A Single Amino Acid Substitution, Gly117His, Confers Phosphotriesterase (Organophosphorus Acid Anhydride Hydrolase) Activity on Human Butyrylcholinesterase†

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ABSTRACT: The G117H mutant of human butyrylcholinesterase (EC 3.1.1.8) was expressed in Chinese hamster ovary cells. Substitution of Gly 117 with His to make the G117H mutant endowed butyrylcholinesterase with the ability to catalyze the hydrolysis of organophosphate esters. G117H was still able to hydrolyze butyrylthiocholine, benzoylcholine, and *o*-nitrophenyl butyrate, but in addition it had acquired the ability to hydrolyze the antiglaucoma drug echothiophate and the pesticide paraoxon. Wild-type butyrylcholinesterase was irreversibly inhibited by echothiophate and paraoxon, but G117H regained 100% activity within 2−3 min following reaction with these compounds. On a polyacrylamide gel, the same bands that stained for activity with butyrylthiocholine also stained for activity with echothiophate. G117H is the only enzyme known that hydrolyzes echothiophate. Diethoxyphosphorylated G117H aged with a half-time of 5.5 h, a rate 600 times slower than the rate of hydrolysis. Echothiophate and paraoxon were hydrolyzed with the same k_{cat} of 0.75 min^{-1}. This calculates to a rate acceleration of 100 000-fold for hydrolysis of echothiophate and paraoxon by the G117H mutant compared to the nonenzymatic rate.

Organophosphate esters (OP)[1] have applicability as pesticides, chemical warfare agents, and drugs for treatment of medical disorders such as glaucoma and parasite infection. These toxic chemicals have a high potency because one molecule of OP inhibits one molecule of cholinesterase at the active-site serine. Loss of acetylcholinesterase function leads to muscle paralysis and seizures and may cause death by asphyxiation. Our goal was to make an enzyme that could destroy OP by catalyzing their hydrolysis.

We made mutants of human butyrylcholinesterase (BChE) in which histidine was placed within 4−10 Å of the active-site serine (Millard et al., 1995). The amino acids which were mutated to histidine were Gly 115 *(G117)*,[2] Gly 117 *(G119)*, Gln 119 *(Y121)*, Gly 121 *(G123)*, Leu 286 *(F288)*, and Val 288 *(F290)* (see Figure 1). The active site has been subdivided into named, functional regions, among which are the oxyanion hole and the acyl binding pocket. The oxyanion hole is formed by the main-chain NH groups of Gly 116 *(G118)* , Gly 117 *(G119)*, and Ala 199 *(A201)* (Harel

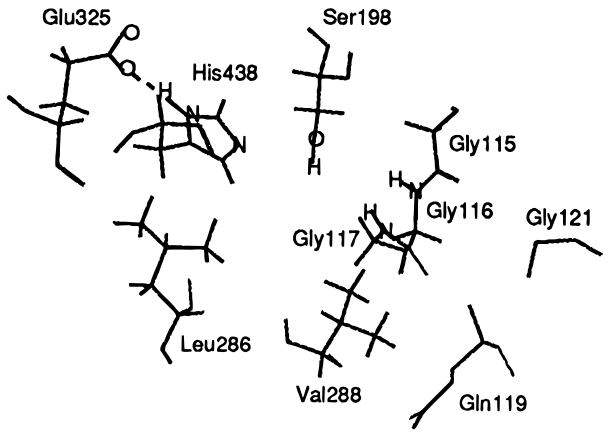

FIGURE 1: Location of amino acids mutated in this study. A model of human BChE structure is shown. Amino acids Glu 325, His 438, and Ser 198 form the catalytic triad. Distances between the hydroxyl group of Ser 198 and the α-carbon atom are 4.7 Å for Gly 115, 4.4 Å for Gly 117, 9.4 Å for Gln 119, 9.2 Å for Gly 121, 8.6 Å for Leu 286, 9.9 Å for Val 288, and 7.6 Å for His 438. The main-chain NH groups of Gly 116 and Gly 117, which form the oxyanion hole, are shown. The figure was made with Sybyl software from Tripos Inc.

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[1] Abbreviations: BChE, butyrylcholinesterase enzyme (EC 3.1.1.8); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ϵ, extinction coefficient; KP_i, potassium phosphate buffer; OP, organophosphate ester; VX, *O*-ethyl *S*-[2-[bis(1-methylethyl)amino]ethyl]methylphosphonothiolate.

[2] By convention, the italicized number in parentheses immediately following a BChE amino acid residue refers to the corresponding amino acid residue in *T. californica* acetylcholinesterase. For example, BChE G117 *(G119)* corresponds to acetylcholinesterase G119.

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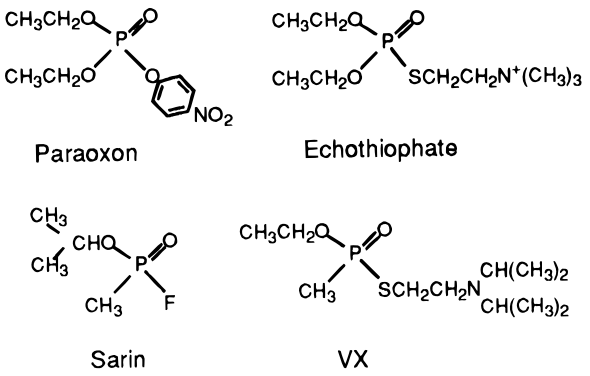

FIGURE 2: Structures of paraoxon, echothiophate, sarin, and VX.

et al., 1996). The acyl binding pocket is formed by Leu 286 and Val 288 (Sussman et al., 1991; Harel et al., 1992).

Human BChE was chosen as the starting enzyme for several reasons. (1) Wild-type human BChE carries out half the reaction necessary for hydrolysis of an OP by forming the equivalent of the acyl-enzyme intermediate (Main, 1979). Organophosphates may be regarded as hemisubstrates for BChE. By introducing a histidine we hoped to enable BChE to carry out the second half of the reaction, in which the phosphorylated enzyme is hydrolyzed. (2) Human BChE is already present in human serum. This means that BChE could be used as a therapeutic agent injected into blood because BChE is well tolerated in blood. (3) Human BChE has a history of use in the clinic. Partially purified human BChE has been injected into 54 patients to treat succinylcholine- and mivacurium-induced apnea (Evans et al., 1953; Borders et al., 1955; Stovner & Stadskleiv, 1976; Scholler et al., 1977; Schuh, 1977; Viby-Mogensen, 1981; Benzer et al., 1992; Ostergaard et al., 1995) and in two patients to treat poisoning by parathion (Goedde & Altland, 1971; Cascio et al., 1988). No adverse effects were noted following these treatments. (4) About 20 different naturally occurring mutants of human BChE have been identified (Primo-Parmo et al., 1996; Bartels et al., 1992). There is no evidence that mutants are antigenic. (5) Human BChE has a large space within its active-site gorge (Harel et al., 1992, Millard & Broomfield, 1992; Radic et al., 1993) which can accommodate a wide variety of OP (Kabachnik et al., 1970). This suggested that a successful mutant would be capable of hydrolyzing a variety of OP.

One mutant, G117H, achieved our goal of hydrolyzing organophosphate esters. Four OP have been tested to date and found to be substrates. Results for paraoxon (pesticide) and echothiophate (antiglaucoma drug) are reported here, while results for hydrolysis of the nerve agents, sarin and VX, are in Millard et al. (1995). Paraoxon and echothiophate are phosphate triesters, whereas sarin and VX are phosphonate esters (Figure 2). The G117H mutant of BChE is the only enzyme ever reported that is capable of hydrolyzing echothiophate. In addition, the G117H mutant is the first example of a serine esterase that has the capacity to hydrolyze OP at a significant rate.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression of Human BChE. The cDNA of human BChE (McTiernan et al., 1987) was modified to give the optimal ATG start site context GCCACCATGG for the ATG of the 28 amino acid leader sequence. Site-directed mutagenesis to make L286H and V288H was performed with the polymerase chain reaction and *Pfu* DNA polymerase (Stratagene, La Jolla, CA) in a protocol similar to that of Chen and Przybyla (1994). Other mutants were made in M13mp19 as described by Millard et al. (1995). The codons for Gly 115, Gly 117, Gln 119, Gly 121, Leu 286, and Val 288 were replaced with the codon for His (CAT). The 1.8-kb PCR fragment was purified with the QIA quick-spin PCR purification kit (Qiagen, catalog no. 28106, Chatsworth, CA) to remove *Pfu* polymerase, then digested with *Hin*dIII and *Apa*I, and purified a second time on a QIA quick-spin column to remove *Hin*dIII and *Apa*I enzymes. The 1.8-kb fragment was ligated into the expression plasmid pGS (Dr. Tyler White, Scios Nova, Mountain View, CA). The cloned gene was completely resequenced to ensure the absence of unwanted mutations. Oligonucleotide synthesis and DNA sequencing were performed by the University of Nebraska Molecular Biology Core Facility. Plasmid DNA, purified with the Qiagen plasmid kit (Qiagen catalog no. 12162), was transfected into CHO K1 cells (ATCC no. CCL 61, Rockville, MD) by calcium phosphate coprecipitation. The pGS plasmid is similar to pRc/CMV (Invitrogen, San Diego, CA) but has rat glutamine synthetase in place of the neomycin resistance gene. Two days after transfection, selection was begun with glutamine-free, serum-free medium (Ultraculture 12-725B, BioWhittaker, Walkersville, MD) containing 50 μM methionine sulfoximine (Sigma, St. Louis, MO). Colonies appeared 2−5 weeks later. Colonies were picked with a sterile cotton-tipped stick for transfer to 24-well cluster plates. Clones with the highest BChE activity were expanded into T150 flasks and into roller bottles. Secreted BChE was collected for 4 days into serum-free medium (Ultraculture, BioWhittaker) and 3 days into serum-free DMEM. Cells continued to secrete BChE at a consistently high level for 6 months without being fed serum. Absence of serum was desired because fetal bovine serum is rich in acetylcholinesterase.

Purification of Recombinant BChE. Four liters of culture medium containing G117H were centrifuged to remove particulate matter and the clear supernatant was poured into dialysis tubing having a flat diameter of 7.7 cm. The tubing was placed on a plastic trough and covered with table sugar for the purpose of concentrating the protein. In 24 h the volume reduced to 10% of the original. The concentrated culture medium was diluted 4−5-fold with 20 mM potassium phosphate and 1 mM EDTA, pH 7.0, to reduce the salt concentration to a sufficiently low level to allow all of the BChE to bind to the affinity column. The sample was loaded onto an 18-mL procainamide-Sepharose affinity column (Lockridge & La Du 1978; Lockridge 1990). The column was washed with buffer before BChE was eluted with 90 mL of 0.2 M procainamide. The BChE in 90 mL of 0.2 M procainamide was diluted to 450 mL with 20 mM KP_i and 1 mM EDTA, pH 7, and loaded onto the regenerated affinity column. Contaminating proteins were eluted by washing with increasing NaCl concentrations, from 0.02 to 0.2 M NaCl in buffer. The column was washed with buffer just before BChE was eluted with 0.2 M procainamide. A final purification step which removed contaminating proteins as well as procainamide was ion-exchange chromatography on DEAE-Sephacel (Pharmacia). A 120-mL column was equilibrated with 20 mM Tris-HCl and 1 mM EDTA, pH 8. The BChE sample was diluted 4× to lower the salt

concentration below 0.05 M, thus allowing the BChE to bind. The column was washed with buffer until the absorbance was zero and then with a salt gradient made from 500 mL of 20 mM Tris-HCl and 1 mM EDTA, pH 8, vs 500 mL of 0.5 M NaCl in buffer. BChE eluted in the fraction from 300 to 600 mL, corresponding to NaCl concentrations of 0.15−0.3 M. A 50-mL fraction containing the highest activity contained the purest enzyme. The 99% pure G117H had a specific activity of 150 units/mg where activity was assayed with 1 mM butyrylthiocholine in 0.1 M KP_i, pH 7.0, and where protein concentration was determined from absorbance at 280 nm (1 mg/mL has an absorbance at 280 nm of 1.8). Wild-type human BChE was purified by procainamide-affinity chromatography to approximately 50% purity.

Native Wild-Type BChE. Wild-type BChE purified from pooled human plasma was a gift from Dr. Christopher Bryant (Armour Pharmaceuticals, Kankakee, IL). The BChE preparation was 90−95% pure.

Activity-Stained Gel. A 4−30% nondenaturing polyacrylamide gradient gel, 1.5 mm thick, was poured in an SE600 apparatus (Hoefer Scientific, San Francisco, CA). The gel was electrophoresed for 3000 V·h and stained for OP hydrolase activity by a modification of the procedure of Karnovsky and Roots (1964). In place of butyrylthiocholine iodide we used 2 mM echothiophate iodide as substrate to stain for echothiophate-hydrolyzing activity.

Activity Assays. Butyrylthiocholine iodide hydrolysis was measured by the Ellman method (Ellman et al., 1961) in 0.1 M KP_i, pH 7.0, 25 °C. Benzoylcholine chloride hydrolysis was measured in 0.1 M KP_i, pH 7.0, 25 °C, at 240 nm where the difference in the extinction coefficient between substrate and product, $\Delta\epsilon$, was 6700 M^{-1} cm^{-1}. *o*-Nitrophenyl butyrate hydrolysis was measured in 0.067 M Na/K phosphate, pH 7.5, 25 °C, at 420 nm where the extinction coefficient (ϵ) = 3430 M^{-1} cm^{-1}. The stock solution of *o*-nitrophenyl butyrate was prepared in methanol and the final methanol concentration in each assay was 5.3%. Units of activity are micromoles per minute.

When the data conformed to Michaelian behavior, K_m and V_{max} were determined by simple weighted nonlinear regression of the Michaelis−Menten equation using the Enzfitter kinetic calculation program (Elsevier Biosoft, Cambridge, U.K.). Non-Michaelian behavior with activation at high butyrylthiocholine concentrations is described as the binding of substrate to two different sites (Radic et al., 1993; Millard et al., 1995; Masson et al., 1996). K_m, K_{ss}, and b values were calculated by nonlinear computer fitting of eq 1 using Sigma Plot (Jandel Scientific, San Rafael, CA). The parameter b reflects the efficiency with which the ternary complex SES forms product. Substrate activation is observed when $b > 1$.

$$v = \frac{k_{cat}e_o}{1 + K_m/[S]}\left(\frac{1 + b[S]/K_{ss}}{1 + [S]/K_{ss}}\right) \qquad (1)$$

Assay of Organophosphate Ester Hydrolysis. Paraoxon (diethyl *p*-nitrophenyl phosphate, Sigma D-9286) hydrolysis was measured as absorbance of the *p*-nitrophenol product at 400 nm, where ϵ at pH 7.0 is 8130 M^{-1} cm^{-1}. The extinction coefficient at pH 7.0 was calculated from the Henderson−Hasselbalch equation using values reported by Bender and Marshall (1968); $pK_a = 7.11$, ϵ = 18 600 M^{-1} cm^{-1} at 400 nm for *p*-nitrophenolate ion in sodium hydroxide; ϵ = 140 M^{-1} cm^{-1} at 400 nm for *p*-nitrophenol in acid. The concentration of paraoxon in our stock solution was determined after an aliquot of paraoxon was boiled in 0.1 M NaOH, followed by reading of the absorbance at 400 nm. It was found that 54% of the stock paraoxon was degraded. Paraoxon was used without removal of the hydrolysis products because the products are not inhibitory. Enzyme-catalyzed rates were corrected for spontaneous hydrolysis of paraoxon.

Scheme 1[a]

$$\mathrm{EOH} + \mathrm{XP(RO)_2O} \underset{K_d}{\rightleftharpoons} \mathrm{EOH{\cdot}XP(RO)_2O} \xrightarrow{k_2} \mathrm{EO\text{-}P(RO)_2O} \xrightarrow[H_2O]{k_3} \mathrm{EOH} + \mathrm{P(RO)_2O(OH)} + \mathrm{HX}$$

[a] EOH is free enzyme; $XP(RO)_2O$ is an OP with leaving group X; $EOH{\cdot}XP(RO)_2O$ is the reversible complex; $EO\text{-}P(RO)_2O$ is the phosphorylated enzyme; k_2 is the phosphorylation rate constant; k_3 is the dephosphorylation rate constant.

Echothiophate iodide (diethoxyphosphinylthiocholine iodide, Wyeth-Ayerst, Philadelphia, PA) hydrolysis was followed by absorbance of the anion of DTNB produced by reaction with thiocholine. Reactions carried out in the Gilford spectrophotometer at 412 nm in 2 mL total volume gave the same V_{max} and K_m values as reactions in a 96-well microtiter plate measured at 405 nm in 0.1 mL total volume in a microtiter plate reader. Observed absorbance changes were corrected for spontaneous hydrolysis. For extinction coefficients, see the Experimental Procedures section on pH dependence.

Determination of k_{cat}. V_{max} was determined from a double-reciprocal plot, $1/v$ vs $1/S$, for hydrolysis of echothiophate iodide, paraoxon, butyrylthiocholine iodide, and benzoylcholine chloride at 25 °C using highly purified G117H. k_{cat} was calculated from V_{max} by dividing V_{max} per milliliter by micromoles of enzyme subunits per milliliter, where subunit concentration was calculated from protein concentration. One milligram of BChE contains 11.8 nmol of subunits. The k_{cat} values measured in 0.1 M potassium phosphate pH 7.0 were lower than in 0.1 M Bis-Tris/Bis-Tris propane pH 7.0.

Determination of k_2 and k_3. The rate constants for phosphorylation, k_2, and dephosphorylation, k_3, in Scheme 1 were measured for G117H reacting with echothiophate and paraoxon.

The k_3 value was determined by measuring the return of butyrylthiocholine hydrolyzing activity for an inhibited preparation of G117H after dilution. A preparation of G117H (0.01 mg/mL) was saturated with OP (10 mM echothiophate or 10 mM paraoxon) so that there was no free enzyme. The enzyme consisted solely of the reversibly bound and covalently bound complexes. A 5-μL aliquot was removed after 1 min of incubation and assayed immediately with 1 mM butyrylthiocholine in 2 mL of 0.1 M KP_i, pH 7.0, and 0.5 mM DTNB at 25 °C. This 400-fold dilution brought the concentration of OP to well below the K_m of the OP, allowing the fraction of BChE in the reversible complex to rapidly dissociate. The activity corresponded to the amount of enzyme in the reversible complex. The remainder of the enzyme in the covalent complex reactivated at the rate k_3, gradually increasing the concentration of free enzyme and thereby increasing the measured activity. The final steady-state rate represented the rate of 100% free

Table 1: Kinetic Parameters for Hydrolysis of Butyrylthiocholine, Benzoylcholine and *o*-Nitrophenyl Butyrate

	butyrylthiocholine[a]					benzoylcholine[a]			*o*-nitrophenyl
BChE	K_m (mM)	K_{ss} (mM) activation	*b* value activation	k_{cat} (min^{-1})	bk_{cat} (min^{-1})	K_m (mM)	K_{ss} (mM) inhibition	k_{cat} (min^{-1})	butyrate[a] K_m (mM)
wild-type	0.02	0.3	2.4	24 000	58 000	0.01	0.2	15 000	0.12
G117H	0.15	0.4	1.7	9000	15 000	0.11	no inhibition	2200	>10[b]
Q119H	0.04	0.3	1.6			0.01	no inhibition		0.26
L286H	0.06	0.8	2.3			0.02	0.1		0.12
V288H	0.03	0.5	1.7			0.01	0.5		0.15

[a] Values were determined in triplicate with standard deviations of 5−30%. Butyrylthiocholine and benzoylcholine hydrolysis was assayed in 0.1 M potassium phosphate, pH 7.0, 25 °C; *o*-nitrophenyl butyrate activity was measured in 0.067 M Na/K phosphate, pH 7.5, 25 °C. Assays with *o*-nitrophenyl butyrate contained 5.3% methanol. [b] K_m for G117H could not be measured accurately because the low solubility of *o*-nitrophenyl butyrate allowed a maximum concentration of 0.8 mM, a concentration well below the estimated K_m value.

enzyme and showed that all of the G117H was released from its covalent association with organophosphate within 2−3 min of dilution. The half-time for reactivation, $t_{1/2}$,was calculated from the lag phase, by plotting log ΔA as a function of time (Hatfield et al., 1970), where ΔA is the difference between the extrapolated steady-state absorbance and the actual absorbance. The dephosphorylation rate constant, k_3, was calculated from the half-time for reactivation using the relationship $k_3 = \ln 2/t_{1/2}$. k_2 was calculated from k_3 and the k_{cat} for Scheme 1, using the relationship $k_{cat} = k_2k_3/(k_2 + k_3)$.

pH Dependence. V_{max} and K_m were measured as a function of pH in a 50:50 mixture of 0.1 M Bis-Tris, $(HOCH_2CH_2)_2NC(CH_2OH)_3$ ($pK_a = 6.5$) and 0.1 M Bis-Tris propane, $(HOCH_2)_3CNHCH_2CH_2CH_2NHC(CH_2OH)_3$ ($pK_a = 6.8$ and 9.0) (Sigma). The pH was adjusted with HCl and measured again at the end of the reaction. Echothiophate iodide and butyrylthiocholine iodide hydrolysis were measured spectrophotometrically with DTNB (Ellman et al., 1961). 2-Nitro-5-thiobenzoic acid, the anion produced by reaction of thiocholine with DTNB, has a pK_a of 4.53 (Riddles et al., 1979); molar absorptivity values at 412 nm as a function of pH are 10 600 M^{-1} cm^{-1} at pH 5.0, 11 150 at pH 5.1; 12 800 at pH 5.5; 13 700 at pH 6.0; 14 100 at pH 7.0; 14 150 at pH 8.0; and 14 150 at pH 9.0. The pH dependence of butyrylthiocholine hydrolysis was measured in a Gilford spectrophotometer at 25 °C. The pH dependence of echothiophate hydrolysis was measured in 96-well microtiter plates where the extinction coefficients at 405 nm of 100-μL volumes were 25.8% of the extinction coefficients listed above. Each 100-μL assay used approximately 5 units (33 μg) of G117H. Autohydrolysis rates (general base catalysis) were significant at pH 8 and higher. Autohydrolysis rates were subtracted from the observed rates.

Data were fitted to eqs 2 and 3 using the curve-fitting program of Sigma Plot. Equation 2 and 3 are for a mechanism in which only the unprotonated enzyme−substrate complex makes product. These equations describe a rapid equilibrium binding model where K_m is identical to the dissociation constant of the enzyme−substrate complex (Segal, 1975). The fitted points and the experimental points were plotted according to Dixon and Webb (1979) where a slope has a value of 1 or 0. Standard errors were calculated by the curve-fitting software of Sigma Plot.

Inhibition by Paraoxon. BChE was incubated with paraoxon (0.05−1 μM) for a fixed time (1−30 min). Then the mixture was diluted into buffer for measurement of activity with butyrylthiocholine. A semilogarithm plot of percent activity remaining versus time yielded a set of first-order constants, representing the apparent rate of inhibition (k_{obs}) at various paraoxon concentrations. A plot of the reciprocal of k_{obs} versus the reciprocal of the paraoxon concentration yielded k_2, the phosphorylation constant, and K_i, the dissociation constant for the enzyme−inhibitor complex (Kitz & Wilson, 1962).

$$K_{mapp} = K_m\left\{1 + \frac{[H^+]}{K_a}\right\} \quad (2)$$

$$k_{catapp} = \frac{k_{cat}}{1 + \frac{[H^+]}{K_a}} \quad (3)$$

RESULTS

Four Mutants Hydrolyze Carboxyl Ester Substrates. Mutants G117H, Q119H, L286H, and V288H resembled wild-type BChE in that they hydrolyzed butyrylthiocholine, benzoylcholine, and *o*-nitrophenyl butyrate. Mutants G115H and G121H had no detectable activity (less than 0.01% that of wild-type). Table 1 shows that the K_m values for butyrylthiocholine were similar for wild type and the four active mutants. Another similarity was that they experienced substrate activation at butyrylthiocholine concentrations in the range 0.4−40 mM. The K_{ss} values measured in the substrate activation range of butyrylthiocholine concentration (0.4−40 mM) were approximately 10-fold greater than the K_m values, a result similar to that for wild type. The *b* value (Radic et al., 1993), defined as the factor by which maximum velocity is increased in the substrate activation range, was similar for G117H, Q119H, L286H, V288H, and wild type.

The substrate benzoylcholine had a 10-fold higher K_m value for G117H than for the other mutants in Table 1. Wild-type BChE has substrate inhibition rather than substrate activation with benzoylcholine (Kalow, 1964). Mutants L286H and V288H behaved like wild type in having substrate inhibition, but G117H and Q119H had no substrate inhibition within the limited range of benzoylcholine concentrations possible in the spectrophotometer (0.2 mM benzoylcholine has an absorbance at 240 nm of 1.874). The K_m values for *o*-nitrophenyl butyrate for wild type, Q119H, L286H, and V288H were similar, but the K_m value for G117H was about 2 orders of magnitude higher (Table 1). The second-order rate constant for hydrolysis of butyrylthiocholine, as measured by k_{cat}/K_m, was 20-fold lower for G117H compared to wild-type BChE.

G117H Hydrolyzes Organophosphate Esters. One mutant, G117H, hydrolyzed OP. Hydrolysis of echothiophate and

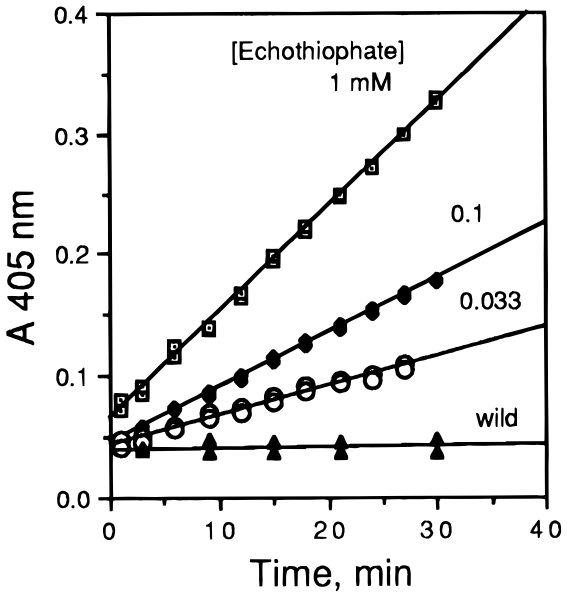

FIGURE 3: Hydrolysis of echothiophate by G117H. Examples of data obtained from a microtiter plate assay are shown. Each 0.1-mL reaction volume contained 1.0, 0.1, or 0.033 mM echothiophate iodide, 0.5 mM DTNB, and 3.9 units of G117H (0.3 nmol) in 0.1 M KP_i, pH 7.0. Wild-type BChE (14 units/assay) did not hydrolyze echothiophate.

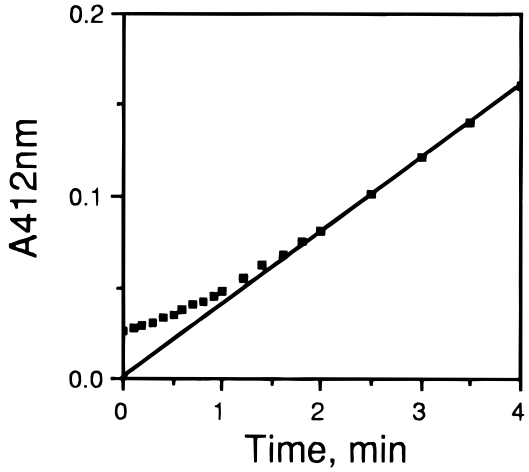

FIGURE 4: One hundred percent recovery of G117H activity. Chart traces of activity assays with 1 mM butyrylthiocholine in 0.1 M KP_i, pH 7.0, 25 °C, are shown for two samples. Squares are for G117H recovering from treatment with 10 mM echothiophate. The solid line is the activity of a control sample of G117H which had not been exposed to echothiophate. Within 2−3 min of dilution, the echothiophate-treated G117H had the same activity as the control, demonstrating 100% recovery of activity. $t_{1/2}$ for dephosphorylation in Table 2 was calculated from the difference in absorbance between the actual and extrapolated absorbance at time points between 0.1 and 1.5 min.

Table 2: Organophosphate Hydrolase Activity of the G117H Mutant of Human BChE

organophosphate	K_m (mM)	K_d (mM)	k_{cat} (min^{-1})	k_2 (min^{-1})	k_3 (min^{-1})	$t_{1/2}$ (min)
paraoxon[a]	0.070		0.75	1.8	1.3	0.54
echothiophate[a]	0.074		0.75	2.0	1.2	0.60
sarin (GB)[b]		0.110	0.0040	0.018	0.0052	133
VX[b]		0.050	0.0066	0.078	0.0072	96

[a] Assays were done 3−10 times with standard deviations of 5−30%. Paraoxon and echothiophate values were measured in 0.1 M potassium phosphate, pH 7.0, 25 °C. k_2 and k_3 are rate constants for phosphorylation and dephosphorylation as in Scheme 1. $t_{1/2}$ is the half-time for dephosphorylation where $t_{1/2} = \ln 2/k_3$. [b] Data for sarin and VX are from Millard et al. (1995) in 0.067 M Na/K phosphate, pH 7.5, 25 °C. k_{cat} was calculated from the relationship $k_{cat} = k_2k_3/(k_2 + k_3)$.

paraoxon was visualized within minutes after the addition of G117H by the appearance of yellow product. Figure 3 gives four examples of data collected in a microtiter plate assay, showing that G117H but not wild-type BChE hydrolyzed echothiophate. Experiments of the type in Figure 3 were used to calculate the K_m and k_{cat} values in Table 2. Paraoxon and echothiophate both had K_m values of 0.07 mM. These values were similar to the K_m values for butyrylthiocholine and benzoylcholine and also similar to the K_d values for sarin and VX. The k_{cat} values for paraoxon and echothiophate were approximately 1 min^{-1}, a rate that is 100−200 times faster than k_{cat} for the nerve agents in Table 2.

The half-time for reactivation was measured in the reaction illustrated in Figure 4. G117H was treated with a concentration of echothiophate that exceeded the K_m value by 100-fold, thus converting 100% of the enzyme into the enzyme−substrate complex and the phosphorylated intermediate. An aliquot was diluted 400-fold into 1 mM butyrylthiocholine for measurement of free enzyme in the Ellman assay. Figure 4 shows a lag phase of about 2 min before the activity reached the same fast rate as control G117H which had never been exposed to echothiophate. The half-time for return to 100% of the original activity was about 0.5−0.6 min for both echothiophate and paraoxon-treated G117H, making the rate constant for dephosphorylation, k_3, equal to 1.3 min^{-1}. k_3 and k_2 were close in value, suggesting that both phosphorylation and dephosphorylation were partly rate-limiting. By contrast, k_3 was 4−10-fold faster compared to k_2 when the substrate was sarin or VX, indicating that the rate-limiting step for hydrolysis of sarin and VX is simply dephosphonylation (Millard et al., 1995).

pH Dependence. That the rate-limiting events in the hydrolysis of echothiophate are different from those controlling hydrolysis of sarin was confirmed by studies of pH dependence. The k_{cat} value for G117H was independent of pH when the substrate was butyrylthiocholine (Figure 5A) or echothiophate (Figure 6A). Butyrylthiocholine hydrolysis was measured in the pH range 5.5−9.0. Echothiophate hydrolysis could not be measured above pH 8 because the autohydrolysis rate was so fast that addition of G117H did not increase the rate. By contrast, hydrolysis of sarin and VX showed an increase in k_3 at low pH values and revealed a pK_a of 6.2 (Millard et al., 1995). Thus the rate-limiting step for hydrolysis of sarin and VX requires participation of a protonated histidine.

Wild-type BChE had a third pattern of pH dependence. Hydrolysis of butyrylthiocholine by wild-type BChE showed a decrease in k_{cat} at low pH and a pK_a of 6.1 ± 0.1 (Figure 7A), suggesting that in wild-type BChE the rate-limiting step required deprotonated histidine, most likely His 438 of the catalytic triad. Our pK_a value of 6.1 ± 0.1 for wild-type human BChE complexed to butyrylthiocholine is similar to the pK_a of 6.2 for wild-type horse BChE complexed to acetylcholine (Dixon & Webb, 1979) and the pK_a of 6.7 for wild-type human BChE complexed to benzoylcholine (Kalow, 1964).

Plots of pK_m vs pH showed pK_a values of 7.2±0.1 (Figure 5B) and 7.0 ± 0.1 (Figure 6B) for G117H. These pK values

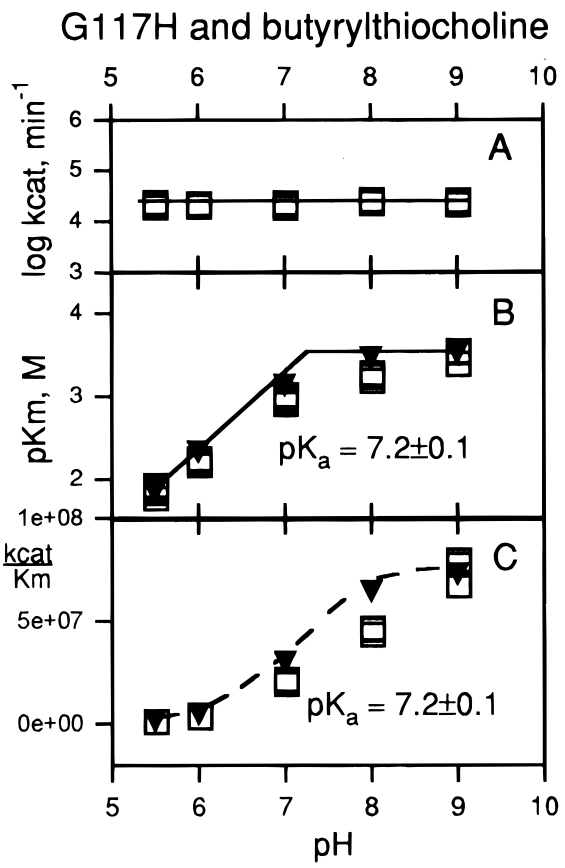

FIGURE 5: pH dependence for G117H hydrolyzing butyrylthiocholine iodide. k_{cat} and K_m values were measured in 0.1 M Bis-Tris/Bis-Tris propane at 25 °C. Slopes of 1 and 0 were fit to the data according to Dixon and Webb (1979). The pH-independent k_{cat} value was 25 000 min^{-1} in this buffer. K_m values ranged from 15 mM at pH 5.5 to 0.3 mM at pH 9. Closed triangles are theoretical points derived by fitting experimental points (open squares) to eq 2.

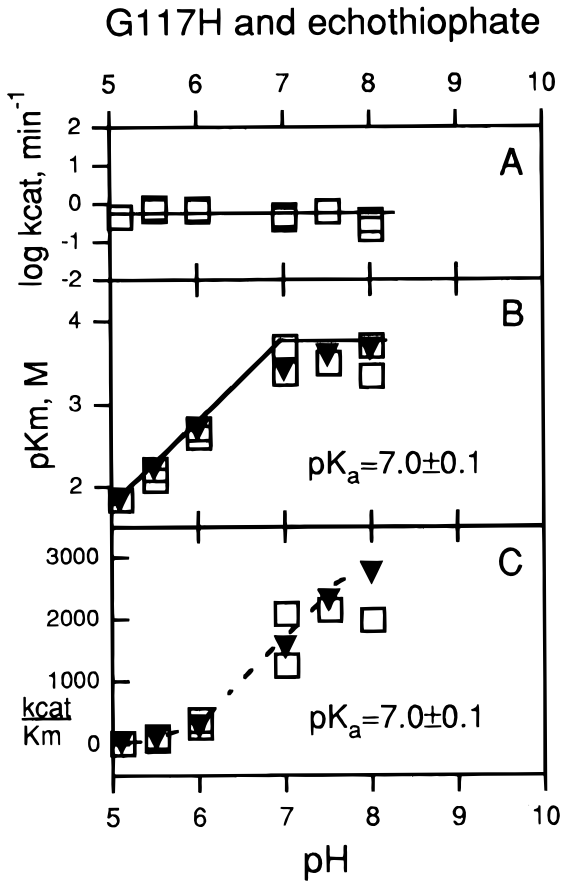

FIGURE 6: pH dependence for G117H hydrolyzing echothiophate iodide. The k_{cat} value of ~ 0.6 min^{-1} was independent of pH in 0.1 M Bis-Tris/Bis-Tris propane at 25 °C. K_m values ranged from 14 mM at pH 5.1 to 0.3 mM at pH 8.0. Closed triangles are theoretical points derived by fitting experimental points (open squares) to eq 2.

are for the free enzyme and probably reflect ionization of histidine. To determine whether this histidine was 117 or 438, we measured the pH dependence of pK_m in wild-type BChE, since wild type has His 438 but not His 117. Figure 7B shows that K_m for wild-type BChE was independent of pH in the pH range 5.5−8.0, suggesting that the pK values observed for G117H reflect His 117. That His 117 is in intimate contact with the bound substrate is also suggested by the weaker affinity of all substrates for G117H than for wild-type BChE and by the 20-fold reduction in the G117H specificity constant, k_{cat}/K_m, compared to that of wild type. The assignment of the pK value of 7 to His 117 is tentative. Another result from Figures 5B and 6B is that binding of butyrylthiocholine and echothiophate to G117H is tighter when His 117 is unprotonated.

A pK value obtained from a plot of pK_m versus pH reflects the pK of the free enzyme when the plot has the shape in Figures 5B and 6B (Dixon & Webb, 1979). A pK value obtained from a plot of log k_{cat} versus pH reflects the pK of the enzyme−substrate complex (Dixon & Webb, 1979). The pK values of approximately 7.0 measured for the G117H mutant are for the free enzyme. The pK value of 6.2 measured by Millard et al. (1995) is the pK for the covalent complex between sarin and the G117H mutant. No pK value for the echothiophate−G117H complex was observed. This difference in pK for the two OP−enzyme complexes can be attributed to the identity of the organophosphates which have different structures and create different environments at the active site.

Table 3: Inhibition by Paraoxon[a]

BChE	K_i (μM)	k_2 (min^{-1})	k_{II}[b] (μM^{-1} min^{-1})
wild type	0.26	0.52	2.0
G117H	no inhibition		
Q119H	0.20	0.55	2.7
L286H	0.54	0.42	0.77
V288H	0.15	1.08	7.2

[a] Measured in 0.1 M phosphate buffer, pH 7.0, 25 °C. Standard deviations were 20−60%. [b] $k_{II} = k_2/K_i$.

Aging. When G117H was incubated with 10 mM echothiophate in 0.1 M potassium phosphate, pH 7.0, 25 °C, the enzyme lost activity over a period of hours (Figure 8). Activity could not be regenerated by incubation with 1 mM pralidoxime chloride in pH 8.0 buffer for 20 h at 25 °C. Therefore, the lost activity was attributed to aging. The half-time for aging of the diethoxyphosphorylated G117H was 5.5 ± 0.2 h (Figure 8).

Inhibition by Paraoxon. Paraoxon is an inhibitor of wild-type BChE, as well as of mutants Q119H, L286H, and V288H (Table 3), all having K_i values less than 1 μM. However, paraoxon did not inhibit the G117H mutant when the paraoxon concentration was 1 μM or less. This agrees with the finding in Table 2 where the K_m value of paraoxon for G117H is 70 μM, indicating that binding of paraoxon to G117H is negligible at 1 μM paraoxon.

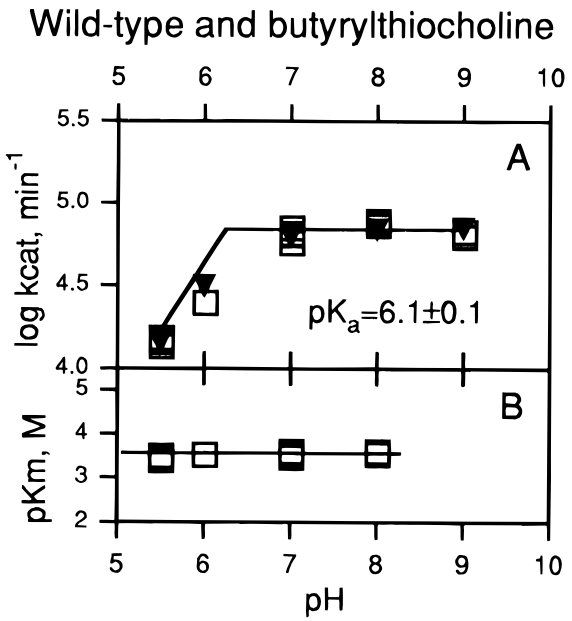

FIGURE 7: pH dependence for recombinant wild-type BChE hydrolyzing butyrylthiocholine iodide. k_{cat} values ranged from 15 000 min^{-1} at pH 5.5 to 70 000 min^{-1} at pH 7−9 in 0.1 M Bis-Tris/Bis-Tris propane at 25 °C. The K_m value of 0.3 mM was independent of pH in the range pH 5.5−8.0. Closed triangles are theoretical points derived by fitting experimental points (open squares) to eq 3.

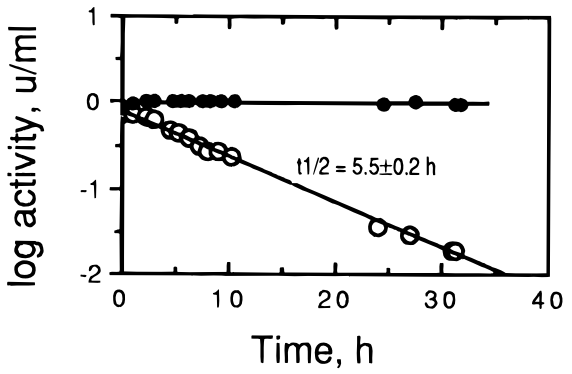

FIGURE 8: Aging of echothiophate inhibited G117H. One unit of G117H (78 pmol) was incubated with 10 mM echothiophate in 1 mL of 0.1 M potassium phosphate buffer, pH 7.0, 25 °C. Aliquots (10 μL) were removed for assay at 25 °C with 1 mM butyrylthiocholine in 2 mL of pH 7 buffer containing DTNB. The activity after various times of incubation with 10 mM echothiophate (○) or with 0 mM echothiophate (●) is shown. The half-time for aging calculated from three experiments was 5.5 ± 0.2 h.

The phosphorylation rate constant, k_2, was similar for wild type, Q119H, L286H, and V288H (Table 3). The apparent bimolecular rate constant k_{II}, where $k_{II} = k_2/K_i$, was similar to values reported by Skrinjaric-Spoljar and Simeon (1993) for wild type and the D70G mutant, and to the value of 1.3 μM^{-1} min^{-1} reported by Millard et al. (1995) for wild-type BChE and sarin.

Activity-Stained Gel. Phosphotriesterase activity in the G117H mutant was confirmed by staining a gel for activity with echothiophate iodide (Figure 9). The same bands that hydrolyzed echothiophate (Figure 9A) also hydrolyzed butyrylthiocholine (Figure 9B). The association of OP hydrolase activity with bands migrating at BChE positions strengthens the argument that the OP hydrolase activity comes from G117H. Wild-type BChE had no activity with echothiophate (Figure 9A), which means the bands did not

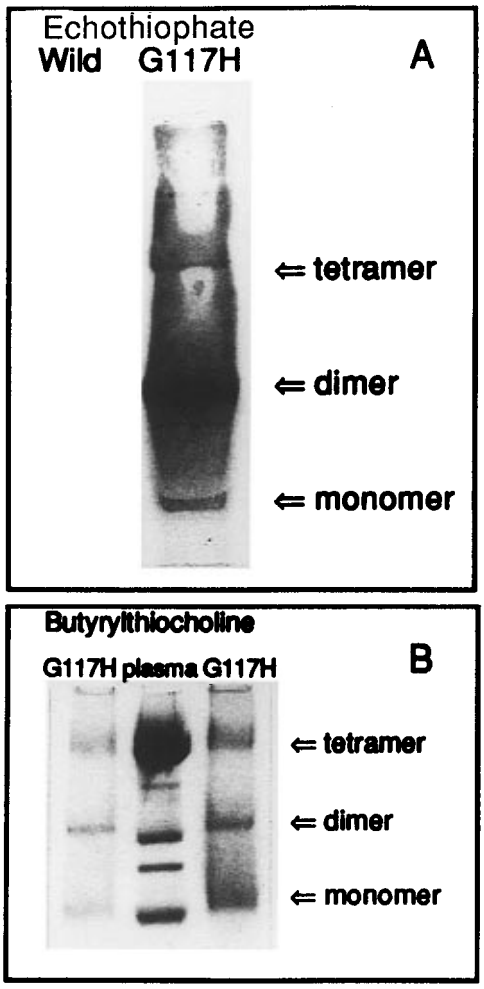

FIGURE 9: Activity-stained gel, using echothiophate as substrate. (A) Nondenaturing 4−30% polyacrylamide gradient gel stained for activity in the presence of 2 mM echothiophate iodide in pH 6.0 buffer. The lane labeled wild contains 0.45 mg of native wild-type BChE (370 units); the lane labeled G117H contains 0.65 mg of G117H (100 units). The dimer and tetramer bands appeared after 1 h and the monomer band after overnight staining. (B) Nondenaturing gel stained for activity with 2 mM butyrylthiocholine. The lanes labeled G117H contain 0.002 and 0.004 unit of G117H. The lane labeled plasma contains 4 μL of plasma, which corresponds to 0.016 unit of wild-type BChE assayed with 1 mM butyrylthiocholine in 0.1 M KP_i, pH 7.0. Plasma contains monomer, dimer, trimer, and tetramer bands of BChE as well as a disulfide-linked BChE/albumin dimer (Masson, 1989). All bands were visible after 40 min of incubation in the staining solution.

come from release of stoichiometric amounts of thiocholine but from turnover. G117H dimers were more abundant than monomers and tetramers. Approximately 10 000 times more G117H had to be loaded into one lane to detect activity with echothiophate as substrate than with butyrylthiocholine as substrate; this agrees with the 10 000-fold difference in k_{cat} for echothiophate (0.75 min^{-1}) and butyrylthiocholine (9000 min^{-1}). The gel in Figure 9A rules out the possibility that echothiophate acts as a tight-binding, reversible inhibitor of G117H rather than as a substrate.

DISCUSSION

Proof of Hydrolysis of Organophosphates. Four different OP have been shown to be hydrolyzed by the G117H mutant of human BChE. We have found that in the presence of G117H both paraoxon and echothiophate were rapidly

hydrolyzed, the amount of product formed being manyfold greater than the amount of enzyme present, suggesting turnover. In contrast, in the absence of G117H or in the presence of wild-type BChE no product was detected. This simple observation is proof that G117H hydrolyzed paraoxon and echothiophate. In addition we have three other findings to support this conclusion: (1) In the presence of G117H, the rate of product formation increased in a saturating fashion as the concentration of paraoxon or echothiophate was increased, indicative of a catalyzed hydrolysis. (2) After inhibition of activity toward butyrylthiocholine by excess paraoxon or echothiophate, 100% of the original activity was rapidly recovered after dilution. The $t_{1/2}$ for spontaneous reactivation of G117H inhibited by echothiophate was 0.6 min at 25 °C in pH 7.0 buffer. The $t_{1/2}$ for the nonenzymatic hydrolysis of echothiophate at 25 °C in pH 7.0 buffer was 50 days (Hussain et al., 1968). Thus the G117H mutant accelerated the rate of echothiophate hydrolysis 120 000-fold. (3) G117H but not wild-type BChE showed bands of activity on a polyacrylamide gel stained with echothiophate.

Millard et al. (1995) used a different method to measure hydrolysis. They reacted G117H with excess sarin or VX to form the phosphonylated enzyme intermediate. The intermediate was rapidly separated from excess sarin and VX, and the rate of return of BChE activity was measured. The rate of reactivation was fast for G117H but unmeasurably slow for wild-type BChE. One hundred percent activity was recovered after treatment of G117H with sarin or VX. Millard et al. (1995) tested and rejected the possibility that reversible binding rather than hydrolysis might be occurring in the G117H mutant. Our gel electrophoresis experiment (Figure 9) also tested and rejected this possibility. We conclude that the G117H mutant of BChE did indeed hydrolyze OP.

Comparison of k_{cat} Values. The G117H mutant of human butyrylcholinesterase hydrolyzed paraoxon with a k_{cat} value of 0.75 min^{-1} at 25 °C in pH 7.0 phosphate buffer. This rate compares to a k_{cat} of 659 min^{-1} at 37 °C in pH 7.4 buffer (Smolen et al., 1991) for human paraoxonase (B-type allele with Arg 191; EC 3.1.8.1) and a k_{cat} of 124 200 min^{-1} at 25 °C in pH 7.0 buffer (Dumas et al., 1990) for bacterial phosphotriesterase (EC 3.1.8.1 aryldialkylphosphatase).

Rate-Determining Step for OP Hydrolysis. We have shown that hydrolysis of the phosphotriesters, echothiophate and paraoxon, had two partially rate-determining steps. The rate constant for phosphorylation was about 2.0 min^{-1} and for dephosphorylation about 1.2 min^{-1}. By contrast a single step, dephosphonylation, was rate-limiting for hydrolysis of sarin and VX. Dephosphonylation was faster at low pH when histidine was protonated (Millard et al., 1995). These results show that the rate-determining events are different for these two classes of OP.

Aging. Aging is the loss of an alkoxy group from the phosphylated enzyme intermediate. (Phosphylated denotes phosphorylation and phosphonylation reactions without distinction.) Aging is catalyzed by His 438, which forms a hydrogen bond with the oxygen of the alkoxy group and thereby facilitates release of a carbonium ion (Kovach, 1988; Qian & Kovach, 1993; Michel et al., 1967). Another amino acid important to the aging process is the negatively charged Glu 197, which stabilizes the developing carbonium ion (Qian & Kovach, 1993; Saxena et al., 1993; Ordentlich et al., 1993). Aged enzyme is irreversibly inhibited as measured by the inability of oximes to reactivate it. Wild-type BChE inhibited with a diethoxyorganophosphate, such as paraoxon or echothiophate, ages with a half-time of 38.9 h at 22 °C in pH 7.4 buffer (Mason et al., 1993). G117H inhibited by echothiophate aged with a half-time of 5.5±0.2 h at 25 °C in pH 7.0 buffer. In G117H the rate of hydrolysis of the diethoxyphosphorylated intermediate was 600 times faster than the rate of aging, thus explaining the observation that a short incubation with echothiophate allowed recovery of 100% of the original activity without the addition of oximes. Similarly, Millard et al. (1995) regained 100% of the original activity following treatment of G117H with sarin or VX.

Twenty hours of treatment with pralidoxime (Wang & Braid, 1967) did not reactivate echothiophate-inactivated G117H. This result supports our interpretation that OP-inactivated G117H contains aged G117H.

Mechanism. A possible mechanism for hydrolysis of OP by G117H is shown in Figure 10. The early steps are adapted from the Kovach mechanism (Kovach, 1988; Qian & Kovach, 1993) for the reaction of soman and cholinesterase. Step 1 shows activation of Ser 198 Oγ by His 438 and Glu 325 and attack of Ser Oγ on the OP. Step 2 leads to formation of a pentavalent transition state in which the leaving group is still attached to the OP. The negatively charged oxygen is stabilized by hydrogen bonding to the main-chain nitrogens of the oxyanion hole. Step 3 forms a tetrahedral phosphorylated enzyme intermediate, which is unstable in G117H but is very stable in wild-type BChE. Ser 198 has formed a covalent bond with the OP and the leaving group has detached from the OP. The tetrahedral oxygen of the OP is in the oxyanion hole where it forms hydrogen bonds with the main-chain NH groups of Ala 199, Gly 116, and His 117. An alkoxy group on phosphorus is hydrogen-bonded to protonated His 438. In the Kovach mechanism for wild-type cholinesterase the species in step 3 is extremely stable and cannot undergo hydrolysis. Instead of hydrolysis it undergoes aging to form an irreversibly inhibited enzyme. G117H was capable of undergoing aging with echothiophate ($t_{1/2}$ = 5.5 ± 0.2 h) but this step was slow compared to hydrolysis ($t_{1/2}$ = 0.5 min). In step 4, His 117 forms a hydrogen bond with the other alkoxy group on phosphorus, thus weakening the hydrogen bond to His 438. After the hydrogen bond to His 438 is broken, His 438 deprotonates in step 5. This is followed by nucleophilic attack on phosphorus by a water molecule in step 6. The water molecule is stabilized by interaction with His 438 and with an alkoxy group on the OP. His 438 is available for activating the water molecule because it has lost its proton due to absence of interaction with the alkoxy group of the OP. In steps 7, 8, and 9 the OP is released from Ser 198, thus regenerating active enzyme. The hydrogen ion that goes back to Ser 198 comes from water.

The key feature of this mechanism is the protonation state of His 438. His 438 must be deprotonated to bind water (Kovach, 1988). In wild-type BChE, His 438 is frozen in the wrong protonation state because of interaction with the OP and is therefore unable to participate in hydrolysis. In the G117H mutant, His 117 facilitates deprotonation of His 438.

The mechanism in Figure 10 assumes that (1) His 117 forms a hydrogen bond with the alkoxy group on phosphorus, (2) His 438 forms only a transient hydrogen bond with the alkoxy group of the OP, (3) His 438 deprotonates and thus

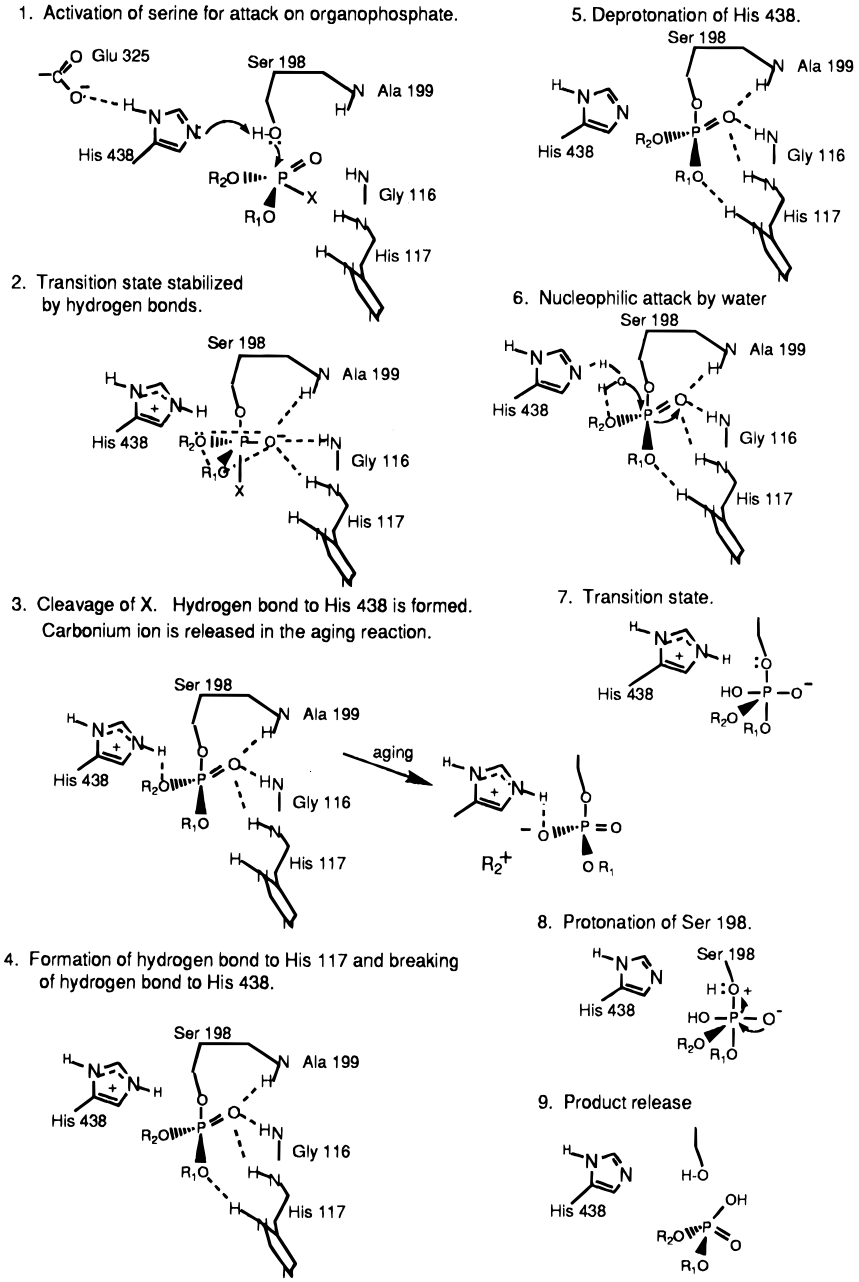

FIGURE 10: Proposed mechanism of organophosphate hydrolysis by the G117H mutant of human BChE. The early steps of this mechanism are from Kovach (1988) and Qian and Kovach (1993). The amino acids in the catalytic triad, Glu 325, His 438, and Ser 198, have a similar orientation as in Figure 1.

becomes available for binding a water molecule, (4) the water molecule binds to His 438, at the same site where it binds for hydrolysis of butyrylthiocholine (the alternative possibility, that water binds to His 117, has not been ruled out), and (5) the proton that goes back to Ser 198 comes from water.

Sarin and VX have only one alkoxy group on phosphorus. Therefore a hydrogen bond to His 117 through a second alkoxy group is not possible. Nevertheless, the protonated His 117 could withdraw electrons from phosphorus leading to a weakening of the hydrogen bond with His 438. This interpretation is consistent with the observation that dephosphonylation of nerve agents is fastest at pH 5.5 (Millard et al., 1995). The rate constant for hydrolysis of echothiophate, k_{cat}, was the same at all pH values tested. The k_{cat} value has contributions from k_2 and k_3. In Table 2 we found that k_2 and k_3 were close in value so that at least two steps were rate-limiting. In Figure 10, steps 4 and 8 are expected to be faster at acid pH, while steps 1, 5, and 6 are expected to be faster at alkaline pH. It is possible that these rates balance each other leading to a constant k_{cat} value at the pH values tested.

An alternative mechanism is a modification of Järv's idea that wild-type enzyme is not dephosphylated because of a steric problem. Järv proposed that water, bound to His 438, is unable to attack the correct face of the phosphorus to bring about dephosphylation (Järv, 1984). According to our model of phosphylated BChE, the His 438 side chain sits right in front of the correct face, sterically blocking attack by water. The G117H mutant causes two changes that alter dephosphylation rates. It distorts the oxyanion hole so that P═O sits askew and His 438 no longer blocks attack by water. The steric effect of G117H is independent of pH. The second change caused by the G117H mutation is that His 117 withdraws electrons through the phosphonyl oxygen and,

thereby, destabilizes the P−O−Ser and P−O−R′ bonds. His 438 can hydrogen-bond with O−R′ to promote aging or with O−Ser to promote reactivation in two competing reactions. In this view the rate-limiting step for hydrolysis of echothiophate is the steric effect of G117H (independent of pH), whereas the rate-limiting step for hydrolysis of the nerve agents is the electron-withdrawing effect (faster at low pH).

In conclusion, the G117H mutant of human BChE accelerates hydrolysis of paraoxon and echothiophate 100 000-fold. The potential usefulness of the G117H mutant of human BChE is for treatment of organophosphate poisoning and for decontamination of eye and skin.

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