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Recombinant Human Butyrylcholinesterase G390V, the Fluoride-2 Variant, Expressed in Chinese Hamster Ovary Cells, Is a Low Affinity Variant*

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Kinetics of recombinant fluoride-2 variant of human butyrylcholinesterase (Gly390 Val) secreted by Chinese hamster ovary cells were compared to recombinant usual and to usual butyrylcholinesterase purified from human plasma. The usual and fluoride-2 variant were distinguishable with regard to hydrolysis of benzoylcholine (Km = 5 μM), neutral esters, and at high concentrations of acetylthiocholine, propionylthiocholine, and butyrylthiocholine. However, at low substrate concentrations Km values for acetylthiocholine and succinylthiocholine were 2-6-fold higher for the fluoride-2 variant. pH rate profiles revealed small differences in pK, that could be attributed to changes in the active site histidine environment. On the other hand, Arrhenius plot analysis of o-nitrophenylbutyrate hydrolysis at pH 7.5 showed no difference in activation energy between fluoride-2 and usual butyrylcholinesterases. Both exhibited an anomalous temperature dependence with a wavelike change in activation energy around 18°C. Affinity of the fluoride-2 variant for sodium fluoride, tacrine, dibucaine, amodiaquine, and succinylcholine was lower than for usual enzyme. Apparent Km for succinylcholine was 125 μM for the fluoride-2 variant and 20 μM for the usual enzyme. Organophosphate inhibition showed equivalent reactivity, indicating that the point mutation altered only gene and the wild-type (usual) BCHE gene in CHO cells. The fluoride-2 variant has a point mutation at nucleotide 728 that changes threonine 243 to methionine. pH rate concentrations of 0.067 M Na/K phosphate buffer, pH 7.4, at 25°C with 50 μM benzoylcholine chloride (Sigma) as the substrate. Benzoylcholine hydrolysis was followed by monitoring decrease in absorbance at 240 nm (A = 6800 m-1 cm-1). The special of the preparation was 180 units/mg protein (1 unit = 1 μmol of benzoylcholine hydrolyzed/min). The enzyme had the "usual" phenotype.

CHO Cell Line Secreting Fluoride-2 Variant of Human Butyrylcholinesterase—Human cDNA encoding a 28-amino-acid signal peptide and 574 amino acids of the mature BuChE enzyme was cloned into the pD5 expression vector where expression is controlled by the adenovirus major late promoter (10). This cDNA had a substitution which changed Gly390 (GTT) to Val (GTT), the mutation responsible for the fluoride-2 variant (8). The mutation was present in cDNA isolated from a human brain library (11) and therefore shows that the donor was a carrier of the fluoride-2 mutation BCHE*390V. A stable cell line expressing human BuChE was created by cotransfecting pD5-BCH and pD5-dihydrofolate reductase into CHO cells doubly deficient for dihydrofolate reductase. The deficient CHO cells, DG44, were kindly provided by L. A. Chasin (12). The BCHE and BuChE expressed in CHO cells was purified by a combination of gel filtration and hydrophobic interaction chromatography. The enzyme activity was determined using the rate of hydrolysis of acetylthiocholine, as described above. The enzyme specific activity of the preparation was 180 units/mg protein (1 unit = 1 μmol of benzoylcholine hydrolyzed/min). The enzyme had the "usual" phenotype.

MATERIALS AND METHODS

Purified Human Plasma BuChE—Human plasma BuChE was purified to 90% purity in two steps (4). The enzyme was assayed according to Kalow and Lindsay (9) in 0.067 M Na/K phosphate buffer, pH 7.4, at 25°C with 50 μM benzoylcholine chloride (Sigma) as the substrate. Benzoylcholine hydrolysis was followed by monitoring decrease in absorbance at 240 nm (A = 6700 m-1 cm-1). The special of the preparation was 180 units/mg protein (1 unit = 1 μmol of benzoylcholine hydrolyzed/min). The enzyme had the "usual" phenotype.

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dihydrololate reductase genes became amplified approximately 100-fold in response to increasing doses of methotrexate. The stable cell line was maintained under selective pressure by growing cells in minimal essential medium-α, with l-glutamine, without ribonucleosides and deoxyribonucleosides (GIBCO Catalog No. 320-2561AJ) containing 10% dialyzed fetal calf serum. BuChE was collected into serum-free medium. CHO-S-FM (GIBCO 260-2561AJ) because it has only 0.4% of the bovine AChE present in fetal calf serum. The benzoylcholine activity of secreted rBuChE fluoride-2 variant was 0.04 units/ml.

CHO Cell Line Securing Secreted Human Butyrylcholinesterase—The cDNA sequence of human BuChE was modified by site-directed mutagenesis to generate recombinant human BuChE (rBuChE). The optimal start site sequence is GCCACCAATGG where ATG is the translation initiation codon. Two BglII recombination sites were introduced at the 5’ and 3’ ends of the coding sequence to have the shortest possible cDNA free of extraneous sequences that might interfere with translation efficiency. The BamHI site at Gly72 was removed. After site-directed mutagenesis, the BuChE gene was completely resequenced to ensure the absence of unwanted mutations. The 1.8-kilobase BCHE gene was cloned into plasmid pRCMV (Invitrogen) where expression is controlled by the cytomegalovirus promoter. Ten μg pRCMV-BCHE was transfected into CHO DG44 cells by calcium phosphate coprecipitation. Cells resistant to 0.8 mg/ml genetin (GIBCO 880-1811; GIBCO 290-2561AJ) were selected over a 3-week period. Clonal cell lines were created by diluting resistant cells into 96-well plates at less than 1 cell/well. Cells secreting the highest level of BuChE activity, as measured with benzoylcholine, were expanded into T75 flasks. Cells were grown to 90% confluence in a T75 flask in the presence of complete medium containing 10% fetal calf serum. Culture medium was replaced with CHO-S-FM containing 0.8 mg/ml genetin for the purpose of collecting BuChE into serum-free medium. Cells were continuously cultured in CHO-S-FM for 3 months. The secreted rBuChE reached an activity of 0.5 units/ml which is nearly the activity in human plasma.

Activity Versus pH—The pH dependence of enzyme activity (kcat) was investigated at 25 and 41°C in 0.067 M potassium phosphate buffer in the pH range 5.5 to 8.98. Various ratios of 0.067 M K2HPO4 and 0.067 M K2HPO4 were mixed to get buffers that varied in pH but had the same concentration. Activity was measured with 50 μM benzoylcholine, a concentration 10 times the Km value determined at pH 7.4, 25°C.

In parallel, the pH dependence of enzyme inhibition by 50 μM NaF was measured in the same pH range in the presence of 50 μM benzoylcholine. The substrate and inhibitor concentrations were chosen on the basis of differential inhibition of usual and fluoride-resistant BuChE phenotypes: with these concentrations, the difference between the percentage inhibition by sodium fluoride of usual and fluoride BuChE is maximum (7, 14). The kcat, Km of BuChE-catalyzed hydrolysis of benzoylcholine was interpreted according to the simplest model which assumes that H+ acts as an uncompetitive inhibitor, i.e. the protonated form of the enzyme is completely unreactive.

\[
k_{\text{cat}} = k_{\text{cat,max}} \frac{K_m}{[S] + [H^+]} \quad \text{(Eq. 1)}
\]

In Equation 1, kcat,max is the maximum value of kcat at high pH, and Km is the ionization constant of an amino acid side chain whose deprotonated form takes part in catalysis.

Apparent kcat values were computer-calculated by nonlinear regression fitting of the experimental data to Equation 1. We used the GraFit (version 1.0) pH GFE curve fitting program (Erithacus Software Ltd, United Kingdom, 1989).

Kinetcs of Substrate Hydrolysis—Kinetic analyses were performed in 0.067 M phosphate buffer, pH 7.0, 7.5, or 8.0 at 25°C. Hydrolysis of acetylthiocholine iodide, propionylthiocholine iodide, butyrylthiocholine iodide, and benzoylcholine was interpreted according to the simplest model which phosphorylation of the enzyme active site serine (E) according to Scheme I:

\[
E + 1 \overset{k_1}{\longrightarrow} E::I \overset{k_2}{\longrightarrow} E::IP \overset{k_3}{\longrightarrow} E + H_2O
\]

Scheme 1

where \( k_i = k_i / k_e \) is the dissociation constant of the enzyme-inhibitor complex and \( k_r \) the rate constant of carbamylation or phosphorylation. The rate constant of hydrolysis, \( k_r \), is very slow. Assuming that the enzyme is irreversibly inhibited under the conditions of the experiment, the activity (e) decreases with time (t) as follows,

\[
e(t) = e^{-k_r t} \quad \text{or} \quad e(t) = e^{-k(t)/k_e} \quad \text{(Eq. 2)}
\]

where \( k_e \) is the apparent inhibition rate constant.

Since \( [I] >> e \) and assuming that the rapid equilibrium condition, \( k_r << k_e \), holds (18), the apparent rate of inactivation can be written as:

\[
k_e = \frac{k_2}{1 + K_m}[I] \quad \text{(Eq. 3)}
\]

The value of the inactivation rate constant \( k_e \) was determined for each inhibitor concentration by weighted exponential regression of the

\[
K_m \text{ and } V_{\text{max}} \text{ Determinations—Kinetic data were plotted using the Lineweaver-Burk double-reciprocal plot. The enzymes had either Michaelian or non-Michaelian behavior depending on the nature of the substrate. For Michaelian behavior, } K_m \text{ and } V_{\text{max}} \text{ were determined by nonlinear regression of the Michaelis-Menten equation using the Enzfitter kinetic calculation program (Biosoft, Cambridge, UK). For non-Michaelian behavior (curves in the Lineweaver-Burk plots), the apparent } K_m \text{ and } V_{\text{max}} \text{ were estimated graphically from the linear sections of the curves.}

Temperature Dependence of α-Nitrophenylbutyrate Hydrolysis Rate—The temperature dependence of BuChE-catalyzed hydrolysis of α-nitrophenyl butyrate (0.8 mM in 0.067 M phosphate buffer, pH 7.5, containing 5.3% methanol) was determined at various temperatures from 10 to 50°C. The reaction rate (k) was measured at intervals of 1-2°C. We operated at nearly saturating substrate concentration, at 0.8 mM which is four times higher than \( K_m \). Observed reaction rates were corrected for spontaneous substrate hydrolysis. Arrhenius plots were obtained by plotting ln k against 1/T, where T is the absolute temperature. The experimental activation energies E were calculated from the slopes of the linear portions of the plots by linear regression analysis.

Reversible Inhibition—Inhibition by NaF was performed in 0.067 M phosphate, pH 7.0 and 8.0, at 25°C with benzoylcholine as the substrate. Inhibition by succinylcholine chloride (Sigma), tacrine (tetrahydroaminoacridine), dibucaine, and amiodarion was carried out in 0.067 M phosphate, pH 7.5, at 25°C with α-nitrophenylacetate as the substrate (all compounds were from Sigma). Inhibition by amiodarion was also carried out at pH 7.0 with butyrylthiocholine iodide as the substrate. Types of inhibition and apparent inhibition constants were determined from Lineweaver-Burk plots and Dixon plots (17) by plotting the reciprocal of velocity [v] against inhibitor concentration [I]. Inhibition constants for nonlinear inhibition were calculated from replots of the reciprocals of slopes and intercepts of the Lineweaver-Burk plots against the reciprocal of inhibitor concentration (17).

Progressive Inhibition—Progressive inhibition by eserine (0.5, 1, 10 μM) was conducted in 0.1 M phosphate, pH 7.0, at 25°C. Residual activity was measured in 0.067 M phosphate, pH 7.5, with α-nitrophenylacetate (0.8 mM) as substrate. Progressive inhibition by tris(mopropylpyrophosphoramide (iso-OMPA) was either performed as above or after 1 h of incubation of BuChE in 0.1 M phosphate, pH 7.0, containing 5 mM EDTA as an inhibitor of metal proteases and organophosphate hydrolyase. The iso-OMPA concentrations ranged from 10 to 200 μM. Residual activity was measured by Ellman's method (15) with butyrylthiocholine iodide (1 mM) as substrate.

Inhibition by eserine or iso-OMPA (1) results in carbamylation or phosphorylation of the enzyme active site serine (E) according to Scheme I:
Equation 2. Then $k_2$ and $K_1$ were determined by weighted nonlinear regression fitting of Equation 3 using the Enzfitter program.

**Molecular Modeling**—Three-dimensional models of wild-type and the fluoride-2 variant of human BuChE were built from the 3-D coordinates of Torpedo californica AChE (19) by molecular replacement and energy minimization using the molecular modeling package TURBO-FRODO 4.0 (20) and the refinement program X-PLOR 3.0 (21). Analysis of 3-D models was carried out on a Silicon Graphics 310 GETX workstation.

3-D structures of succinylcholine (22) and dibucaine (23) were obtained from the Cambridge Structural Database (24, 25). The 3-D structure of o-nitrophenylacetate was built from the 3-D structure of $p$-nitrophenylacetate (26) which is in the Cambridge Structural Database. Topology and energy parameters of these compounds were computed from Cambridge Structural Database data and by using the X-PLOR "prolsq.pro" file. This file allowed optimization of substrate and ligand geometry in the BuChE active site. Structures of substrate and ligand BuChE complexes were refined using the X-PLOR program after inspection of substrate and ligand noncovalent bonds with the solvent-accessible molecular surface being represented as a smooth continuous lattice of points using Connolly's program MS (27). All these refinements were carried out by molecular dynamics at 300 K and conjugate gradient method with a convergence criterion of 1.5 kcal/niol/Å. These refinements were carried out by molecular dynamics at 300 K and conjugate gradient method with a convergence criterion of 1.5 kcal/niol/Å.

### RESULTS

**Kinetics of Substrate Hydrolysis**—Hydrolysis of neutral esters followed Michaelis-Menten kinetics, giving a linear relationship between the reciprocal of activity and the reciprocal of ester concentration for both fluoride-2 and usual BuChE. Table I shows that fluoride-2 BuChE (rFF) had a slightly lower affinity for neutral substrates, with $K_m$ at most 1.5 times higher for fluoride-2 BuChE than for usual BuChE.

Hydrolysis of charged esters was more complex, with benzoylcholine giving the simplest kinetics. The kinetics of hydrolysis of benzoylcholine were Michaelian, but as previously shown, usual BuChE displayed substrate inhibition at benzoylcholine concentrations greater than 50 μM when assayed at pH 7.0 (34, 35). The substrate inhibition constant $K_s$ was determined according to the Haldane equation:

$$v = \frac{V_{max}}{1 + \frac{K_s}{[S]} + \frac{[S]}{K_m}} \quad (\text{Eq. 4})$$

$K_s$ was found to be 500 μM at pH 7.0 and at pH 8.0 for both recombinant usual and purified usual plasma BuChE. No substrate inhibition was observed for fluoride-2 BuChE except at pH 8.0 in the presence of 0.05–2 mM NaF. Usual and fluoride-2 BuChE had nearly the same affinity for benzoylcholine, both having $K_m$ values of approximately 5 μM (Table II).

Hydrolysis of thiocholine esters was the most complex. Usual and fluoride-2 BuChE both deviated from the simple Michaelis-Menten model when the substrates were thiocholine esters. For example, Fig. 1 shows biphasic kinetics for the hydrolysis of butryrylthiocholine. It has been known for many years (40) that these biphasic kinetics reflect activation at high substrate concentration. A least two mechanistic models can account for this activation: the first one assumes binding of an additional substrate molecule to the acyl-enzyme intermediate (41); the second hypothesizes the existence of a peripheral regulatory binding site for charged ligands or substrates (42). Lineweaver-Burk plots allowed us to estimate apparent $K_m$ values for high and low substrate concentrations (Table II). At high substrate concentration the $K_{act}$ values for acetetylthiocholine, propionylthiocholine, butryrylthiocholine, and succinylthiocholine were practically the same for both usual and fluoride-2 BuChE. A more refined description of the kinetics of acetetylthiocholine, propionylthiocholine, and butryrylthiocholine hydrolysis was possible using the general mathematical expression proposed for the two above-mentioned models (42):

$$V = \frac{V_{max}[S] + V_{max}[S]^2}{K_i + [S] + [S]^2} \quad (\text{Eq. 5})$$

where $V_{max}' = \alpha V_{max}$ with $\alpha > 1$. Equation 5 was used to calculate $V_{max}$, $V_{max}'$, $K_1$, and $K_2$ in Table III. A comparison of $K_{act}$ and $K_{max}$ in Table II with $K_1$ and $K_2$ in Table III shows that values for $K_{act}$ are very close to values for $K_1$, and values for $K_{max}$ are very close to values for $K_2$. Thus, the graphic analysis to calculate $K_{act}$ and $K_{max}$ in Table II yielded similar results as the computer analysis to calculate $K_1$ and $K_2$ in Table III. In Table III the $K_1$ and $K_2$ ratios decrease in parallel as the size of the acid moiety of the substrates increases. The conclusion from the data in Tables II and III is that there are no major kinetic differences between usual BuChE and fluoride-2 BuChE. The point mutation in the fluoride-2 variant does not change the mechanism of hydrolysis of thiocholine esters.

The $K_m$ values for succinylthiocholine for usual and fluoride-2 BuChE are of special interest because of the clinical problems associated with use of succinylcholine in people who have the fluoride-2 variant. Table II shows that the $K_{act}$ values for fluoride-2 BuChE are 3.5- and 1.6-fold higher than for usual BuChE at pH 7.0 and 8.0, respectively. It is reason-

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH</th>
<th>UU</th>
<th>rFF</th>
<th>ratio</th>
<th>rUU</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>o-Nitrophenylacetate</td>
<td>7.5</td>
<td>0.48±0.16</td>
<td>0.45±0.19</td>
<td>1</td>
<td>0.38±0.01</td>
<td>In the presence of 5.3% methanol</td>
</tr>
<tr>
<td>o-Nitrophenylbutyrate</td>
<td>7.5</td>
<td>0.125±0.02</td>
<td>0.185±0.03</td>
<td>1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Naphthylacetate</td>
<td>7.0</td>
<td>0.34</td>
<td>0.41</td>
<td>1.2</td>
<td></td>
<td>In the presence of 3.33% ethanol</td>
</tr>
<tr>
<td>α-Naphthylbutyrate</td>
<td>7.0</td>
<td>0.044</td>
<td>0.068</td>
<td>1.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 0.067 M phosphate, 25 °C.
* Literature values for $K_m$ for o-nitrophenylbutyrate: 0.14 mM in 0.067 M phosphate, pH 7.4 (28, 29); 0.19 mM in 50 mM phosphate, pH 7.6 (30); 0.14 mM in 50 mM phosphate, pH 7.6, 25 °C (31); 0.33 mM in 0.1 M Tris-Cl, pH 7.4, 25 °C (33).
* Literature values for $K_m$ for α-naphthylacetate: 0.76 mM in 50 mM Tris-Cl, pH 7.4, 37 °C (32); 1.0 mM in 0.1 M Tris-Cl, pH 7.4, 25 °C (33).
Kinetics of a Recombinant Human Cholinesterase Variant

**Table I**

Kinetic parameters for positively charged substrates of purified BuChE from human plasma (UU), recombinant usual BuChE (rUU), and recombinant fluoride-2 BuChE (rFF)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH*</th>
<th>Michaelian behavior $K_m$</th>
<th>Non-Michaelian behavior $K_m$, low [S]</th>
<th>Non-Michaelian behavior $K_m$, high [S]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>UU</td>
<td>rUU</td>
<td>rFF</td>
</tr>
<tr>
<td>Benzylocholine</td>
<td>7</td>
<td>0.0044</td>
<td>±0.0007</td>
<td>0.0034</td>
</tr>
<tr>
<td>Benzylocholine</td>
<td>8</td>
<td>0.0041</td>
<td>±0.0006</td>
<td>0.0034</td>
</tr>
<tr>
<td>Acetyliothiocholine</td>
<td>7</td>
<td>0.0064</td>
<td>±0.0012</td>
<td>0.0034</td>
</tr>
<tr>
<td>Propionyliothiocholine</td>
<td>7</td>
<td>0.0022</td>
<td>±0.0007</td>
<td>0.0034</td>
</tr>
<tr>
<td>Butyryliothiocholine</td>
<td>7</td>
<td>0.0026</td>
<td>±0.0007</td>
<td>0.0034</td>
</tr>
<tr>
<td>Succinylidithiocholine</td>
<td>7</td>
<td>0.0014</td>
<td>±0.0004</td>
<td>0.0034</td>
</tr>
<tr>
<td>Succinylidithiocholine</td>
<td>8</td>
<td>0.0019</td>
<td>±0.0000</td>
<td>0.0034</td>
</tr>
</tbody>
</table>

*0.067 M phosphate, 25 °C.

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<tr>
<td>Benzylocholine</td>
<td>7</td>
<td>0.0044</td>
<td>±0.0007</td>
<td>0.0034</td>
</tr>
<tr>
<td>Benzylocholine</td>
<td>8</td>
<td>0.0041</td>
<td>±0.0006</td>
<td>0.0034</td>
</tr>
<tr>
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<td>7</td>
<td>0.0064</td>
<td>±0.0012</td>
<td>0.0034</td>
</tr>
<tr>
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<td>0.0034</td>
</tr>
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*0.067 M phosphate, 25 °C.

**FIG. 1. Lineweaver-Burk plot for BuChE-catalyzed hydrolysis of butyryliothiocholine.** Activity was measured in 0.1 M phosphate buffer, pH 7.0, at 25 °C. Velocity is expressed as the reciprocal (1/V) of the change in absorbance at 420 nm/min recombinant fluoride-2 BuChE, FF (A); purified usual plasma BuChE, UU (O). At high substrate concentration, $K_m = 0.25$ mM for both recombinant FF and plasma UU.

The observed $K_m$ values at 25 and 41 °C are in agreement with the known $K_m$ of the histidine ring.

The apparent enthalpy of ionization, $\Delta H$, was calculated from $pK_a$ values at 25 and 41 °C using the Van't Hoff equation, by assuming a linear temperature dependence of $pK_a$. The $\Delta H$ value for usual BuChE was 27.9 kJ mol$^{-1}$, a value consistent with $\Delta H$ for ionization of histidine, which is known to have $\Delta H$ values of 28–31 kJ mol$^{-1}$. The $\Delta H$ value for fluoride-2 BuChE was significantly lower, 15.8 kJ mol$^{-1}$, and may reflect a change in the immediate environment of histidine to a more hydrophobic environment, thus tending to destabilize the imidazole ring. The histidine measured by these $pK_a$ and $\Delta H$ values was assumed to be the histidine in the catalytic triad, His$_{210}$, by analogy with Torpedo AChE (18, 43, 44).

**Temperature Dependence of the Hydrolysis of o-Nitrophenylbutyrate**—The temperature-dependence of BuChE-catalyzed hydrolysis of o-nitrophenyl butyrate at $v = $ approximate $V_{max}$, measured at o-nitrophenylbutyrate concentration about 5-fold higher than $K_m$, is identical for the usual enzyme (plasma or recombinant) and fluoride-2 BuChE. As shown in Fig. 3, the Arrhenius plot is nonlinear and exhibits a wavelike discontinuity at 18 °C. In an earlier experiment with usual BuChE, a clear break in the Arrhenius plot was observed at 21 °C in the presence of 0.5% methanol (29). This was ascribed to a temperature-induced conformational change. In Fig. 3 the wavelike break may be interpreted as the coexistence of two active conformations in equilibrium in a narrow temperature interval around 18 °C. A break at 18 °C rather than at 21 °C is due to the higher methanol concentration of 5.3%. The activation energy ($E_a$) required for substrate hydrolysis is different on both sides of the discontinuity but there is no significant difference between the three enzymes. In the temperature range 10–17 °C, $E_a$ is 71.8 ± 9.4 kJ mol$^{-1}$; between 18 and 38 °C it is 44.8 ± 4.5 kJ mol$^{-1}$. Beyond 38 °C the activity of the recombinant usual enzyme drops rapidly. The heat sensitivity of this enzyme is due to proteolytic nicks. The fact that the three enzymes exhibit the same temperature dependence indicates that their posttranslational modifications (glycosylation and proteolytic nicks) do not affect the catalytic step. Moreover, it appears that the G390V mutation has no effect on the activation step that precedes enzyme acylation by o-nitrophenylbutyrate.

**Inhibition by Sodium Fluoride**—The effect of sodium fluoride (50 μM) upon the pH dependence of benzylocholine hydrolysis was examined. NaF shifted the pH activity curve in opposite directions for UU and rFF (Fig. 2, panels 1 and 2).
Kinetics of a Recombinant Human Cholinesterase Variant

Table III

Kinetic parameters of purified usual (UU) plasma BuChE and recombinant fluoride-2 BuChE (rFF) calculated by nonlinear regression using equation 5

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH*</th>
<th>UU</th>
<th>rFF</th>
<th>K1</th>
<th>K2</th>
<th>K1</th>
<th>K2</th>
<th>ratio</th>
<th>ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylthiocholine</td>
<td>7.0</td>
<td>19.2</td>
<td>51.9</td>
<td>0.003</td>
<td>0.62</td>
<td>11.3</td>
<td>26.8</td>
<td>0.072</td>
<td>1.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.067 m phosphate, 25 °C.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionylthiocholine</td>
<td>7.0</td>
<td>34.8</td>
<td>77.5</td>
<td>0.024</td>
<td>0.41</td>
<td>8.8</td>
<td>67.3</td>
<td>0.027</td>
<td>0.63</td>
</tr>
<tr>
<td>Butyrylthiocholine</td>
<td>7.0</td>
<td>42.9</td>
<td>213.7</td>
<td>0.014</td>
<td>0.21</td>
<td>13.5</td>
<td>65.6</td>
<td>0.011</td>
<td>0.23</td>
</tr>
</tbody>
</table>

2) At 25 °C the apparent pK_a for UU shifted slightly to a lower pH (pK_a = 7.12 ± 0.10), whereas the apparent pK_a for rFF shifted to a higher pH (pK_a = 7.24 ± 0.06). At 41 °C (Fig. 2, panels 3 and 4), the apparent pK_a for UU shifted slightly to a lower pH (pK_a = 6.89 ± 0.11), whereas the apparent pK_a for rFF did not shift. Using acetylcholine, rather than benzoylcholine, and 5 mM NaF rather than 0.05 mM NaF, Heilbronn (45) as well as Brestkii and Fruentova (46) got results for usual BuChE that were the opposite of our results for UU in Fig. 2, panel 1. Heilbronn (45) found that in the presence of 5 mM NaF the pH activity curve was displaced toward a higher pH. This indicates a substrate-dependent kinetic behavior of BuChE.

As shown in Fig. 4, pH inhibition profiles differed widely for usual and fluoride-2 BuChE. At 25 °C usual BuChE was maximally inhibited by sodium fluoride at pH 8.0, whereas fluoride-2 BuChE was maximally inhibited at pH 7.5, a difference of 0.5 pH units. At 41 °C usual BuChE was maximally inhibited at pH 8.0, whereas fluoride-2 BuChE was maximally inhibited at pH 7.0, a difference of 1.0 pH units. For both usual and fluoride-2 BuChE percent inhibition by NaF was far greater at 25 than at 41 °C, confirming that inhibition by NaF is temperature sensitive (6, 7). At 25 °C and pH 7.4, the inhibition percentage (fluoride number) of usual BuChE in Fig. 3 is 67%, a value similar to the average value of 60 for usual plasma BuChE (6).

Dixon plots for the inhibition of benzoylcholine hydrolysis.
Kinetics of a Recombinant Human Cholinesterase Variant

at pH 7.0 suggested that at low benzoylcholine concentration inhibition of fluoride-2 BuChE by sodium fluoride is uncompetitive with apparent $K_i = 0.3$ mM (Fig. 5, panel 1). However, at 25 μM benzoylcholine there was a deviation from simple uncompetitive inhibition in a fashion suggestive of a negative cooperative interaction similar to that seen by Page et al. (35) for usual BuChE. Page et al. (35) found that the binding of benzoylcholine is enhanced in the presence of fluoride ion with a cooperativity factor of 30. The inhibition mechanism of usual BuChE also appeared to be uncompetitive (apparent $K_i = 0.09$ mM) at low substrate concentration ([S] ≤ 10 μM), but the mechanism again changed at 25 μM benzoylcholine (Fig. 5, panel 2) in a manner indicative of cooperativity in the binding of fluoride ion and benzoylcholine, as expected for usual BuChE (35). Inspection of the 25 μM traces in panels 1 and 2 of Fig. 5 showed that the rates up to 75 μM NaF are slower than would be expected for a simple uncompetitive mechanism. At NaF concentrations higher than 75 μM the traces differ, with the rFF enzyme showing a more shallow slope than the UU enzyme. This behavior is consistent with the switch point in the cooperative mechanism occurring at a higher fluoride concentration for the rFF enzyme. A weaker interaction between rFF and fluoride ion is also seen in the uncompetitive $K_i$, values of 0.3 mM for rFF and 0.09 mM for UU. Fig. 5 (panels 3 and 4) shows that at pH 8.0, both usual and fluoride-2 BuChE exhibited substrate inhibition in the presence of NaF. At pH 8.0 inhibition was partially uncompetitive at low substrate concentration, but there was a synergistic effect between benzoylcholine and fluoride ion at high substrate concentration that amplified the inhibition. Due to the strong cooperativity in mutual binding of fluoride ion and benzoylcholine, Dixon plots were abnormal and could not lead to $K_i$ estimation; nevertheless, they suggested that usual and fluoride-2 BuChE displayed a similar degree of cooperativity. Although inhibition by sodium fluoride has been known for many years (45), its mechanism has not yet been resolved and remains disconcerting.

Reversible Inhibition by Positively Charged Ligands—Inhibition of o-nitrophenylacetate hydrolysis by dibucaine, tacrine, succinylcholine, and amodiaquin was nonlinear for both usual and fluoride-2 BuChE, i.e. Dixon plots of $v' = v/[I]$ versus [I] were downwardly curved, as shown for dibucaine in Fig. 6. This means that high inhibitor concentrations did not drive the reaction rate to zero. Apparent inhibition constants at low inhibitor concentration were estimated from Dixon plots and are given in Table IV. Fluoride-2 BuChE was less inhibited by these compounds than usual BuChE by a factor of 2.7-6.2 as shown by $K_i$ ratios. Due to the strong deviation from linearity of these plots at high [I], neither the type of inhibition nor actual $K_i$ could be simply determined. Partial nonlinear inhibition indicates that both ES and ternary complexes ESI yield products. Nonlinear reversible inhibition may be depicted by the following minimum reaction scheme II.
Kinetics of a Recombinant Human Cholinesterase Variant

**Fig. 5.** Dixon plots for inhibition by NaF of BuChE-catalyzed hydrolysis of benzoylcholine. Benzoylcholine concentrations were 5 µM (C, ○), 10 µM (Δ, △), 25 µM (□, □). In panels 1 and 2 the buffer was 0.067 M potassium phosphate, pH 7.0, at 25 °C. In panels 3 and 4 the buffer was 0.067 M potassium phosphate, pH 8.0, at 25 °C. Recombinant fluoride-2 BuChE, rFF, was used in panels 1 and 3. Purified plasma usual BuChE, UU, was used in panels 2 and 4. At low benzoylcholine concentration, uncompetitive $K_i = 0.3$ mM for rFF and uncompetitive $K_i = 0.09$ mM for UU.

When $\alpha > 1$ and $0 < \beta < 1$ the reciprocal form of this equation gives a nonlinear plot as shown in Fig. 7. Inhibition constants and coefficients $\alpha$ and $\beta$ were determined for dibucaine from secondary replots of $1/$slope and $1/$intercept versus $1/[I]$, where $\Delta$slope and $\Delta$intercept are the change in slope and in $y$ intercept of the reciprocal of Equation 6, i.e. $1/v$ versus $1/[S]$ plots at different fixed inhibitor concentrations. This analysis showed nonlinear mixed inhibition of BuChE by dibucaine. The values of $K_i$ were calculated to be 0.6 µM ($\alpha = 5.0, \beta = 0.5$) for the purified plasma usual enzyme, 0.45 µM ($\alpha = 4.0, \beta = 0.44$) for the recombinant usual enzyme, and 12.5 µM ($\alpha = 1.6, \beta = 0.7$) for the recombinant fluoride variant. Since $\alpha_{exo} > \alpha_{FF}$, the competitive inhibition component is higher for the usual enzyme than for the fluoride-2 variant; inhibition of fluoride-2 BuChE is nearly nonlinear noncompetitive. The fact that the $\beta$ inhibition coefficients for the two enzymes are of the same order of magnitude indicates that the ternary complexes $ESI$ of both enzymes release product with a similar efficiency. This suggests that mutation G390V does not affect the covalent steps of enzyme catalysis. On the other hand, the actual affinity of the fluoride variant for dibucaine, which is 21-fold lower than that of the usual enzyme, confirms that the point mutation G390V strongly altered the binding of charged ligands to the enzyme active site gorge.
Inhibition of butyrylthiocholine hydrolysis by amodiaquin was linear for both variants (Table IV). Thus, with the neutral ester, o-nitrophenylacetate, inhibition was nonlinear, but with the positively charged ester, butyrylthiocholine, inhibition by amodiaquin was linear. Substrate-dependent inhibition kinetic behavior has been observed for horse serum BuChE (41, 49, 50) and for AChE (55). The present data, showing that cationic ligands produced a partial nonlinear inhibition of BuChE-catalyzed hydrolysis of the neutral ester o-nitrophenylacetate, are consistent with the mechanistic models proposed for hydrolysis of positively charged substrates. They suggest that these ligands affect the enzyme reaction scheme by binding to vacant “anionic” site(s). Finally, whatever the intimate molecular mechanism of this inhibition, it is noteworthy that usual and fluoride-2 BuChE exhibited similar behavior and differed only in affinity for the inhibitors, with fluoride-2 BuChE consistently having weaker binding.

Kinetics of Progressive Inhibition—Progressive inhibition of usual plasma BuChE by iso-OMPA at pH 7.0 followed Scheme I and Equations 2 and 3 (Fig. 8). The calculated inhibition constants for usual BuChE were: \( K_i = 60 \pm 16 \mu M \) and \( k_i = 0.53 \pm 0.06 \text{ min}^{-1} \). These values are in accordance with previously reported data (56). On the other hand, loss of activity of recombinant fluoride-2 BuChE (Fig. 9) did not follow reaction Scheme I. Instead, inhibition could be described by a two-exponential decay model:

\[
\frac{e_t}{e_0} = A e^{k_1 t} + (1 - A) e^{k_2 t} \quad (\text{Eq. 7})
\]

A plot of \( k_{il} \) against iso-OMPA concentration (Fig. 8, inset, dotted line) gave a curve which was virtually superimposable on the curve for usual plasma BuChE (Fig. 8, inset, solid line). The inhibition constants for fluoride-2 BuChE were estimated to be \( K_i = 72 \pm 35 \mu M \) and \( k_i = 0.51 \pm 0.13 \text{ min}^{-1} \). Thus, the inhibition constants by iso-OMPA for usual and fluoride-2 BuChE are very similar.

As regards the curved portion of Fig. 9, \( k_{il} \) appeared to be roughly constant whatever the iso-OMPA concentration. When plots of log \( e_t/e_0 \) versus \( t \), such as the plot in Fig. 9, are curvilinear rather than linear, the curve can be interpreted as a reflection of inhibition of a multiple enzyme system (57). However, recombinant fluoride-2 BuChE is mostly a tetramer, arguing against multiple forms of BuChE being the source of catalytic heterogeneity. Thus, the curved plot suggests the presence in the CHO culture medium of competing enzymes which consume iso-OMPA. These enzymes could be organophosphohydrolases and/or other targets of iso-OMPA such as serine enzymes. Evidence supporting the presence of several serine enzymes in the CHO cell culture medium was the observation of several \([\text{H}]\text{diisopropylfluorophosphate}-\text{labeled proteins on sodium dodecyl sulfate gels (data not shown). Additional support for the presence of competing enzymes came from the observation in Fig. 9 (dashed line) that preincubation of the CHO cell culture medium for 1 h with 5 mM EDTA significantly increased the inhibitory power of iso-OMPA. The EDTA might have neutralized an organophosphohydrolase or a metal protease. Another possible explanation for the curvilinear plot, i.e. that the rate of reactivation (\( k_i \) in Scheme I) was increased in the fluoride-2 variant, has not been ruled out.

Despite this uncertainty over the later portions of the time course in Fig. 9, the results from early time points show that reactivity of recombinant fluoride-2 BuChE with iso-OMPA was similar to reactivity of the usual plasma enzyme. Progressive inhibition by eserine confirmed this conclusion (data not shown). Finally, these results suggested that the reactivity
Kinetics of a Recombinant Human Cholinesterase Variant

TABLE IV

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Substrate</th>
<th>pH*</th>
<th>Kapp ( \mu M )</th>
<th>K, ratio</th>
<th>Type of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tacrine</td>
<td>o-NPA (^a)</td>
<td>7.5</td>
<td>0.0004(^c)</td>
<td>0.0014</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nonlinear; competitive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>at low [I]</td>
</tr>
<tr>
<td>Dibucaine</td>
<td>o-NPA</td>
<td>7.5</td>
<td>5(^d)</td>
<td>30</td>
<td>6</td>
</tr>
<tr>
<td>Succinylcholine</td>
<td>o-NPA</td>
<td>7.5</td>
<td>20(^e)</td>
<td>125</td>
<td>6.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nonlinear; competitive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>at low [I]</td>
</tr>
<tr>
<td>Amodiaquin</td>
<td>o-NPA</td>
<td>7.5</td>
<td>25</td>
<td>80</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nonlinear; competitive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>at low [I]</td>
</tr>
<tr>
<td>Amodiaquin</td>
<td>BuSCW</td>
<td>7.0</td>
<td>6</td>
<td>16</td>
<td>2.66</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Linear; competitive</td>
</tr>
</tbody>
</table>

*0.067 M phosphate, 25 °C.

Literature values for tacrine inhibition: complex inhibition of acetylcholine hydrolysis at pH 8.0 and 25 °C with competitive \( K_c = 0.0015 \) \( \mu M \) at high substrate concentration and noncompetitive \( K_c = 0.0056 \) \( \mu M \) at low substrate concentration (47).

Literature value for dibucaine inhibition: \( K_c = 2.7 \) \( \mu M \) in 0.067 M phosphate, pH 7.4, with 50 \( \mu M \) benzoylcholine (48).

Literature value for succinylcholine inhibition: \( K_c = 97.7 \) \( \mu M \) in 0.067 M phosphate, pH 7.4, with 50 \( \mu M \) benzoylcholine (49).

\( BuSCW \), butyrylthiocholine.

\(^a\) o-NPA, ortho-nitrophenylacetate.

\(^c\) Literature values for tacrine inhibition: complex inhibition of acetylcholine hydrolysis at pH 8.0 and 25 °C with competitive \( K_c = 0.0015 \) \( \mu M \) at high substrate concentration and noncompetitive \( K_c = 0.0056 \) \( \mu M \) at low substrate concentration (47).

\(^d\) Literature value for dibucaine inhibition: \( K_c = 2.7 \) \( \mu M \) in 0.067 M phosphate, pH 7.4, with 50 \( \mu M \) benzoylcholine (48).

\(^e\) Literature value for succinylcholine inhibition: \( K_c = 97.7 \) \( \mu M \) in 0.067 M phosphate, pH 7.4, with 50 \( \mu M \) benzoylcholine (49).

\( BuSCh \), butyrylthiocholine.

![Figure 7](http://example.com/figure7.png)

**FIG. 7.** Secondary plots of data from Lineweaver-Burk plots for BuChE-catalyzed hydrolysis of o-nitrophenylacetate in the presence of various, fixed concentrations of dibucaine. Lineweaver-Burk plots yielded intercepts for \( 1/V_{\text{max}} \) and slopes for \( K_{\text{app}}/V_{\text{max}} \), which were plotted against dibucaine concentration. This plot shows that dibucaine gives nonlinear inhibition of o-nitrophenylacetate hydrolysis. Dibucaine concentrations above 25 \( \mu M \) for UU and 75 \( \mu M \) for rFF have an activating effect. \( 1/V_{\text{max}} \), continuous curves; slope, dashed curves. Panel 1, purified usual BuChE; panel 2, recombinant fluoride-2 BuChE.

![Figure 8](http://example.com/figure8.png)

**FIG. 8.** Progressive inhibition of purified usual plasma BuChE by iso-OMPA. The semi-log plot shows residual activity toward butyrylthiocholine after incubation of BuChE with 10-200 \( \mu M \) iso-OMPA in 0.1 M phosphate, pH 7.0, at 25 °C, for various lengths of time. Each reaction contained 0.01 \( \mu M \) active sites of purified usual plasma BuChE, UU. Numbers on lines are iso-OMPA concentrations (\( \mu M \)). The lines were drawn by fitting the data to Equation 2, thus yielding an apparent \( k_a \) value for each iso-OMPA concentration. The open circles \((O)\) are apparent \( k_a \) values for fluoride-2 BuChE, calculated from the initial slopes of the lines in Fig. 9. The dotted line is a fit of these apparent \( k_a \) values to Equation 3. For usual BuChE, \( K_a = 60 \pm 16 \) \( \mu M \), \( k_a = 0.53 \pm 0.06 \) min\(^{-1}\).
The G390V mutation induces no steric hindrances in the model of the fluoride-2 variant, and there is no indication that it could affect either folding or stability of the variant. Molecular Modeling—Modeling of human BuChE by Harel et al. (58) gave a structure superimposable on the 3-D crystallographic structure of Torpedo AChE. The Gly³⁹⁰ Val mutation is located in the αF helix (Fig. 10). In an attempt to explain the reduced affinity of the fluoride-2 variant, we have used the BuChE model to simulate the interaction of BuChE with succinylcholine (Fig. 11) and of the mutant. These changes were not observed. The second hypothesis to explain the reduced affinity of the fluoride-2 variant, G390V, is located on the αF helix. Amino acids are numbered from the N-terminal of mature human BuChE (43). Corresponding amino acid numbers for Torpedo AChE are S200, H440, E327, G119, W84, W233, Y442, and D392.

Since these hydrogen bonds stabilize the acid turn (60) on which Glu³⁷⁵ is located, a weakening of these bonds may alter the position and/or reactivity of His⁴⁴⁰. Thus, the G390V mutation could change the relationship between Glu³⁷⁵ and His⁴⁴⁰. This view could explain the observed slight shift in pKᵣ of His⁴⁴⁰.

As described in Fig. 11 amino acids Trp⁸², His⁴⁴⁰, Gly⁴⁴⁰, and Tyr⁴⁴⁰ are involved in the binding of one of the choline heads of succinylcholine. A displacement of His⁴⁴⁰ could move Tyr⁴⁴⁰ away from Trp⁸², with the result of a weaker affinity for substrates and ligands. However, this hypothesis is doubtful. It supposes significant movement of His⁴⁴⁰, which would imply important changes in the catalytic properties of the mutant. These changes were not observed. The second choline head of succinylcholine interacts with Trp⁵³¹. Molecular modeling provides no evidence that the orientation of Trp⁵³¹ is changed in the mutant enzyme.

A second hypothesis to explain the low affinity of this mutant suggests that the G390V mutation weakens the interaction between Asp⁷⁰ and Tyr⁷³² and alters the transducer function of these amino acids. The G390V mutation could change the hydrogen bond network around Thr⁷³⁷ and thus change the beginning of the αF helix on which are located Ala⁷²⁸, Phe⁷⁷⁹, and Tyr⁷³². In Torpedo and electric eel AChE, the amino acids in equivalent positions (Phe⁷³⁰, Phe⁷³¹, and Tyr⁷³⁰) are assumed to play an important role in the binding of substrate and ligand (19, 61).

Ala⁷²⁸, Phe⁷³⁰, and Tyr⁷³² appear to be more than 5 Å from the Trp⁸² aromatic cluster and the substrate choline head. It could nevertheless be supposed that displacement of the αF helix could affect Tyr⁷³² which is hydrogen-bonded to Asp⁷⁰ in the gorge surface 10 Å distant from the Trp⁸² aromatic...
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Fig. 11. Stereo view of succinyldicholine in the active site gorge of modeled human BuChE. a, close-up view of the G390V mutation and its relation to the active site. One choline head of succinyldicholine interacts with Trp52, with the peptide bond carbonyl of Gly117, and with the peptide bond carbonyl of His118. Trp52 is hydrogen-bonded to Tyr406. The presence of Leu180 and Val189 in human BuChE in place of Phe180 and Phe189 in Torpedo AChE reduces steric constraints in the BuChE active site gorge and allows a weak (4 Å) cation-aromatic interaction between the second choline head and Trp52. The two carbonyls of succinic acid are hydrogen-bonded to NH in the peptide bonds of Gly117 and Gly118. These carbonyls form a solid angle of 80°. The carbonyl susceptible to nucleophilic attack by Ser196 has been placed in a tetragonal conformation at 1.6 Å from OG2 of Ser196. Number under dashed line are distances expressed in Angstroms. b, photograph. Amino acids are colored according to their type: acidic, red; basic, blue; aromatic, magenta; aliphatic, yellow; and green for other residues. Succinyldicholine is dark yellow. The Connolly's surface of the enzyme is represented by red points to a 1.6 Å probe sphere, while the Van der Waals surface of succinyldicholine is green.

binding site. It should be remembered that mutation of Asp70 to Gly in human BuChE determines the "atypical" variant (62). People carrying the Asp70 Gly mutation experience hypersensitivity to succinyldicholine because of reduced affinity for this drug (4, 6). As suggested from site-directed mutagenesis of human AChE (63), these two amino acids (Tyr41 and Asp74 in human AChE) could be involved in signal transduction of substrate-induced conformational changes following initial binding to the cholinesterase active surface. Thus, displacement of the beginning of helix αF'1 could affect the conformational plasticity of amino acids involved in the binding process (Fig. 11). Dislocation of the interaction between Asp70 and Tyr41 and weakening of the signal transduction mechanism could explain the reduced affinity of the fluoride-2 variant for charged substrates and ligands. As modeling does not allow a clear understanding of the properties of the fluoride-2 variant, crystallization of human BuChE is underway.

DISCUSSION

The purpose of this work was to investigate the effect of the point mutation Gly390 to Val on the kinetic properties of BuChE. This point mutation is naturally present in the fluoride-2 variant of human BuChE, and its presence is associated with an abnormal response to succinyldicholine (reviewed in Ref. 6).

The fluoride number and dibucaine number (percent inhibition of benzoylcholine hydrolysis by 50 μM NaF or 50 μM dibucaine) of the recombinant fluoride-2 BuChE were 36 and 66, respectively. These values are in agreement with fluoride
numbers of 34–35 and dibucaine numbers of 64–68 for the three published homozygous fluoride plasma samples (64–66). This indicates that the kinetic behavior of the recombinant enzyme is not dependent on posttranslational modifications, such as glycosylation, that occurred in CHO cells. No other comparisons are possible because no other kinetic data for the fluoride variants of plasma BuChE have been published.

The secreted recombinant fluoride-2 BuChE acquired proteolytic nicks during storage of serum-free culture medium. These proteolytic nicks did not appear to affect kinetic parameters. A similar conclusion regarding the unimportance of proteolytic nicks was made by Caet et al. (67) who determined that the trypsin-generated monomer of horse serum BuChE behaved with identical kinetic parameter values as the native tetrameric enzyme. Our results are for the tetrameric form of recombinant fluoride-2 BuChE, since despite proteolysis most of the recombinant BuChE was a tetramer.

The kinetic data we report show that the Gly' to Val mutation did not dramatically change the behavior of the enzyme. Although the $pK_a$ of the fluoride-2 BuChE was slightly lower than the $pK_a$ of the usual plasma BuChE, the reactivity of fluoride-2 BuChE with the irreversible organophosphate inhibitor iso-OMPA was similar to that of usual BuChE, suggesting that catalytic activity of usual and fluoride-2 BuChE did not differ markedly. In fact, the only significant differences between usual and fluoride-2 BuChE concerned their $K_s$, $K_a$, and $a$ coefficient of mixed-type inhibition for dibucaine inhibition of $p$-nitrophenylbutyrate hydrolysis. Ratios of these apparent parameters fluctuated between 1.5 and 6 depending on the substrate and inhibitor nature. This indicated that the fluoride-2 BuChE bound neutral and charged substrates and ligands less tightly than the usual BuChE.

Molecular modeling to understand the molecular basis of the remote effect of the Gly' to Val mutation upon the binding affinity of BuChE suggested two hypotheses. One hypothesis is that the mutation exerts an indirect effect on the position of Glu+, which in turn slightly alters the $pK_a$ of His'. The altered position of Glu+ could induce displacement of Tyr'' which is hydrogen bonded to Trp'. A second, more likely, hypothesis is that the mutation alters the beginning of helix aF1, which in turn could dislocate the hydrogen bond between Asp' and Tyr. This may weaken the binding of charged substrates and reduce signal transduction capability. These tentative explanations from molecular modeling need to be verified by x-ray analysis of the BuChE crystal structure.

As regards the question of hypersensitivity to succinylcholine, one can calculate that after intravenous injection of a clinical dose of succinylcholine (1 mg/kg), the initial plasma concentration of succinylcholine is close to 20 μg/liter, that is 50 μM (68). Since the apparent $K_s$ of fluoride-2 BuChE for this compound is 125 μM (Table IV), the rate of hydrolysis of succinylcholine by this variant should be slow when the concentration of the muscle relaxant is in the pharmacologic range. Thus, the low affinity of fluoride-2 BuChE for succinylcholine could explain the moderate succinylcholine hypersensitivity of people carrying this variant.

Acknowledgments. We thank Dr. J. Sussman and Dr. M. Harel (Weizmann Institute of Science, Rehovot, Israel) for the coordinates of the three-dimensional structure of T. californica AcChE and for the coordinates of their human BuChE model. Expression plasmid pD5 was a gift from Dr. K. Berker, ZymoGenetics, Seattle, WA.