

MUTANTS OF HUMAN BUTYRYLCHOLINESTERASE WITH ORGANOPHOSPHATE HYDROLASE ACTIVITY; EVIDENCE THAT HIS117 IS A GENERAL BASE CATALYST FOR HYDROLYSIS OF ECHOTHIOPHATE

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ABSTRACT

Human butyrylcholinesterase (BChE, EC 3.1.1.8) is an efficient scavenger of nerve agents and organophosphorus (OP) pesticides; one molecule of BChE inactivates one molecule of OP in a suicide reaction that irreversibly inhibits BChE. By contrast the BChE mutant, G117H, inactivates many molecules of OP. The OP makes a covalent bond with the active site serine and then the serine is dephosphorylated by the action of His117. In an effort to understand the mechanism by which His 117 achieves dephosphorylation, 62 new mutants of human BChE were tested for OP hydrolase activity, using a new screening assay. It was found that not only G117H, but also G117D, G117E, and L286H mutants were OP hydrolases. These results support the hypothesis that a hydrogen-bond acceptor acts as a general base to activate a water molecule which in turn dephosphorylates the active site serine. The screening assay provides a convenient means for identifying cholinesterase mutants with OP hydrolase activity.

INTRODUCTION

Organophosphorus cholinesterase inhibitors are widely used as pesticides and have been stockpiled and sometimes used as chemical warfare agents. Each year thousands of people are exposed to these compounds by accident or in attempts at suicide. The threat of the use of anticholinesterases in warfare or in terrorist attacks has increased greatly in recent years. Progress in protection against OP toxicity was achieved by using human butyrylcholinesterase (BChE) as a scavenger (Broomfield et al., 1991; Raveh et al., 1997). Laboratory animals including monkeys are protected from up to 4 LD₅₀ doses of

nerve agent by pretreatment with BChE (Raveh et al., 1997; Wolfe et al., 1992). A genetically engineered human BChE containing a single amino acid substitution, Gly 117 His, has the unusual property of being resistant to OP inhibition by virtue of its ability to hydrolyze OP (Millard et al., 1995; Lockridge et al., 1997; Millard et al., 1998). The G117H mutant hydrolyzes paraoxon (the active form of the common pesticide parathion) 120,000 times faster than spontaneous hydrolysis of paraoxon in water at pH 7 occurs (Lockridge et al., 1997). The G117H mutant also hydrolyzes acetylcholine. Transgenic mice expressing human G117H BChE are resistant to OP toxicity (Wang et al., 2004).

Our goals were to identify additional mutations in BChE that would provide resistance to OP and to determine the mechanism of OP resistance. A new OP hydrolase screening assay was developed; activity was inhibited with echothiophate [CASRN: 6736-03-4], and, the rate at which activity reappeared was taken as the rate of dephosphorylation. Sixty-two mutants were tested. Twenty-nine were single mutants, 31 were double mutants and two were triple mutants. Of these, 13 were found to be resistant. The OP resistant mutants all had a nucleophile at a distance less than 6—8 Angstroms from the phosphorylated active site serine. The nucleophile was a histidine, aspartic acid, or glutamic acid. This led to the conclusion that an activated water molecule was responsible for the dephosphorylation.

MATERIALS AND METHODS

Mutagenesis and Expression of Recombinant BChE. Mutagenesis and expression were performed as previously described (Xie et al., 1999; Josse et al., 1999). Briefly, mutations in human BChE were made by PCR with Pfu polymerase. Fragments containing the mutation were cloned into the plasmid pGS and the DNA sequenced to ensure that no unwanted mutations were present. Both stably transfected and transiently transfected cell lines were used. Stably transfected CHO-K1 cells (American Type Culture Collection, No CCL61) were selected in 50 μ M methionine sulfoximine. Expressed BChE was secreted from these cells and collected into serum free medium (Ultraculture 12-725B, BioWhittaker). For transient transfections, the plasmid was transfected into 293T/17 human embryonic kidney cells (American Type Culture Collection, No CRL 11268, used with permission of David Baltimore (Pear et al., 1993), by calcium phosphate-DNA coprecipitation as previously described (Josse et al., 1999). After four days, there was enough transiently expressed BChE to test. Untransfected media had no background hydrolysis of butyrylthiocholine (BTC) or OP.

Purification of BChE. In some instances, mutant forms of BChE were purified from culture medium taken from permanent cell lines, as previously described (Xie et al., 1999). Briefly, 1 to 10 liters of culture medium were passed over a 100 to 300 ml procainamide-Sepharose affinity column, which retained the BChE. Contaminating proteins were eluted with 0.1 M NaCl. BChE was selectively eluted with 0.2 M procainamide hydrochloride. Further purification was obtained by ion exchange chromatography on 400 ml of DE52 (Whatman) using a sodium chloride gradient (0 to 0.5 M NaCl in 20 mM Tris/Cl buffer pH 7.4) for elution. Purified enzyme was dialyzed into 20 mM potassium phosphate buffer pH 7.0, containing 0.3 mM EDTA. Enzyme was

either stored at 4 °C, or after addition of glycerol to 30%, was frozen at –80 °C. Wild-type BChE, used in this work, was purified from human serum using ion exchange chromatography at pH 4.0, followed by affinity and DE52 chromatography at neutral pH (Lockridge, 1990).

Organophosphatase screening assay. Reacting small amounts of OP with cholinesterases results in a gradual decrease in cholinesterase activity (Aldridge and Davison, 1952), which is referred to as progressive inhibition (Main, 1979). Main first introduced the idea of including substrate in the inhibition reaction so that the progress of the inhibition could be continuously monitored (Main and Dauterman, 1963). We have adapted Main's assay for progressive inhibition in the presence of substrate, to screen for OP resistance and OP hydrolysis activity in BChE mutants. After the enzyme, the inhibitor (echothiophate iodide: from Wyeth Ayerst, Rouses Point, NY), and the substrate (BTC and 5,5'-dithiobis-2-nitrobenzoic acid [DTNB]) were mixed in a cuvette, product formation was monitored spectrophotometrically at 412 nm. Progressive decrease in the rate of product formation reflected inhibition of the enzyme. All assays were performed at 25 °C, in 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mM BTC, 0.5 mM DTNB and variable amounts of echothiophate. Variable amounts of echothiophate (1 to 90 µM, depending upon the enzyme under study) were used to improve detection of dephosphorylation. When phosphorylation was too fast, the assay failed to detect OP hydrolysis. Therefore, the rate of phosphorylation was minimized by adjusting the concentration of echothiophate to require about 30 minutes to achieve maximum inhibition. If, after about 30 minutes of reaction with echothiophate, a linear steady rate of BTC hydrolysis was observed, that linear rate was taken to be an indication of OP turnover. As an illustration of this point, the reaction of the double mutant G117H/E197Q and the resulting linear rate of BTC hydrolysis is shown in figure 1.

The lower limit for detection was a dephosphorylation rate of 0.001 min⁻¹. A dephosphorylation rate as low as 0.001 min⁻¹ could be observed when the rate of phosphorylation was not faster than 0.1 min⁻¹. The rate of phosphorylation was about 0.1 min⁻¹ when the echothiophate concentration was low enough so that 30 minutes elapsed before a linear steady state level of BTC hydrolysis was observed. The exact concentration on echothiophate needed to achieve this condition varied from 1 to 90 µM, depending upon the enzyme under study.

No attempt was made to correct the observed rate of product formation from BTC for contribution from hydrolysis of echothiophate. Though both compounds generate thiol products, which would be detected in the Ellman assay, the rate for echothiophate turnover was found to be four orders of magnitude slower than for BTC (Lockridge et al., 1997). In addition, any residual product formation found in the screening assay, regardless of the origin, would qualify the candidate enzyme as a potential OP-hydrolase and make it subject to additional study.

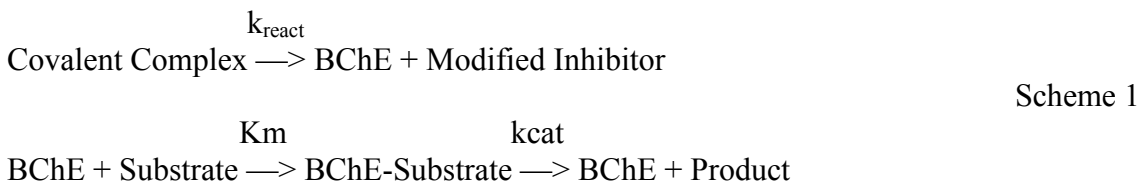
Spontaneous reactivation in the presence of BTC. Mutants that gave an indication of OP hydrolysis in the screening assay were directly assayed for spontaneous reactivation. For this assay, echothiophate-inhibited enzyme was diluted into buffer containing BTC and

DTNB, without removing excess inhibitor. Reactivation was monitored by the increase in rate of product formation. This method is similar to the approach described by Skrinjaric-Spoljar et al. (1973), and was chosen because some of the mutants reactivated so quickly that the conventional sampling method was impractical.

The first step in the spontaneous reactivation assay was to prepare inhibited BChE. BChE mutant was mixed with the minimum concentration of echothiophate needed to inhibit activity by 90-95% in 1-2 hours (1 to 90 μ M echothiophate depending upon the enzyme). It should be noted that active organophosphatases, such as G117H, could be inhibited only about 80% because the phosphorylation and dephosphorylation rate constants were similar. The amounts of enzyme and inhibitor used depended on the activity of the particular enzyme under investigation. The inhibition mixture was incubated at pH 7.0, 25 °C, in a sealed plastic microfuge tube, and aliquots were withdrawn periodically to measure residual activity. When maximal inhibition had been achieved, a 10-50 μ L aliquot was removed and added to a cuvette containing 2 mL of 0.1 M potassium phosphate buffer, pH 7.0, plus 1 mM BTC and 0.5 mM DTNB, at 25 °C. Product formation was monitored continuously at 412 nm. Reactivation was allowed to proceed until a constant rate of product formation was obtained (see figure 2 for an example).

This assay relies on the assumption that the magnitude of the dilution and competition from BTC are sufficient to prevent re-inhibition by residual echothiophate. This assumption was validated by control experiments in which the reactivation time course was shown to be unaffected by the concentration of echothiophate.

The mechanism for spontaneous reactivation in the presence of substrate is illustrated in Scheme 1.



Equation 1 describes the time dependence of product formation for scheme 1. It was derived by the general principles outlined by Tsou (1988).

$$[P] = Vt - \frac{V}{k_{\text{react}}} (1 - e^{-k_{\text{react}} t}) \quad \text{Equation 1}$$

k_{react} is the reactivation rate constant. V is the linear, steady state turnover rate (delta in absorbance at 412 nm per min) at the end of the reactivation portion of the time course. V/k_{react} is the y-axis intercept, extrapolated from the linear portion of the time course. Values for V and k_{react} were obtained by fitting the observed time course for reactivation to equation 1, using SigmaPlot (Jandel Scientific). Equation 1 is identical to the reactivation expression reported by Tsou if the re-inhibition terms in that equation are set to zero (see equation 13 in Tsou, 1988). The only assumptions involved in deriving

equation 1 are a) that the rates of substrate binding and turnover are very fast relative to the dephosphorylation rate, and b) that the substrate concentration is much larger than the enzyme concentration.

Aging in the absence of substrate. Aging kinetics were determined directly for BChE mutants that showed reactivation rates $>0.01 \text{ min}^{-1}$ because, at 0.01 min^{-1} , the reactivation process is well separated from aging, and the kinetics of the two do not interact significantly. An aging assay consisted of a series of spontaneous reactivation assays from a single inhibition reaction. The inhibition mixture was prepared as described in the reactivation section, and was incubated at $25 \text{ }^\circ\text{C}$ for up to several days. Reactivation assays were conducted periodically to determine the amount of residual activity remaining after reactivation. The limiting steady state rate of substrate turnover at the end of each reactivation assay (V) is directly proportional to the concentration of active (unaged) enzyme remaining. A parallel incubation of uninhibited enzyme was assayed periodically to control for spontaneous loss of enzymatic activity.

RESULTS

Design of BChE mutants. A model (Millard and Broomfield, 1992) of the three-dimensional structure of human BChE, shown later to be consistent with the crystal structure of human BChE (Nicolet et al., 2003; Protein Data Bank 1P0i), was used to design mutants that might have OP hydrolase activity. Histidine was moved to various locations in the active site to test the effect of the position of the positive charge. Acidic residues were added to ion pair with the extra histidines, thereby creating more protonated histidine character at neutral pH. The G117D mutation was introduced to correspond with the naturally occurring mutation in the sheep blowfly, whose carboxylesterase is an OP hydrolase (Newcomb et al., 1997; Claudianos et al., 1999). The mutations in the OP resistant acetylcholinesterase (AChE) of *Drosophila melanogaster* (Mutero et al., 1994) were introduced into BChE. A variety of amino acid residues at position 117 tested the absolute requirement for a positive charge in that location. Many of these mutants hydrolyzed echothiophate, but none had better echothiophate-hydrolysis activity than G117H.

Figure 3 is a schematic representation of the active site of BChE showing the catalytic triad, S198, H438, and E325, at the bottom of the active site gorge. G117 is part of the oxyanion hole that stabilizes the transition state intermediate. The acyl binding pocket is defined by L286 and V288. Two tryptophan residues, W231 and W82, are prominent components of the wall of the active site gorge. Positively charged choline is oriented toward W82 and A328. The leaving group of echothiophate is oriented toward the mouth of the gorge.

Screening for resistance to OP inhibition. When echothiophate was added to wild-type BChE, product formation came to a complete stop, indicating complete inhibition of the BChE by OP. When the mutant was OP resistant, product formation slowed to a constant rate, reflecting a steady state level of active enzyme. For example in figure 1, the double mutant G117H/E197G was still able to hydrolyze BTC in the presence of $100 \text{ } \mu\text{M}$

echothiophate. The rate slowed to a steady state at 55 minutes, which is about 30 minutes after addition of echothiophate.

Screening for reactivation of echothiophate-inhibited mutants. An example of the reactivation assay results is shown in figure 2, where the L286H mutant achieved a constant rate of product formation after 300 minutes. Sixty-two BChE mutants were screened for reactivation after echothiophate inhibition. Thirteen, including G117H, showed significant rates of reactivation in the organophosphatase screening assay. Spontaneous reactivation rate constants were determined for these thirteen mutants by fitting the time course data to equation 1. Results are summarized in table 1.

The G117H mutant exhibited the fastest reactivation rate constant, $1.15 \pm 0.15 \text{ min}^{-1}$. This is identical to the value reported by Lockridge et al., 1.2 min^{-1} , pH 7.0, 25 °C (Lockridge et al., 1997); and it is slightly faster than that reported by Broomfield et al., 0.48 min^{-1} , pH 7.4, 25 °C (Broomfield et al., 1998). Echothiophate creates a diethylphosphoryl-adduct of BChE. Therefore, these dephosphorylation rate constants can be compared to that for reactivation of wild-type BChE, after inhibition by the diethylphosphoryl-organophosphate paraoxon ($0.000016 \text{ min}^{-1}$, pH 7.8, 37 °C, Davison, 1955). The G117H mutant reactivates about 70,000-fold faster than wild-type BChE.

Addition of other mutations to G117H slowed reactivation. Even attempts at ion-pairing, which were designed to enhance the nucleophilicity of the histidine at position-117, were unproductive, e.g., G117H/Q119E, G117H/A199E, and G117H/F329E/V288A.

With the exception of L286H, moving the histidine to other locations in the active site was also unproductive, e.g., V288H, Q119H, T120H, and A277H. Though the L286H mutant reactivated 400-fold more slowly than G117H, it was still 250-fold faster than wild-type BChE. Adjusting the position of histidine 286 by adding glycine residues to the sequence, e.g., L286GH, L286HG, and L286GHG, did not improve the reactivation rate.

Changing the residue at position-117 revealed that acidic residues G117E and G117D were significantly reactive, but less nucleophilic residues such as G117S, G117C, and G117Y were not. The mutation G117S is homologous to the mutation G119S found in *ace-1* of the insecticide resistant mosquitoes *Anopheles gambiae* and *Culex pipiens* (Weill et al., 2003). Mosquitoes were resistant to the carbamate propoxur but were not shown to be resistant to OP.

The following 49 mutants gave no convincing evidence for reactivation: G117H/G115H, G117H/G439H, G117H/V288H, G117H/A199H, G117H/L286H, G117H/A328H, G117H/F329H, V288H/F329D, V288H/F329E, V288H/G291D, G117H/F329E/V288A, V288H, V288GH, V288GHG, Q119H, T120H, A277H, G117C, G117K, G117S, G117Y, G117L, G117H/Q119E, G117H/A199E, G117H/L286Y, G117H/F329Y, F329E, F329S, K339M, F329S/E197Q, F398I, G117H/F398I, P285L, P285L/F398I, G117H/P285L, G117H/P285L/F398I, G225A, L286Y, G225A/L286Y, E197Q,

G117H/W82A , G117H/Y332A, G117H/E197H, W430A, F329S/E197Q, E325Q, G117H/E325Q, G117H/A328W, and G117H/L286R.

Determination of the dephosphorylation rate constants from the spontaneous reactivation rate constants. Spontaneous reactivation is the combination of two parallel processes: dephosphorylation and aging. Aging is defined as dealkylation of the phosphoryl adduct to give a negatively charged adduct that is stabilized by interaction with the catalytic histidine (Millard et al., 1999). Aging irreversibly prevents dephosphorylation and limits the amount of enzyme that becomes reactivated. The fraction of enzyme that becomes reactivated is described by equation 2.

$$[E] = \frac{k_{\text{dephos}} * [Eo]}{k_{\text{dephos}} + k_{\text{aging}}} (1 - e^{-(k_{\text{dephos}} + k_{\text{aging}}) * t}) \quad \text{Equation 2}$$

[E] is the concentration of reactivated enzyme at time t. [Eo] is the total concentration of enzyme. k_{dephos} is the dephosphorylation rate constant; k_{aging} is the aging rate constant; and $(k_{\text{dephos}} + k_{\text{aging}})$ is the observed rate constant for spontaneous reactivation (k_{react}). This equation comes directly from the theory of parallel first-order reactions (Frost and Pearson, 1961) and was described first in the cholinesterase literature by Skrinjaric-Spoljar et al. (1973). If the reactivation is allowed to run to completion, then the exponential term becomes very small relative to one, and equation 2 can be re-arranged to give equation 3.

$$\frac{[E]}{[Eo]} (k_{\text{react}}) = k_{\text{dephos}} \quad \text{Equation 3}$$

The ratio [E]/[Eo] is the fraction of enzyme, which becomes reactivated. This fraction is equal to the measured, linear, steady state velocity at the end of reactivation (V) divided by the velocity for an equal amount of uninhibited enzyme. Multiplying the observed k_{react} by the fraction of reactivated enzyme gives k_{dephos} . Calculated values for k_{dephos} are listed in table 1. For most of the mutants k_{dephos} was essentially equal to k_{react} .

A complication for the values reported in table 1 derives from the fact that reactivation of echothiophate-inhibited BChE is slowed by the presence of 1 mM BTC. For example, with G117H reactivation was slowed by about 30%, i.e., 1.4 min^{-1} at zero BTC and 1.0 min^{-1} at 1 mM BTC (see table 2). Therefore, the values reported in table 1 must be considered as approximate.

Aging. In all cases, the concentration of reactivatable enzyme decreased in a first-order manner with time, yielding the half-life values for aging. Aging for all six mutants studied was substantially slower than aging for G117H ($t_{1/2} = 5.5$ hour, (Lockridge et al., 1997), see table 1.

Characterization of the OP hydrolysis properties of G117H. In order to obtain a better appreciation of the capabilities of G117H, kinetics for phosphorylation,

dephosphorylation and aging were determined with: methyl-paraoxon (which forms a dimethylphosphoryl-adduct), echothiophate (which forms a diethylphosphoryl-adduct) and diisopropylfluorophosphate (which forms a diisopropylphosphoryl-adduct).

Dephosphorylation rate constants were calculated from the reactivation rate constants and the fractions of G117H reactivated, using equation 3. Reactivation measurements were made in the presence of BTC because the reactivation rates of G117H were too rapid to allow the measurements to be made in the absence of substrate. In order to determine the reactivation rate constant in the absence of BTC, a series of experiments at different BTC concentrations were made and the value for the apparent reactivation rate constant was extrapolated to zero BTC (see figure 4 for an example and details of the extrapolation). In addition to providing a basis for calculating the dephosphorylation rate constant at zero BTC, these experiments gave the dissociation constant for BTC binding to the phosphoryl-enzyme adduct, and the dephosphorylation rate constant in the presence of saturating BTC. The results are summarized in table 2.

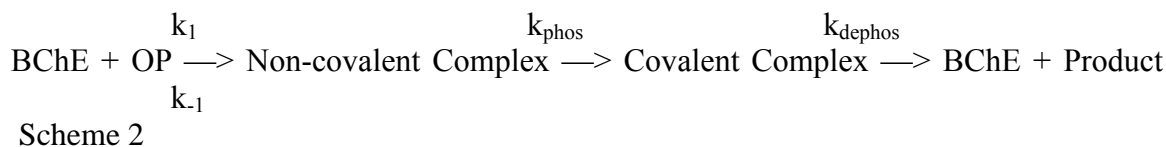
Aging was determined separately, in the absence of substrate. The results are shown in table 3. The aging reactions of G117H all followed first-order time courses for more than three half-lives. The spontaneous reactivation rate constants were at least 20-fold faster than their respective aging rate constants, therefore use of spontaneous reactivation to determine the fraction of unaged enzyme remaining at each time point, did not bias the measurements. The results are shown in table 3.

Phosphorylation rate constants were calculated rather than measured directly. This is because the classical method for measuring phosphorylation rate constants was unworkable. In the classical method, OP is mixed with enzyme and the mixture is sampled periodically to determine the amount of uninhibited enzyme remaining (Main, 1979). Preliminary experiments indicated that with G117H the rate constant for dephosphorylation was about the same as that for phosphorylation. This meant that when the concentration of inhibitor was high enough so that phosphorylation could compete with dephosphorylation, the rate of inhibition was too fast to measure. But, when the concentration of inhibitor was lowered, the rate for dephosphorylation became large relative to the rate for phosphorylation, and the fraction of inhibited G117H became too small to measure. Consequently, we turned to calculation.

For this calculation, we used the maximum fraction of covalently inhibited G117H, together with the rate constant for dephosphorylation in the absence of substrate (measured separately). This analysis is conceptually similar to that used by Froede and Wilson (1984) to determine the fraction of acetyl-enzyme present during turnover.

The fraction of covalently inhibited G117H was determined by diluting the inhibited enzyme into a solution containing substrate, and measuring the residual activity. Dilution and competition from substrate dissociated the non-covalently inhibited complex, leaving only the covalently inhibited form. The maximum fraction of covalent complex was determined by increasing the concentration of OP until no further increase in the extent of inhibition was detected.

Calculations were based on the general mechanism for turnover of OP (Main, 1979) diagrammed in scheme 2.



The expression for the time dependent change in the concentration of the covalent complex for this mechanism can be derived following the general principles described by Strickland et al. (1975).

$$[C] = \frac{\text{Et} * [\text{OP}] * k_{\text{phos}}}{k_{\text{dephos}} * [\text{OP}] + k_{\text{dephos}} * K_d + k_{\text{phos}} * [\text{OP}]} (1 - e^{-k_{\text{app}} * t})$$

Equation 4

[C] is the concentration of covalent complex. Et is the total concentration of BChE. [OP] is the concentration of OP. k_{app} is the apparent rate constant for phosphorylation. K_d is k_{-1}/k_1 , k_{phos} is the phosphorylation rate constant, and k_{dephos} is the dephosphorylation rate constant.

The inhibition reaction was allowed to reach equilibrium before the fraction of covalent complex was measured. The time required to reach equilibrium was determined from preliminary experiments. At equilibrium, the exponential term becomes small relative to unity. When the concentration of OP is saturating, $[\text{OP}] \gg K_d$ and the concentration of covalent complex is maximal. Then, equation 4 can be reduced and re-arranged into equation 5.

$$k_{\text{phos}} = k_{\text{dephos}} \left(\frac{[C]_{\text{max}}/\text{Et}}{1 - [C]_{\text{max}}/\text{Et}} \right)$$

Equation 5

$[C]_{\text{max}}/\text{Et}$ is the maximum fraction of covalent complex.

DISCUSSION

Advantages of this screening assay. Following the progressive inhibition of BTC turnover by echothiophate is a simple and sensitive method for evaluating mutants of BChE for OP-hydrolase activity. The inability of echothiophate to completely inhibit BTC hydrolysis is highly suggestive of echothiophate hydrolysis by the mutant. The full time course of progressive inhibition provides an immediate, visual depiction of the level of residual activity. With a time course of 30-45 minutes, the assay is relatively rapid. The assay can be performed on sub-nanomolar levels of candidate enzyme—where 0.1 nanomolar (nM) wild-type BChE yields a readily detectable rate of product formation in the Ellman assay, when using 0.1 M BTC at pH, 25 °C. The assay can be performed on

crude enzyme preparations, since BTC is highly selective for BChE. Thus, it is suitable for testing mutants made via transient transfection, without purification. In contrast, direct turnover of echothiophate is not a suitable tool for mutant screening. Though turnover of echothiophate would provide definitive evidence for OP-hydrolase activity, the rates for echothiophate turnover are too slow to be practical. Lockridge et al. (1997) have shown that G117H, the most reactive of the BChE mutants, turns over echothiophate with a rate constant of 0.75 min^{-1} . Three μM G117H were required to give reliable rates of product formation. In addition, use of crude preparations for echothiophate turnover creates a real potential for interference from A-esterases, such as paraoxonase. Direct turnover is more suited for confirmation of candidate mutants, which have been purified in micromolar concentrations.

Validity of the assay. Resistance of BChE to inhibition by OP can theoretically take two forms. Either the reactivity toward OP can be reduced, making inhibition more difficult, or reactivation of the inhibited enzyme can be enhanced, making recovery from inhibition faster. The screening assay employed in this work tested for the latter form of resistance, enhancing the reactivation of the inhibited enzyme.

The reactivation rate was used to calculate the rate of dephosphorylation of the active site serine. This calculation assumed that the active site serine became phosphorylated. Since OP was not used as a substrate, but only as an inhibitor, the assay leaves open the possibility that the OP bound somewhere other than to the active site serine and that dissociation from this noncovalent binding site accounted for the return of BChE activity. The assay described in this report does not rule out this possibility. Additional experiments have to be performed, after the screening assay is complete, to confirm OP hydrolase activity. For example, OP hydrolase activity for G117H BChE was demonstrated by Millard et al. (Millard et al., 1995; Millard et al., 1998) by measuring consumption of OP substrate. Lockridge et al. (1997) measured release of thiocholine from echothiophate and release of para-nitrophenol from paraoxon to demonstrate OP hydrolase activity of G117H. Claudianos et al. (1999) measured hydrolysis of a radiolabeled OP, C14-chlorfenvinphos, by the sheep blowfly carboxylesterase mutant G117D ortholog. The advantage of the present assay is that only small amounts of enzyme are required. This makes it possible to screen many mutants produced by transient expression in 293T or COS cells.

Mechanistic interpretation of the dephosphorylation enhancement promoted by G117H. The results indicate that position-117 plays a critical role in enhancing reactivation of echothiophate-inhibited BChE. There was a histidine at this position in six out of thirteen mutants that showed significant dephosphorylation rate constants. Two of the remaining seven reactive mutants had an acidic group at this position.

Mutation G117H was originally conceived as a means of introducing a nucleophilic residue into BChE, at a position which was geometrically favorable for activating water to attack the face of the phosphoryl-adduct opposite the position of the serine ligand (Broomfield et al., 1995). Jarv had earlier proposed that phosphoryl-adducts of serine esterases were stable because their active sites were not capable of activating water for

attack on the phosphorus at this position (Jarv, 1984; Jarv, 1989). The 70,000-fold increase in the dephosphorylation rate constant upon introduction of histidine at position-117 supports the original hypothesis. Increase in the dephosphorylation rate constant upon substitution of acidic groups into this position (G117D and G117E) further supports the proposal, because the major feature which histidine, aspartate, and glutamate have in common is their ability of serve as a hydrogen-bond acceptor for water.

It is interesting to note, that a naturally occurring, homologous mutation (G137D) has been reported for carboxylesterase in OP resistant strains of both the blowfly (*Lucillia cuprina*) and the housefly (*Musca domestica*) (Newcomb et al., 1997; Claudianos et al., 1999). The authors in this case suggested that the increase in OP hydrolase activity was due to aspartate-137 acting as a base to orient a water molecule for attack on the phosphorylated enzyme intermediate (Newcomb, 1997), a hypothesis in agreement with the present results.

Four of the five remaining mutants that showed enhanced dephosphorylation rate constants, also carried a histidine. For those mutants, the histidine was at position-286. As was the case with position-117, inspection of the 3-dimensional structure of human BChE suggested that a histidine at position-286 might be favorably positioned to activate a water molecule for attack on the phosphoryl-adduct. Enhancement of dephosphorylation by L286H was 300-fold less than for G117H, but the mere existence of an enhancement supports the principle.

Dephosphorylation, phosphorylation and aging with G117H. The phosphorylation rate constants for G117H (4.1 min⁻¹ with diisopropylfluorophosphate and 2.4 min⁻¹ with echothiophate) are within an order of magnitude of those for wild-type BChE (11 min⁻¹ with diisopropylfluorophosphate (Main and Iverson, 1966) and 0.23 min⁻¹ with echothiophate (Broomfield et al., 1995). The t_{1/2} values of aging for G117H (0.68 hour with the dimethylphosphoryl-adduct, 5.5 hour with the diethylphosphoryl-adduct (Lockridge et al., 1997), and 0.46 hour, with the diisopropylphosphoryl-adduct) are less than 10-fold lower than the corresponding values for wild-type BChE (3.9 hour with the dimethylphosphoryl-adduct (Worek et al., 1999), 11.6 hour with the diethylphosphoryl-adduct (Masson et al., 1997), and 0.96 hour with the diisopropylphosphoryl-adduct (Masson et al., 1997). Allowing for the differences in experimental conditions, the differences in phosphorylation and aging between G117H and wild-type BChE are minor. On the other hand, the dephosphorylation rate constants for G117H (0.23 min⁻¹ with the dimethylphosphoryl-adduct, and 1.4 min⁻¹ with the diethylphosphoryl-adduct) are respectively, 200- and 87,500-fold faster than those for wild-type BChE (0.0013 min⁻¹ with the dimethylphosphoryl-adduct (Worek et al., 1999), and 0.000016 min⁻¹ with the diethylphosphoryl-adduct (Davison, 1955). Thus the primary effect of introducing a histidine into position-117 of BChE has been to increase the rate constant for dephosphorylation.

It is noteworthy that the dephosphorylation rate constants for G117H are highly dependent upon the nature of the adduct. There is a 6-fold difference in rate constant between the dimethylphosphoryl-adduct (0.23 min⁻¹) and the diethylphosphoryl-adduct (1.4 min⁻¹).

The dephosphorylation rate constants for the highly toxic nerve agents are substantially slower: sarin (methyl, O-isopropylphosphonyl-adduct; 0.018 min^{-1}), VX (methyl, O-ethylphosphonyl-adduct; 0.07 min^{-1}), and soman (methyl, O-pinacolylphosphonyl-adduct; $<0.00005 \text{ min}^{-1}$) (Millard et al., 1995; Millard et al., 1998).

Significance. The significance of these findings is in understanding the mechanism of OP hydrolase activity in a BChE mutant. The G117H mutant is a rare example of an enzyme that was designed to have a new catalytic activity. The design relied on a hypothesis for how the active site serine could be dephosphorylated. This report supports the original hypothesis for the mechanism of dephosphorylation. An understanding of the mechanism of action could be useful for the design of additional OP hydrolase mutants. A second significant outcome of this work is the introduction of an assay that allows screening for apparent OP hydrolase activity.

REFERENCES

Aldridge, W. N. and Davison, A. N. (1952) The inhibition of erythrocyte cholinesterases by tri-esters of phosphoric acid. *Biochem J.* **51**, 62–70.

Broomfield, C.A., Maxwell, D.M., Solana, R.P., Castro, C.A., Finger, A.V., and Lenz, D.E. (1991) Protection by butyrylcholinesterase against organophosphorus poisoning in nonhuman primates. *J Pharmacol Exp Ther.* **259**, 633–638.

Broomfield, C. A., Millard, C. B., Lockridge, O., and Caviston, T. L. (1995) in *Enzymes of the Cholinesterase Family* (Quinn, D. M., Balasubramanian, A. S., Doctor, B. P., and Taylor, P., Eds.) pp. 169–175, Plenum Press, New York, NY.

Broomfield, C. A., Mills, K. V., Meier, B. M., Lockridge, O., and Millard, C. B. (1998) in *Structure and Function of Cholinesterases and Related Proteins* (Doctor, B. P., Taylor, P., Quinn, D. M., Rotundo, R.L., and Gentry, M. K., Eds.) pp. 223–226, Plenum Press, New York, NY.

Claudianos, C., Russell, R.J., and Oakeshott, J.G. (1999) The same amino acid substitution in orthologous esterases confers organophosphate resistance on the house fly and a blowfly. *Insect Biochem Molec Biol.* **29**, 675–686.

Davison, A. N. (1955) Return of cholinesterase activity in the rat after inhibition by organophosphorus compounds. 2. A comparative study of true and pseudo cholinesterase. *Biochem J.* **60**, 339–346.

Fersht, A. (1977) *Enzyme Structure and Mechanism*, p 135, W.H. Freeman and Co., San Francisco.

Froede, H. and Wilson, I. B. (1984) Direct determination of acetyl-enzyme intermediate in the acetylcholinesterase-catalyzed hydrolysis of acetylcholine and acetylthiocholine. *J Biol Chem.* **259**, 11010–11013.

Frost, A. A. and Pearson, R. G. (1961) *Kinetics and Mechanism 2nd ed.*, pp. 160–161 John Wiley & Sons, New York, NY.

Jarv, J. (1984) Stereochemical aspects of cholinesterase catalysis. *Bioorg Chem.* **12**, 259–278.

Jarv, J. (1989) in *Enzymes Hydrolyzing Organophosphorus Compounds* (Reiner, E., Aldridge, W. N., and Hoskin, F. C. G., Eds.) pp. 221–225, Ellis Horwood Ltd, Chichester, UK.

Josse, D., Xie, W., Renault, F., Rochu, D., Schopfer, L. M., Masson, P., and Lockridge, O. (1999) Identification of residues essential for human paraoxonase (PON1) arylesterase/organophosphatase activities. *Biochemistry* **38**, 2816–2825.

Lockridge, O. (1990) Genetic variants of human serum cholinesterase influence metabolism of the muscle relaxant succinylcholine. *Pharmacol Ther.* **47**, 35–60.

Lockridge, O., Blong, R.M., Masson, P., Froment, M.T., Millard, C.B., and Broomfield, C.A. (1997) A single amino acid substitution, Gly117His, confers phosphotriesterase (organophosphorus acid anhydride hydrolase) activity on human butyrylcholinesterase. *Biochemistry.* **36**, 786–795.

Main, A. R. and Dauterman, W. C. (1963) Determination of the bimolecular rate constant for the reaction between organophosphorous inhibitors and esterases in the presence of substrate. *Nature.* **198**, 551–553.

Main, A. R. and Iverson, F. (1966) Measurement of the affinity and phosphorylation constants governing irreversible inhibition of cholinesterases by di-isopropyl phosphorofluoridate. *Biochem J.* **100**, 525–531.

Main, A. R. (1979) Mode of action of anticholinesterases. *Pharmacol Ther.* **6**, 579–628.

Masson, P., Fortier, P. L., Albaret, C., Froment, M. T., Bartels, C., and Lockridge, O. (1997) Aging of di-isopropyl-phosphorylated human butyrylcholinesterase. *Biochem J.* **327**, 601–607.

Millard, C. B. and Broomfield, C.A. (1992) A computer model of glycosylated human butyrylcholinesterase. *Biochem Biophys Res Commun.* **189**, 1280–1286.

Millard, C.B., Lockridge, O., and Broomfield, C.A. (1995) Design and expression of organophosphorus acid anhydride hydrolase activity in human butyrylcholinesterase. *Biochemistry* **34**, 15925–15933.

Millard, C.B., Lockridge, O., and Broomfield, C.A. (1998) Organophosphorus acid anhydride hydrolase activity in human butyrylcholinesterase: synergy results in a somanase. *Biochemistry*. **37**, 237–247.

Millard, C.B., Koellner, G., Ordentlich, A., Shafferman, A., Silman, I., and Sussman, J.L. (1999) Reaction products of acetylcholinesterase and VX reveal a mobile histidine in the catalytic triad. *J Am Chem Soc*. **121**, 9883–9884.

Mutero, A., Pralavorio M., Bride, J.M., and Fournier D. (1994) Resistance-associated point mutations in insecticide-insensitive acetylcholinesterase. *Proc Natl Acad Sci USA*. **91**, 5922–5926.

Newcomb, R.D., Campbell, P.M., Ollis, D.L., Cheah, E., Russell, R.J., and Oakeshott, J.G. (1997) A single amino acid substitution converts a carboxylesterase to an organophosphorus hydrolase and confers insecticide resistance on a blowfly. *Proc Natl Acad Sci USA*. **94**, 7464–7468.

Nicolet, Y., Lockridge, O., Masson, P., Fontecilla-Camps, J.C., and Nachon, F. (2003) Crystal structure of human butyrylcholinesterase and of its complexes with substrate and products. *J Biol Chem*. **278**, 41141–41147.

Pear, W. S., Nolan, G. P., Scott, M. L., and Baltimore, D. (1993) Production of high-titer helper-free retroviruses by transient transfection. *Proc Natl Acad Sci USA*. **90**, 8392–8396.

Raveh, L., Grauer, E., Grunwald, J., Cohen, E., and Ashani, Y. (1997) The stoichiometry of protection against soman and VX toxicity in monkeys pretreated with human butyrylcholinesterase. *Toxicol Appl Pharmacol*. **145**, 43–53.

Skrinjaric-Spoljar, M., Simeon, V., and Reiner, E. (1973) Spontaneous reactivation and aging of dimethylphosphorylated acetylcholinesterase and cholinesterase. *Biochim Biophys Acta*. **315**, 363–369.

Strickland, S., Palmer, G., and Massey, V. (1975) Determination of dissociation constants and specific rate constants of enzyme-substrate (or protein-ligand) interactions from rapid reaction kinetic data. *J Biol Chem*. **250**, 4048–4052.

Tsou, C. L. (1988) Kinetics of substrate reaction during irreversible modification of enzyme activity. *Adv Enzymol Relat Areas Mol Biol*. **61**, 381–436.

Wang, Y., Boeck, A.T., Duysen, E.G., Saunders, T.L., Van Keuren, M., and Lockridge, O. (2004) Resistance to organophosphorus agent toxicity in transgenic mice expressing the G117H mutant of human butyrylcholinesterase. *Toxicol Appl Pharmacol*. In press.

Weill, M., Lutfalla, G., Mogensen, K., Chandre, F., Berthomieu, A., Berticat, C., Pasteur, N., Philips, A., Fort, P., Raymond, M. (2003) Insecticide resistance in mosquito vectors. *Nature*. **423**, 136–137.

Wolfe, A.D., Blick, D.W., Murphy, M.R., Miller, S.A., Gentry, M.K., Hartgraves, S.L., and Doctor, B.P. (1992) Use of cholinesterases as pretreatment drugs for the protection of Rhesus monkeys against soman toxicity. *Toxicol Appl Pharmacol*. **117**, 189–193.

Worek, F., Diepold, C., and Eyer, P. (1999) Dimethylphosphoryl-inhibited human cholinesterases: inhibition, reactivation, and aging kinetics. *Arch Toxicol*. **73**, 7–14.

Xie, W., Altamirano, C.V., Bartels, C. F., Speirs, R. J., Cashman, J. R., and Lockridge, O. (1999) An improved cocaine hydrolase: the A328Y mutant of human butyrylcholinesterase is 4-fold more efficient. *Mol Pharmacol*. **55**, 83–91.

Table 1. Kinetic constants for reactivation and aging of echothiophate inhibited BChE

Number	Enzyme *	k_{react} (min^{-1}) measured †	% reactivated measured	k_{dephos} (min^{-1}) calculated ‡	aging $t_{1/2}$ § (hr) measured
0	WT ‖	<0.0003	≈3	id	nd
1	G117H	1.15±0.15	99±4	1.15	nd
2	L286H	0.0040±0.00001	79±0.1	0.0032	nd
3	L286GHG	0.0032±0.0009	50±14	0.0016	nd
4	L286HG	0.0023±0.00005	44±1.0	0.0010	nd
5	L286GH	0.0024±0.00003	96±1.1	0.0024	nd
6	G117E	0.0073±0.00007	50±0.5	0.0037	nd
7	G117D	0.025±0.00016	95±0.6	0.025	44±2
8	W231A	0.0015±0.00001	63±0.4	0.0009	nd
9	G117H/E197Q	0.027±0.00014	89±0.4	0.024	14.8±0.7
10	G117H/E197D	0.086±0.0008	100±1.0	0.084	17±0.1
11	G117H/E197G	0.015±0.00008	100±0.5	0.014	>50±0.5
12	G117H/W82F	0.20±0.0088	85±3.7	0.17	13±0.1
13	G117H/A328G	0.79±0.011	76±1.0	0.60	33±3

* Purity of the enzymes varied. WT, G117H, G117D and G117H/E197Q were purified, as described in Materials and Methods. W231A, G117H/W82F, and G117H/A328G were partially purified by procainamide affinity chromatography. L286H, L286GHG, L286HG, L286GH, G117E, G117H/E197D, and G117H/E197G were in cell culture media (Ultraculture plus 25 μM methionine sulfoximine).

† Values for k_{react} were measured in 0.1 M potassium phosphate buffer at pH 7.0 and 25 °C, in the presence of 1 mM BTC and 0.5 mM DTNB, as described in Materials and Methods.

‡ Values for k_{dephos} were calculated as described in Results (equation 3). “id” indicates that there was insufficient data to allow calculation.

§ Aging measurements were performed in the absence of substrate. “nd” indicates that the value was not measured.

‖ WT refers to wild-type BChE.

Table 2. Effect of BTC concentration on the measured rate constant for reactivation of G117H*

Inhibitor	Kd for BTC (mM)	k _{react} (min ⁻¹)		
		zero BTC	1 mM BTC	saturated BTC
methyl-paraoxon	0.9±0.6	0.26±0.05	0.13	0.008±0.03
echothiophate	1.3±0.54	1.4±0.07	1.0	0.35±0.12
diisopropylfluorophosphate	0.66±0.08	4.1±0.11	2.0	0.57±0.07

*See the legend to figure 4 for details of the calculations that yielded Kd, k_{react} at zero BTC and k_{react} at saturating BTC. All data were taken in 0.1 M potassium phosphate buffer, pH 7.0, at 25 °C.

Table 3. Rate constants for reaction of G117H with OP, in the absence of BTC *

OP	k _{dephos} [†] (min ⁻¹)	k _{phos} [‡] (min ⁻¹)	t1/2 for aging (hr)
methyl-paraoxon	0.23±0.05	0.98 [‡]	0.68±0.08
echothiophate	1.4±0.07	2.4 [‡]	5.5±0.2 [§]
diisopropylfluorophosphate	3.7±0.11	3.7 [‡]	0.46±0.04

*. All data were taken in 0.1 M potassium phosphate buffer, pH 7.0, at 25 °C.

[†] Calculated from the % reactivation at zero BTC (i.e. 89 for methyl-paraoxon, 99 for echothiophate, and 90 for diisopropylfluorophosphate) and k_{react} extrapolated to zero BTC concentration (taken from table 2), using Equation 3 in Results.

[‡] Calculated from the maximum fraction of covalent inhibition (i.e. 0.81 for methyl-paraoxon, 0.63 for echothiophate, and 0.50 for diisopropylfluorophosphate) and k_{dephos}, in the absence of BTC, using Equation 5 in Results.

[§] Taken from Lockridge et al. (1997)

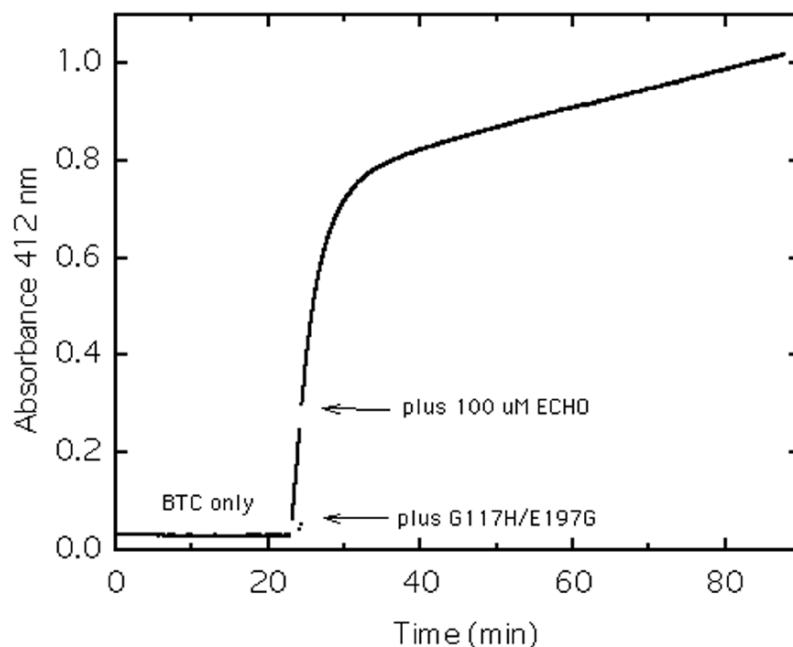


Figure 1. Organophosphatase screening assay. Mutants resistant to inhibition by echothiophate were identified in this assay. Two mL of 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mM BTC and 0.5 mM DTNB were incubated at 25 °C to establish a background rate for spontaneous hydrolysis (marked: BTC only). Ten μL of G117H/E197G were then added (marked: plus G117H/E197G) to establish an uninhibited reaction rate. Then 10 μL of 20 mM echothiophate (100 μM final concentration) were added (marked: plus 100 μM ECHO) to develop inhibition. Product formation was followed by the increase in absorbance at 412 nm.

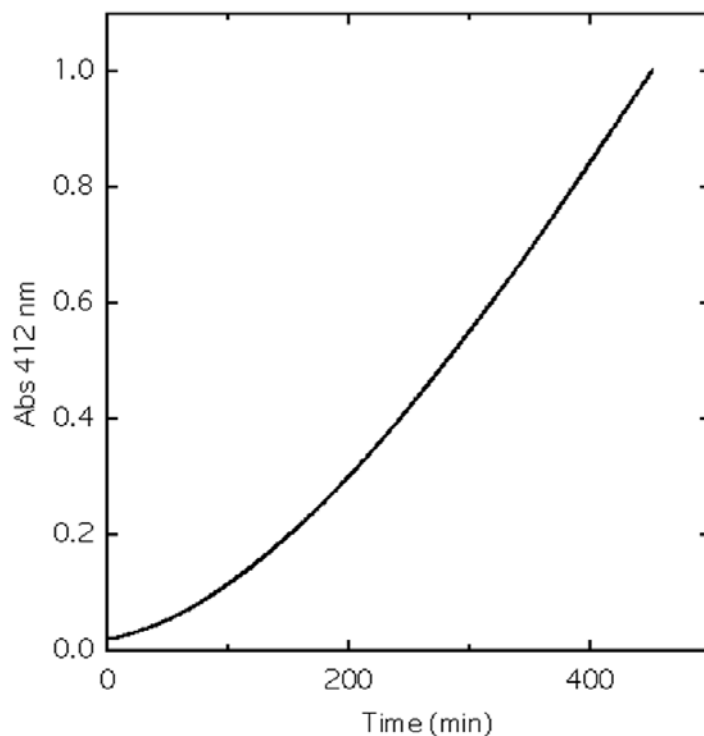


Figure 2. Reactivation of echothiophate-inhibited BChE mutant. This assay yielded the rate constant for reactivation, k_{react} , and a value for V , the linear steady-state turnover rate in Equation 1. Fifty μL of L286H were mixed with 5 μL of 20 mM echothiophate, then incubated at 25 °C in a sealed microfuge tube for 172 minutes. Greater than 90% inhibition was achieved. A 10 μL aliquot of the inhibition mixture was then diluted into 2.0 ml of 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mM BTC and 0.5 mM DTNB, at 25 °C. Product formation was followed by the increase in absorbance at 412 nm. The data were fit to Equation 1. The fitted and actual data were so close that they fell on the same curve.

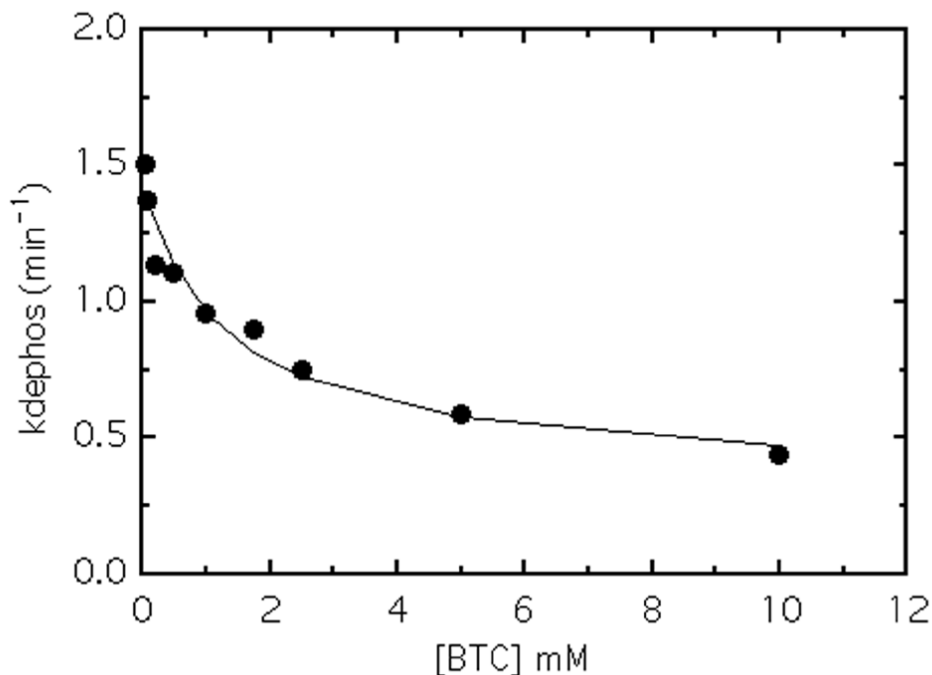


Figure 4. The dependence of the apparent dephosphorylation rate constant for echothiophate-inhibited G117H on the concentration of BTC. Ninety μM echothiophate was mixed with $0.85 \mu\text{M}$ G117H in 0.1 M potassium phosphate buffer, pH 8.0, and incubated at $25 \text{ }^\circ\text{C}$. After 5 minutes, inhibition was about 80%. Three μL aliquots were diluted into 2.0 mL of 0.1 M potassium phosphate buffer, pH 7.0 and $25 \text{ }^\circ\text{C}$, containing 0.5 mM DTNB and various concentrations of BTC. Product formation was followed spectrophotometrically at 412 nm . The apparent reactivation rate constant was determined by fitting the time course to Equation 1. The points are the measured reactivation rate constants. The line is a fit of apparent rate constant versus BTC concentration data to an equation for a single dissociation process (Fersht, 1977):

$$L = \frac{\text{Lab} * [\text{BTC}] + \text{Lf} * \text{Kd}}{\text{Kd} + [\text{BTC}]}$$

where, L is the measured reactivation rate constant; Lab is the reactivation rate constant for the G117H-BTC complex; Lf is the reactivation rate constant for the unliganded G117H; $[\text{BTC}]$ is the concentration of BTC; and Kd is the dissociation constant for BTC binding to G117H.