_R stereoisomers by steric hindrance; the C_R-soman pinacolyl group was modeled to interfere with optimal proton transfer from the active site His440 to Ser200 during acid-catalyzed reactivation.

Reactivation of racemic i-PrMP− or EMP-G117H/E197Q was biphasic. With prolonged incubation in excess VX, the fast-reactivating component of the EMP-enzyme converted entirely to a slow-reactivating form (Figure 6). One explanation for this observation is different reactivation rates for the two phosphorus stereoisomers. Short incubation times of enzyme with VX may have resulted in a mixture of P_S- and P_R-EMP-G117H/E197Q, but reactivation became slower and essentially monophasic as the slow-reactivating stereoisomer accumulated. Oxime-induced reactivation of i-PrMP− and EMP−BChE also was biphasic, and this result was attributed to OP stereochemistry (*66*, *67*). More complex explanations remain plausible, however, because cholinesterase reactivation can be heterogeneous, even in the absence of stereochemical effects (*68*, *69*), and processes other than aging can interfere with reactivation (*38*).

Comparison with Theoretical Models. Cholinesterases rapidly bind nerve agents and stabilize the phosphonylation transition state, but are unable to overcome the kinetic stability of the phosphoenzyme intermediate. One problem is steric hindrance; the active site is optimized for carbon ester deacylation, and His438(*440*) is not positioned to steer a water molecule to the correct face of the phosphorus to catalyze dephosphylation (*42*, *70*). Rapid dealkylation (aging) creates a second problem by introducing a negative charge near His438(*440*). Anionic phosphoesters are inherently resistant to nucleophilic attack (*71*), and by analogy with serine proteases, the anion may form a salt bridge with the His438(*440*) imidazolium to further stabilize aged AChE or BChE (*72*).

Somanase activity was developed in human BChE by making two synergistic changes that simultaneously promote spontaneous reactivation and slow aging, without eliminating cholinesterase activity. Spontaneous reactivation depended upon the presence of His117. The mechanism is unknown, but molecular models predict that His117 is positioned to effect dephosphylation by electron withdrawal (*7*). This may involve either a unique hydrogen bond between His117 and the phosphonyl oxygen or an indirect steric effect that creates a better hydrogen bonding position for His438(*440*) (*9*). Additional hydrogen bond(s) can increase catalysis by reducing $\Delta G°$ for the rate-limiting dephosphylation transition state (*73*).

Slow aging of BChE E197Q mutants is consistent with the Kovach model for PMP-AChE that predicts that Glu199 provides an electrostatic "push" during dealkylation (aging), while the developing negative charge on the phosphonate monoester is stabilized by the His440 imidazolium (*10*, *57*). Replacement of Glu197(*199*) with Gln removes the negatively charged electrostatic catalyst. The mutation also distorts the trimethyl subsite, perhaps misaligning the P−O-alkyl oxygen atom relative to the His438(*440*) imidazolium. Aging of PMP-G117H/E197Q is slowed enough to allow the enzyme to catalyze hydrolysis of soman as a slow substrate. From the wide variation of rate constants observed during reactivation (Table 4; *7*, *9*), however, the cost of removing Glu197(*199*) may be slower dephosphylation of the double mutant (relative to G117H) for some specific OP-

enzyme complexes. Ongoing comparison of BChE His117 enzymes with different alterations in the trimethyl subsite is expected to address the possibility that Glu197(*199*) also participates in dephosphylation.

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