Comparison of Atypical and Usual Human Serum Cholinesterase

PURIFICATION, NUMBER OF ACTIVE SITES, SUBSTRATE AFFINITY, AND TURNOVER NUMBER*

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Atypical and usual human serum cholinesterases were purified and studied with the fluorescent probe, N-methyl-(7-dimethylcarbamoxy)quinolinium iodide. Four active sites per tetramer were found in each enzyme. The turnover numbers of usual and atypical cholinesterases were the same: 15,000 μmol of benzoylcholine hydrolyzed/min/μmol of active site; 48,000 min⁻¹ for o-nitrophenylbutyrate; and 0.0025 min⁻¹ for N-methyl-(7-dimethylcarbamoxy)quinolinium iodide. They had identical rate constants for carba-mylation, (5.0 min⁻¹) and for decarbamylation (0.15 h⁻¹). The major difference between the two genetically determined forms of the enzyme was substrate affinity, Kᵣ, being 0.16 mM for usual and 5.4 mM for atypical cholinesterase, for the fluorescent probe substrate. Kᵣ, for the uncharged ester, o-nitrophenylbutyrate, was 0.14 mM for both enzymes, whereas Kᵣ, for benzoylcholine was 0.005 mM for usual and 0.024 mM for atypical cholinesterase. We interpret these data to mean that the two enzymes differ only in the structure of their anionic site.

Atypical human serum cholinesterase is of interest clinically because it is associated with a protracted duration of action of normal doses of the muscle relaxant succinylcholine. Atypical differs from usual cholinesterase (acylcholine acyl-hydrolase EC 3.1.1.8) in that atypical has a higher Kᵣ for all choline ester substrates (1), atypical has a lower affinity for quaternary nitrogen containing inhibitors (2), and atypical cholinesterase affecting the anionic site. Of the above possible reasons for slower hydrolysis of succinylcholine and o-nitrophenylbutyrate were calculated using the number of active sites obtained from the quinolinium titration and these turnover numbers were the same for both enzymes. We therefore conclude that there is a structural alteration in atypical cholinesterase affecting the anionic site. Of the above possible reasons for slower hydrolysis of succinylcholine by atypical serum, reduced affinity but a lower turnover number explains the reduced activity in our two patients.

MATERIALS AND METHODS

Activity Assay — Activity was measured at a benzoylcholine chloride (Sigma) concentration of 0.05 mM in 0.067 M phosphate, pH 7.4, according to the method of Kalow and Lindsay (7) with a Gilford recording spectrophotometer. The temperature of the cell compartment was maintained at 25° with a Lauda circulating water bath. The extinction coefficient at 240 nm for the difference in absorbance between substrate and product was 6700 M⁻¹ cm⁻¹. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 μmol of benzoylcholine/min. Units were measured at 0.05 mM benzoylcholine; at this substrate concentration the activity of atypical cholinesterase was only 60 to 70% of V₅₀ while usual cholinesterase activity was 90 to 94% of V₅₀. Dibucaine number was determined by the method of Kalow and Genest (8) using dibucaine hydrochloride ("Nupercaine") donated by CIBA Pharmaceutical Co. Fluoride number was determined by the method of Harris and Whittaker (9). Protein concentration for purified enzyme was calculated from absorbance at 280 nm, using an extinction coefficient of 1.8 cm⁻¹ for a 1 mg/ml solution (10).

Acrylamide Gel Electrophoresis — Six per cent acrylamide gels, cast in glass tubing holding about 1 ul, were run at 5 mA/gel at constant current and then stained for protein with Coomassie blue, or stained for esterase activity by the method of Jull (11).
The three-step overall yield was 60%.

**o-Nitrophenylbutyrate** - The rate of hydrolysis of o-nitrophenylbutyrate was measured at 0.008 M phosphate, pH 7.4, containing 0.6% methanol. A stock solution of 15 mM o-nitrophenylbutyrate was made up in 100% methanol and was stable for at least 1 week (it decomposed rather rapidly when stored in aqueous solution). The extinction coefficient of o-nitrophenol at pH 7.4 in 0.008 M phosphate containing 0.6% methanol was 1800 M⁻¹ cm⁻¹ at 414 nm. Observed rates were corrected for nonenzymatic hydrolysis of the ester. From 0.002 to 0.008 unit of enzyme was used per assay in a total volume of 3.0 ml. Substrate concentrations ranged from 0.15 to 1.0 mM. The procedure was modified from Main et al. (12).

**Purification of Usual Cholinesterase** - Plasma from the blood bank was used which had an activity in the order of 0.7 units/ml, a dibucaine number of 80, and a fluoride number of approximately 60. Plasma from 1 pint of whole blood, taken from a single donor, was purified at a time. A two step purification method was used, resulting in pure enzyme. In the first step, dialyzed plasma was chromatographed on 280 ml of DEAE-cellulose at pH 4.0 in the cold as described by Das and Liddell (13) with two modifications suggested by Muench (14): the buffers contained 1 mM EDTA and 1 mM mercaptoethanol to preserve enzyme activity, and a linear salt gradient from 0.02 to 0.4 M NaCl in a small protein shoulder preceding the major protein peak, in agreement with the result obtained by Muensch (14). The first step typically yielded 150 units with a specific activity of 5 to 8 units/mg. Enzyme was concentrated to 20 ml in an Amicon ultrafiltration apparatus fitted with a Diaflo PM 30 membrane, under the low nitrogen pressure of 2 P.S.I. Overnight dialysis in 2 liters of 0.02 M potassium phosphate, pH 6.9, containing 1 mM EDTA was followed by affinity column chromatography at room temperature.

Three to four milliliters of the affinity gel was packed in a column (0.9 x 14 cm) and equilibrated with 0.02 M potassium phosphate, pH 6.9, containing 1 mM EDTA. A flow rate of 1 ml/min was used. The step was containing 1 mM NaCl in buffer; enzyme with a specific activity of 170 units/mg was eluted by 0.2 to 0.4 M NaCl in buffer with an overall yield of 75%. The flow rate was 1 ml/min.

**Purification of Atypical Cholinesterase** - Plasma was obtained from two atypical donors, who were relatives of patients who had suffered prolonged paralysis from succinylcholine following surgery. The term "homologous atypical" was assigned on the basis of their characteristic inhibitor indices: dibucaine and fluoride, and low esterase activity. The donors, F.G. and N.K., had dibucaine numbers of 19 and 17, fluoride numbers of 23 and 27, and activities of 0.3 and 0.4 unit/ml of serum, respectively. The two donors lived in different geographical areas (New York and Michigan) and were unrelated, Caucasian, one male and one female.

The first step in the purification was the same DEAE-cellulose chromatography procedure as used for the usual enzyme. The elution pattern and degree of purification were identical for the atypical and usual cholinesterases. This is contrary to the results of Das who reported a lower fold purification for atypical cholinesterase with this step (15). Affinity chromatography on 5 ml of swollen gel, yielded enzyme with specific activity of 75 units/mg. The enzyme was eluted at 0.1 M NaCl in buffer.

**Fluorimetry** - The "ratio recording" spectrofluorimeter used in these experiments was designed and built by David Ballou and Gordon Ford of the University of Michigan Biochemistry Department. It had a highly stabilized Hewlett-Packard power supply and the lamp output was stabilized by a magnetic arc stabilizer. The system was stabilized and irregularities in the lamp energy spectrum were corrected by a rhodamine B quantum counter in conjunction with an electronic ratio circuit. The temperature of the sample compartment was regulated by a circulating water bath. A strip chart recorder with variable scale expansion was used.

**RESULTS**

**Purity of Usual Cholinesterase** - The usual cholinesterase was pure by the criteria of specific activity and evidence of a single protein band on acrylamide electrophoresis. The specific activity of 170 units/mg is equivalent to that found by Das and Liddell (13) for pure enzyme. The enzyme was concentrated to 160 units/ml in a Minicon A25 concentrator (Amicon Corp.) prior to electrophoresis and 50 µg were applied to ensure that any protein contaminants could be detected. A single protein band corresponding to the C₃ species in the nomenclature of Harris (17) was present. Zymogram staining showed that the protein band was associated with a high degree of cholinesterase activity. A second activity band, containing less than 1% of the total activity and corresponding to the lower molecular weight C₂ species, was present. The ultraviolet absorbance spectrum of the pure enzyme was unremarkable with a peak at 280 nm and a trough at 234 nm. There was a small shoulder at 290 nm. The ratio of 280 to 260 nm absorbance was 1.67.

**Purity of Atypical Cholinesterase** - The specific activity of the purified atypical cholinesterase was 97 units/mg measured under standard assay conditions. At V₅₀, this specific activity was 136 µmol of benzoylcholine hydrolyzed/min/mg. This degree of purity, although higher than the maximum previously reported by Das of 8.5 units/mg (15) probably is not the maximum possible for this enzyme. If the specific activity at infinite substrate concentration of fully active, pure atypical cholinesterase is the same as that of usual cholinesterase, the atypical enzyme was only 72% pure. Acrylamide gel electrophoresis of the ester.
phoresis showed two contaminating bands of lower molecular weight than the enzyme. The ultraviolet spectrum of purified atypical was the same as that of the usual cholinesterase. The 280 to 260 nm absorbance ratio was 1.68.

**Stability** – The purified enzymes were stored at 4°C in 0.02 M phosphate, pH 6.9, containing 1 mM EDTA. The activity was stable for at least 1 year. Stability was the same whether or not 1 mM mercaptoethanol was included. Dilute solutions (0.02 units/ml) were as stable as concentrated enzyme (8 units/ml). Storage for 1 year at pH 4.0 resulted in 98% loss of activity, and storage for 1 year in water resulted in 50% loss of activity. As much as 75% of enzyme activity was lost when the affinity column was operated at a slow flow rate of 2 ml/h. Probably some of the affinity ligand, procainamide, which is an inhibitor of cholinesterase, was released from the Sepharose backbone and inhibited the enzyme. Affinity column chromatography experiments were therefore completed within 1 day. Buffers applied to the affinity column contained no mercaptoethanol because mercaptoethanol appeared to accelerate this release.

**Number of Active Sites** – Cholinesterase reacts with the substrate, N-methyl-(7-dimethylcarbamoyl)quinolinium iodide, as shown in Scheme 1 (6, 18). The Michaelis complex,

\[
E + S \xrightarrow{k_2} ES \xrightarrow{k_3} E + P
\]

**ES**, is formed in a rapid equilibrium defined by the dissociation constant \(K_d\). The enzyme is then carbamylated with a rate constant \(k_2\) to give a carbamyl enzyme intermediate containing a covalent bond between the active site serine and carbamic acid, and the fluorescent quinolinium ion. The carbamyl enzyme intermediate slowly decarbamylates with the rate constant \(k_3\). Because \(k_3\) is slow compared to \(K_d\), the fluorescent quinolinium ion accumulates in a burst which is titrated with various concentrations of pure usual cholinesterase. The buffer was 0.067 M phosphate, pH 7.4, containing 1 mM EDTA and 1 mM mercaptoethanol. The number of active sites was calculated from fluorescence burst; the decarbamylation rate constant, \(k_3\), was calculated from the slow steady state fluorescence increase. A high ester concentration was chosen so that the half-time for reaching the burst fluorescence was about 0.5 min.

**Fig. 1 (left).** Titration of active sites. Traces are shown of fluorescence increase, on a linear scale, at 510 nm, in an experiment where 57 \(\mu\)M N-methyl-(7-dimethylcarbamoyl)quinolinium iodide was titrated with various concentrations of pure usual cholinesterase. The buffer was 0.067 M phosphate, pH 7.4, containing 1 mM EDTA and 1 mM mercaptoethanol. The number of active sites was calculated from fluorescence burst; the decarbamylation rate constant, \(k_3\), was calculated from the slow steady state fluorescence increase. A high ester concentration was chosen so that the half-time for reaching the burst fluorescence was about 0.5 min.

**Fig. 2 (right).** Experiment for the determination of \(K_d\) and \(k_3\). Fluorescence traces are shown for the reaction of a constant amount of pure usual cholinesterase (11 \(\mu\)g/ml) with various concentrations of N-methyl-(7-dimethylcarbamoyl)quinolinium iodide. The reactions were much slower than in Fig. 1 because the ester concentrations were lower. The same fluorescence endpoint was reached in all three reactions. Buffer and temperature were the same as in Fig. 1. The initial small rise in fluorescence was due to the presence of small quantities of hydrolysis product in the ester solution. Fluorescence began to be monitored 20 to 30 s after the addition of ester; mixing and insertion of the cuvette into the fluorimeter took place during that time.

**Table 1**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Patient</th>
<th>Specific activity [^a]</th>
<th>Active sites [^b]</th>
<th>Turnover number [^c]</th>
</tr>
</thead>
<tbody>
<tr>
<td>UU</td>
<td>UU</td>
<td>84</td>
<td>4.0</td>
<td>14,700</td>
</tr>
<tr>
<td>UU</td>
<td>UU</td>
<td>16</td>
<td>4.0</td>
<td>14,800</td>
</tr>
<tr>
<td>UU</td>
<td>X.Y.</td>
<td>47</td>
<td>4.5</td>
<td>13,300</td>
</tr>
<tr>
<td>UU</td>
<td>X.Y.</td>
<td>63</td>
<td>4.0</td>
<td>15,000</td>
</tr>
<tr>
<td>UU</td>
<td>X.Y.</td>
<td>160</td>
<td>3.9</td>
<td>15,200</td>
</tr>
<tr>
<td>UU</td>
<td>X.H.</td>
<td>160</td>
<td>4.3</td>
<td>13,800</td>
</tr>
<tr>
<td>AA</td>
<td>P.G.</td>
<td>3</td>
<td>4.0</td>
<td>14,700</td>
</tr>
<tr>
<td>AA</td>
<td>P.G.</td>
<td>3</td>
<td>4.3</td>
<td>13,700</td>
</tr>
<tr>
<td>AA</td>
<td>N.K.</td>
<td>4</td>
<td>4.0</td>
<td>14,900</td>
</tr>
<tr>
<td>AA</td>
<td>N.K.</td>
<td>97</td>
<td>3.5</td>
<td>16,700</td>
</tr>
</tbody>
</table>

\[^a\] UU, homozygous usual cholinesterase; AA, homozygous atypical.

\[^b\] Activity was measured at 0.05 mM benzoylcholine; protein was calculated from \(E_{410}^{\text{mm}}\) = 1.8.

\[^c\] Moles of enzyme were calculated by assuming \(M_r = 340,000\) and activity for pure enzyme of 188 \(\mu\)mol of benzoylcholine hydrolyzed min\(^{-1}\) mg\(^{-1}\) at \(V_{\text{max}}\) for both the atypical and usual enzymes.

\[^d\] No assumptions were made for this calculation. Benzoylcholine hydrolysis rate was the extrapolated \(V_{\text{max}}\) in a Lineweaver-Burk plot.

\[^e\] Moles of enzyme were calculated from protein concentration using the extinction in footnote b at 280 nm and \(M_r = 340,000\). colored contaminants had been removed, as in DEAE-purified cholinesterase.

In the second method, enzyme molarity was calculated from milligrams of protein by measuring absorbance at 280...
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The purified enzymes had the same $K_m$ of 0.14 mM for o-nitrophenylbutyrate. Main et al. (12) reported a $K_m$ of 0.11 mM, and Szasz (21) reported a $K_m$ of 0.15 mM for cholinesterase in human serum. McComb et al. (22) reported a 2-fold higher $K_m$ for atypical human cholinesterase for o-nitrophenylbutyrate compared to usual cholinesterase, but our results do not show a difference in $K_m$.

Kinetic Constants — Kinetic constants for the reaction of carbamyl ester with cholinesterase were calculated from experiments of the type illustrated in Fig. 2, which shows traces from an experiment with purified usual cholinesterase. All three reactions in Fig. 2 reached the same fluorescent endpoint. Observed rate constants, $k_{obs}$, for each substrate concentration were evaluated from plots of log fluorescence change versus time. The reciprocals of $k_{obs}$ were then plotted, as in Fig. 3, against reciprocal ester concentration. The intercept on the $y$ axis is equivalent to $1/k_2$; the slope divided by the intercept gives the dissociation constant, $K_D$. Rosenberry and Bernhard (6) as well as Stuckland et al. (23) explain the theoretical basis of these evaluations. Results are summarized in Table III. The usual cholinesterase had a first order rate constant for carbamylation $k_2$, of 5.0 min$^{-1}$ and a $K_D$ of 0.16 mM. Atypical cholinesterase also had a $k_2$ of 5.0 min$^{-1}$, but its $K_D$ was 5.4 mM.

The carbamylamion rate constant, $k_1$, was determined from the steady state increase in fluorescence in experiments of the type shown in Fig. 1. The very slow linear fluorescence increase observed after the burst was the sum of the rate of decarboxylation and of the nonenzymatic hydrolysis of ester.

**TABLE II**

Comparison of turnover numbers and $K_m$ values of purified human usual and atypical serum cholinesterases for three compounds

<table>
<thead>
<tr>
<th>Cholinesterase</th>
<th>Substrate</th>
<th>Turnover number</th>
<th>$K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>usual</td>
<td>N-methyl-(7-dimethylcarbamoyl)quino-&lt;br&gt;linium iodide</td>
<td>0.0025</td>
<td>0.00008</td>
</tr>
<tr>
<td>usual</td>
<td>Benzoylcholine chloride</td>
<td>15,000</td>
<td>0.005</td>
</tr>
<tr>
<td>usual</td>
<td>o-Nitrophenylbutyrate</td>
<td>48,000</td>
<td>0.14</td>
</tr>
<tr>
<td>atypical</td>
<td>N-methyl-(7-dimethylcarba-&lt;br&gt; moyloxy)quinolinium iodide</td>
<td>0.0025</td>
<td>0.002</td>
</tr>
<tr>
<td>atypical</td>
<td>Benzoylcholine chloride</td>
<td>15,000</td>
<td>0.024</td>
</tr>
<tr>
<td>atypical</td>
<td>o-Nitrophenylbutyrate</td>
<td>48,000</td>
<td>0.14</td>
</tr>
</tbody>
</table>

**TABLE III**

Kinetic constants for reaction with N-methyl-(7-dimethylcarbamoyl)quinolinium iodide at 25°

The constants were determined for purified enzyme from three different usual and two atypical sera in experiments such as those of Fig. 2. The buffer was 0.067 M phosphate, pH 7.4, containing 1 mM EDTA and 1 mM mercaptoethanol.

<table>
<thead>
<tr>
<th>Human serum</th>
<th>$K_0$</th>
<th>$k_2$</th>
<th>$k_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>usual type</td>
<td>0.16 ± 0.01</td>
<td>5.0</td>
<td>0.15 ± 0.05</td>
</tr>
<tr>
<td>atypical type</td>
<td>5.4 ± 0.1</td>
<td>5.0</td>
<td>0.15 ± 0.05</td>
</tr>
</tbody>
</table>
by buffer. The contribution of the nonenzymatic rate was eliminated by plotting observed fluorescence increase/h against milliliters of enzyme in the reaction. The slope of the line, when divided by the concentration of active sites which is equivalent to the height of the burst, resulted in kₙ. For both usual and atypical cholinesterase kₙ was found to be 0.15 h⁻¹.

**DISCUSSION**

In order to determine how atypical human serum cholinesterase differed from usual serum cholinesterase, we purified the enzyme from four usual and two atypical donors and studied the purified enzymes with the fluorescent probe substrate. We found the rate constants for carbamylation and decarbamylation to be the same for both enzymes and, therefore, the turnover number for this compound, N-methyl-(7-dimethylcarbamoyl)quinolinium iodide, was the same for both genetic forms of the enzyme. However, we found that the dissociation constant was 33 times higher for atypical cholinesterase. This indicated that the difference between the two enzymes was confined to a difference in affinity for substrate. The binding site for substrate, the "anionic site" in the model proposed by Wilson and Bergmann (24), was altered, but the hydrolytic site, also called "esteratic site," was not. To test this conclusion, we measured the turnover numbers with two other compounds and found that atypical and usual cholinesterases hydrolyzed benzoylcholine with the same maximal rate per active site, and this was also true for o-nitrophenylbutyrate. The Kᵣ for benzoylcholine was lower for usual cholinesterase as compared to atypical cholinesterase, but the Kᵣ for o-nitrophenylbutyrate was the same for both enzymes. These results are consistent with the interpretation that atypical enzyme has an alteration affecting only the anionic site. Possibly the glutamic acid within the anionic site (25) is replaced in atypical enzyme by a neutral amino acid. Such a change would explain why the positively charged quaternary nitrogen-containing compounds were bound more tightly by atypical enzyme, whereas the uncharged o-nitrophenylbutyrate was bound equally well to both enzymes. Kalow and Davies (2) from their studies with differential inhibitors of atypical and usual cholinesterases also concluded that the esteratic sites were the same, whereas the anionic sites differed in the two enzymes.

Our finding of 4 active sites/molecule of atypical as well as usual cholinesterase indicates that the alteration in atypical enzyme does not affect the subunit organization of the molecule. Four active sites agrees with La Du and Choi's (26) observation that heterozygote serum contained five different iso enzymes, separable by affinity chromatography. The five iso enyzmes were thought to have arisen from combination of atypical and usual subunits into tetramers. This interpretation required that the enzyme be a tetramer. Yoshida and Motulsky (27) found 3 or 2 active sites in human serum cholinesterase, while Muensch et al. (14) reported 2 disopropyl fluorophosphate binding sites/tetramer. The present result of 4 active sites is consistent with the findings of other investigators that the molecule is a tetramer (28, 29). Horse serum cholinesterase has been found to be a tetramer (30-33) with 4 active sites (30) or 2 active sites (33).

Four active sites/molecule is conclusive for the usual cholinesterase and probably so for atypical cholinesterase. From our data one could argue that 3 active sites/tetramer is the correct answer for atypical cholinesterase. The atypical molecule would then have to be made up of three active subunits and one totally inactive subunit. However, we know that the atypical enzyme was not completely pure, and therefore the data seem to fit best the interpretation that atypical cholinesterase has 4 active sites/tetramer and that the specific activity at Vₘₐₓ of pure atypical cholinesterase is the same as it is for usual cholinesterase.

One should consider other possibilities with regard to the nature of the variation in atypical cholinesterase. There may be as much genetic heterogeneity in atypical cholinesterase as there is in hemoglobin. The most common hemoglobin variant in the United States is the sickle cell hemoglobin S which differs from hemoglobin A by a single amino acid, glutamic acid having been replaced by valine at the sixth position from the NH₂-terminal end of the β chain. Over 200 much less common hemoglobin variants have been identified, the great majority having only a single amino acid substitution (34, 35). It seems quite likely that a similar type of genetic heterogeneity exists in atypical cholinesterase and that our two atypical patients may represent the most common atypical variant, but others may be found with characteristics very different from those described here. The fluoride (9) and the silent cholinesterase (36) and the quantitative variant (37) are three known examples of genetic heterogeneity in serum cholinesterase.

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