Investigation of the role of a second conserved serine in carboxylesterases via site-directed mutagenesis

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Abstract
Carboxylesterases are enzymes that catalyze the hydrolysis of ester and amide moieties. These enzymes have an active site that is composed of a nucleophile (Ser), a base (His), and an acid (Glu) that is commonly known as a catalytic triad. It has previously been observed that the majority of carboxylesterases and lipases contain a second conserved serine in their active site [Proteins, 34 (1999) 184]. To investigate whether this second serine is also involved in the catalytic mechanism, it was mutated to an alanine, a glycine or a cysteine. Site-directed mutagenesis of this conserved serine resulted in a loss of specific activity, in both the S247G and S247A mutants (5- to 15-fold), which was due to a decrease in the rate of catalysis ($k_{cat}$). Due to the instability of the S247C mutant no reliable data could be attained. A carbamate inhibitor, carbaryl, was then employed to investigate whether this decrease in the $k_{cat}$ was due to the rate of formation of the acyl–enzyme intermediate ($k_2$) or the rate of deacylation ($k_3$). The S247A mutant was found only to alter $k_2$ (2.5-fold decrease), with no effect on $k_3$. Together with information inferred from a human carboxylesterase crystal structure, it was concluded that this serine provides an important structural support for the spatial orientation of the glutamic acid, stabilizing the catalytic triad so that it can perform the hydrolysis.

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Keywords: Carboxylesterase; Site-directed mutagenesis; Catalytic triad; Serine

Carboxylesterases (EC 3.1.1.1) are members of the $\alpha/\beta$ hydrolase fold family and are enzymes that catalyze the hydrolysis of a wide range of endogenous and xenobiotic ester-containing compounds [2]. The $\alpha/\beta$ hydrolase fold family all contain a catalytic triad consisting of a nucleophile, a base, and an orientating acid [2]. The existence of the catalytic triad was first established with acetylcholinesterase (AchE)\(^1\) a well-studied member of the $\alpha/\beta$ hydrolase fold family. It has been known for some time that both the serine and the histidine are members of the catalytic triad in AchE [3–5]. Further investigation via mutagenesis of these amino acids in AchE (Torpedo californica) confirmed that they were essential for catalysis [6]. The third member of the catalytic triad, the glutamic acid, was identified by crystal structure analysis of AchE [7]. This was again supported biochemically via the mutation of the Glu334 in human AchE in which all catalytic activity was lost [8]. This procedure has been repeated for many lipases and esterases (e.g., [9–11]), including the highly specific juvenile hormone esterase [12].

Carboxylesterases cleave esters via a two-step process that involves the formation and degradation of an acyl–enzyme intermediate. The hydrolysis initially occurs through a nucleophilic attack via the serine onto the

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\(^1\) Abbreviations used: AchE, acetylcholinesterase; PCR, polymerase chain reaction; pNPA, p-nitrophenyl acetate; IEF, isoelectrofocusing; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.
carbon of the carbonyl group (Fig. 1A) [2]. The catalytic serine is able to accomplish this with the assistance of the histidine acting as a general base. In turn, the protonated-histidine is stabilized via a hydrogen bond to the glutamic acid. These two catalytic amino acids, His–Glu, are often called a ‘charge relay’ system because they work together to activate the serine. The initial nucleophilic attack produces what is thought to be the first of two tetrahedral intermediates (Fig. 1A, 1) that are stabilized by the presence of two glycines in the active site (oxyanion hole). This tetrahedral intermediate rapidly collapses, aided by the protonated-histidine acting as a general acid, to produce an acyl–enzyme complex (Fig. 1A, 2) while the alcohol component of the ester is released. The acyl–enzyme complex then undergoes an attack by a histidine-activated water molecule (Fig. 1A, 3) which produces the second tetrahedral intermediate (Fig. 1A, 4). After rapid rearrangement of this intermediate, the enzyme is regenerated and the acid component released. Much debate still surrounds the exact nature of the mechanism described above, which includes the possibility of a short, strong bond or low barrier hydrogen bond (LBHB).

Fig. 1. (A) Detailed mechanism of the hydrolysis of esters by carboxylesterases. (B) Mechanism of the two-step carbamate cholinesterase inhibition model [25]. Compounds in boxes are the products of the hydrolysis.
between the glutamic acid and the histidine during catalysis [13–16]. By lowering the activation energy required for the formation of the first tetrahedral intermediate (Fig. 1A, I), the LBHB is believed to assist in the activation of the catalytic serine.

During a study involving the comparison of several esterase and lipase sequences, a second absolutely conserved serine was identified in all the sequences analyzed [1]. It was therefore hypothesized that this serine may be positioning the water molecule required for the second nucleophilic attack [1]. Not only was this second serine conserved in sequence but its absolute three-dimensional position was conserved in all published structures [1]. In this present study, rat carboxylesterase pl 6.1 (ES10) [17] was employed to explore the role of the second serine by converting it via mutagenesis to an alanine, a glycine or a cysteine. This carboxylesterase was chosen because it was one of the first carboxylesterases to be isolated, and it is a well-characterized enzyme [17–19]. It also appears to be a good representative of carboxylesterases in general. To directly explore the hypothesis that the serine orients the water molecule, both the catalytic and kinetic properties of these expressed mutants were measured and compared to that of the recombinant wildtype enzyme. In particular, a carboxylesterase inhibitor, carbaryl, was used to measure the specific kinetic parameters, \( k_d \), \( k_2 \), and \( k_3 \). We selected these compounds as models because the decarbamylation rate constant \( k_3 \) (Fig. 1B) is slow compared to the deacylation rate constant \( k_2 \). Thus, we hoped to use the carboxylesterases to dissect the role of the second serine.

Materials and methods

Chemicals

Carbaryl was purchased from Chem Services (West Chester, PA). All other chemicals used in this study were purchased from Sigma–Aldrich (St. Louis, MO), Fisher Scientific (Pittsburgh, PA), or synthesized as described below.

Synthesis of naphthalen-5-yl ethylcarbamate

Naphthol (1.0 g, 6.94 mmol) was ground to a fine powder using a mortar and pestle and placed into a small round bottomed flask, followed by the addition of ethyl isocyanate (0.5 mL, 6.32 mmol). A catalytic amount of triethylamine was then added, producing an immediate exothermic reaction. The resulting solid was recrystallized twice from methanol to give the product in 39% yield (0.53 g, 2.46 mmol): mp 100–101°C; \(^1\)H NMR (DMSO-\(d_6\)) \( \delta \) 1.12 (t, 3H, J=7.2 Hz), 3.14 (m, 2H), 3.33 (t, 1H, J=6.9 Hz), 7.26 (d, 1H, J=7.5 Hz) 7.57 (m, 3H), 7.86 (m, 3H).

Site-directed mutagenesis

Rat esterase pl 6.1 [17] was isolated by PCR screening a cDNA library prepared from Sprague–Dawley rat livers using specific primers (5*-CACAAATGC3CTG TCTACCTCCTG-3' and 5*-AACATGGCTGACCCCT CCTGATC-3') and a high fidelity Taq polymerase, Advantage-HF (BD Biosciences, San Jose, CA). The PCR product (1.7 kb) was cloned into a pT-Adv vector and sequenced to verify that it was the correct gene (DNA Sequencing Facility, UC Davis). Mutants of rat esterase pl 6.1 (S247G, S247C or S247A) were constructed using the QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). Briefly, this introduced the desired mutations using the following primers:

For
S247G (5'-GCCATTCTGAGGTTGTTGTTGTC3'),
S247C (5'-GCCATTCTGAGTGTGTTGTTGTC3'), and
S247A (5'-GCCATTCTGAGCTGTGTTGTTGTC3') and their complementary strands.

These primers were used in a Pfu polymerase initiated PCR. The original methylated, un-mutated DNA strand was digested with \( \text{DpnI} \), and the remaining DNA was transformed into \textit{Escherichia coli}. These mutated clones were verified by sequencing (DNA Sequencing Facility, UC Davis). The modified gene was then ligated into a pACUW21 baculovirus expression vector via the EcoRI site.

Expression and preliminary analysis

The recombinant baculovirus was generated in Sf21 cells [20]. The viral DNA was extracted using the High Pure Viral Nucleic acid Kit (Roche, Indianapolis, IN) and a PCR was performed to ensure that the gene had been incorporated. The recombinant baculovirus was expressed in \textit{Trichophasia ni} High five cells (2L, 1×10^6/mL) with a multiplicity of infection of approximately 0.1 at 28°C. At 72h post-infection the cells were pelleted (4000g, 20 min) and resuspended in 50 mM Tris–HCl, pH 8.0, containing 1 mM dithiothreitol, 1 mM EDTA, and 1 mM 1-phenyl-2-thiourea (buffer A). The cells were then homogenized (2 × 30 s; 10,000 rpm, Polytron homogenizer, Brinkmann, Westbury, NY) and the microsomes and cellular debris were pelleted by centrifugation (100,000 g, 1 h). The pellet was resuspended in buffer A including 1% (w/v) of n-octyl-\(\beta\)-D-glycopyranoside and placed on a rotating wheel for 1 h according to the method of Huang [21]. The solution was centrifuged (100,000 g, 1 h) and the supernatant, dialyzed over 24 h (4°C). The wildtype rat carboxylesterase 6.1 and each...
of the mutations (30 µg) were analyzed by SDS–PAGE/Western.

Carboxylesterase purification

All procedures were performed at 4°C unless otherwise stated. Following the expression and homogenization of the rat esterase 6.1 as described above, the cellular debris and microsomes were pelleted by centrifugation (100,000g, 1h). The supernatant was diluted 5-fold loaded onto a DEAE ion exchange column (3 × 15 cm, Amersham Biosciences, Piscataway, NJ), and washed with buffer B (20 mM Tris–HCl, pH 8.0, and 1 mM dithiothreitol). This was followed by a second wash with 30 mM NaCl in buffer B and the carboxylesterase was eluted with 50 mM NaCl in buffer B. The carboxylesterase containing fractions were detected by measuring p-nitrophenyl acetate (pNPA) hydrolysis activity at 405 nm according to methods of Ljungquist and Augustinsson [22] as described in Wheelock et al. [23]. Following concentration by filtration (50 kDa cutoff; Millipore, Billerica, MA), the protein was further purified using a preparative isoelectric focusing unit (Bio-Rad, Hercules, CA; pH 5–8). The esterase containing fractions were re-applied onto the preparative IEF unit for refocusing and then loaded onto a gel filtration column (Superose 12 10/300, FPLC, Amersham Biosciences, Piscataway, NJ). The protein was eluted in buffer B, fractions were pooled, concentrated by filtration (50 kDa cutoff; Millipore, Billerica, MA), and stored at −80°C until used.

Antibody preparation

Rat carboxylesterase pI 6.1 was purified from rat liver microsomes according to a previously reported procedure [19]. The purified protein was N-terminally sequenced (Molecular Structure Facility, UC Davis) to confirm its identity. Antibodies were raised against this purified rat esterase 6.1 (ES10) according to the method previously described [24].

Protein analysis

Protein concentrations were determined using the Pierce BCA assay (Pierce, Rockford, IL) with BSA as the standard. SDS–PAGE and native PAGE were performed using 12% Tris-glycine gels (Invitrogen, Carlsbad, CA) whilst IEF–PAGE was performed using IEF 3–7 gels (Invitrogen). Westerns were analyzed with polyclonal antibodies raised against rat esterase 6.1 (ES10) (Rabbit No. 13053). Esterase activity was detected by the hydrolysis of pNPA to its corresponding alcohol by measuring the production of p-nitrophenolate anion at 405 nm as described above [22,23].

Kinetics

All kinetic analysis was performed in 0.1 mg/mL BSA in 20 mM Tris–HCl, pH 8.0. The inhibitor dissociation constant ($K_d$) and the rate of carbamylation ($k_2$), the enzyme was inhibited with a range of inhibitor (carbaryl) concentrations (0–250 µM final concentration) in the presence of excess substrate (pNPA). The substrate was added either 10, 20, 30 or 60 s after the introduction of the inhibitor. The initial rates of the acyl–enzyme formation at $t_0$ ($\rho$) were generated by plotting each concentration of $\ln (A_{t_0}/A_t)$ vs. time. The slopes ($\rho$) that were generated were then plotted against the inhibitor concentration, where $k_2$ is the maximum rate of formation at $t_0$ ($\rho$) ($s^{-1}$) and $K_d$ is the inhibitor concentration at a half of the $\rho_{max}$ ($\mu$M).

To determine the pH profile for the wildtype and mutations, specific activity ($p$NPA) was measured every 0.2 pH units using 20 mM Tris–HCl from pH 7.0 to 8.4. The inhibitor dissociation constant ($K_d$) and rate of carbamylation ($k_2$) were also calculated at pH 7.0, 7.5, 8.0, and 8.5 of 20 mM Tris–HCl buffer.

Results

Construction of rat carboxylesterase 6.1 (ES10) mutants

All mutants were generated using site-directed mutagenesis which incorporated the desired mutation (S247A, G or C) via PCR. Pfu, a high fidelity polymerase, was employed to limit the possible sequencing error produced during PCR. Verification by DNA sequencing was performed both after cloning into pACUW21 and following production of the baculovirus. All baculoviruses produced had incorporated the desired mutation with no unwanted changes.

Preliminary analysis of mutants

The rat esterase 6.1 and each of the mutants were expressed in insect cells (T. ni) that had been infected
with the recombinant baculovirus. The recombinant rat esterase mutants were initially analyzed by SDS–PAGE and IEF–PAGE/Westerns to verify that they were behaving in the same manner as the recombinant wildtype esterase (data not shown). SDS–PAGE/Western blot revealed one band at approximately 60kDa in all the expressed proteins, that corresponded to the size of rat carboxylesterase 6.1 (ES10) [17]. IEF/Western showed two bands of approximately pI 6.0 in the wildtype with an extra band in each of the mutants. As based on both IEF and SDS–PAGE, it was therefore judged that the mutants were apparently intact and could be used for comparison to the wildtype.

Following expression of the recombinant carboxylesterase, microsomes were produced, solubilized, and the soluble fraction was analyzed by SDS–PAGE/Western blot (Fig. 2). Activity assays (pNPA) performed with this crude protein indicated that both the S247A (14±1 nmol/min/mg) and S247G (22±1 nmol/min/mg) mutants had reduced hydrolysis activity compared to that of the wildtype (87±8 nmol/min/mg). The cysteine mutant (S247C) did not appear as stable or was not expressed as well as the other mutants (Fig. 2).

**Purification**

Because it was not possible to generate meaningful kinetic data with crude enzyme preparations, the rat esterase 6.1 and its mutants were purified before further analysis. During purification development, it was found that a considerable amount of the enzyme was located in the cytosolic fraction following the 100,000g centrifugation. The enzyme was therefore purified from this fraction. The carboxylesterase was purified using classical purification techniques (Table 1) in which a preparative isoelectrofocusing unit was largely responsible for obtaining purified protein. Because it is necessary to use ampholytes during the isoelectrofocusing step, a gel filtration step was added to the purification to remove the bulk of these ampholytes. During the course of the purification, the rat carboxylesterase 6.1 was purified 320-fold indicating that the expression from baculovirus was poor (approximately 0.3 % of all protein present). Hence, baculovirus expression may not be the best expression system for this enzyme.

Following the purification it was found that although the S247C mutant was the same size as the wildtype on SDS–PAGE, it was not identical to either the wildtype or the S247A mutant by IEF–PAGE analysis (data not shown). The introduction of a cysteine may have produced properties that shifted the relative pI of the protein or destroyed the native structure. As a result the S247C mutant was not used for any detailed kinetic analysis. IEF/Western blot analysis also revealed that the extra third band that was observed in all the crude mutants was still present after purification.

**Kinetic analysis**

Specific activity for pNPA was measured for both the wildtype and the mutants (Table 2). The purified mutants indicated that the specific activity had indeed been suppressed (5- to 15-fold) by the introduction of the glycine or alanine in place of the serine. Both general kinetic constants (K_m and k_cat) were determined with pNPA to evaluate whether the mutant was affecting the binding of the substrate or the rate of catalysis (Table 2). Very little variation was observed in the K_m

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**Table 1**

<table>
<thead>
<tr>
<th>Purification of wildtype rat carboxylesterase 6.1 (ES10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total protein (mg)</strong></td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td>Crude</td>
</tr>
<tr>
<td>DEAE</td>
</tr>
<tr>
<td>Preparative IEF</td>
</tr>
<tr>
<td>Preparative IEF 2</td>
</tr>
<tr>
<td>Gel filtration</td>
</tr>
</tbody>
</table>

* Enzyme activity measured using pNPA as described in Materials and methods.
between the wildtype and mutants; however, there was a 2.5- to 15-fold decrease in the $k_{\text{cat}}$.

To determine what affect the mutant was having on either the formation of the acyl–enzyme intermediate (Fig. 1A, 2) or the deacylation of this intermediate, kinetic constants of inhibition ($k_2$ and $k_3$) by carbaryl or naphthalen-5-yl ethylcarbamate (the ethyl derivative of carbaryl) were measured for the wildtype and the S247A mutant (Table 3). If this serine was involved in the positioning of the water molecule in the active site, only the rate of deacylation ($k_3$) of the acyl–enzyme intermediate should be affected. The S247A mutant was found to have a similar $k_3$ as the wildtype, only the $k_2$ was significantly different from the wildtype (2.5-fold decrease).

It is also possible that the S247A mutant may have altered the $pK_a$ of the active site. To investigate whether a change in the $pK_a$ of the active site of the mutant was responsible for the change in the $k_{\text{cat}}$ and $k_2$ an experiment was designed to compare the pH profile of the mutant to that of the wildtype (Fig. 3 and Table 4). No significant change in the ratio between the mutant and the wildtype was observed after analysis of the specific activity at various pH units (Fig. 3). Because the largest change observed between the wildtype and the S247A mutant was a decrease in the $k_2$, we also measured $k_2$ at different pH units. Very little relative change was observed for $k_2$ (Table 4).

**Discussion**

The possibility of the catalytic tetrad, which includes a serine in addition to the histidine, serine, and acid of the accepted triad, was first suggested in an unrelated

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**Table 2**

Kinetic parameters determined for the hydrolysis of pNPA for wildtype rat carboxylesterase 6.1 and mutants at pH 8.0

<table>
<thead>
<tr>
<th></th>
<th>Specific activity (µmol/min/mg)</th>
<th>$K_m$ (µM)</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$k_{\text{cat}}/K_m$ (µM$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype</td>
<td>9.1 ± 0.7$^a$</td>
<td>12 ± 1</td>
<td>9.9 ± 0.7</td>
<td>0.86 ± 0.08</td>
</tr>
<tr>
<td>S247A</td>
<td>0.61 ± 0.04</td>
<td>22 ± 3</td>
<td>0.66 ± 0.05</td>
<td>0.030 ± 0.002</td>
</tr>
<tr>
<td>S247G</td>
<td>1.8 ± 0.8</td>
<td>15 ± 4</td>
<td>4.1 ± 0.3</td>
<td>0.27 ± 0.04</td>
</tr>
<tr>
<td>S247C</td>
<td>0.055 ± 0.005</td>
<td>ND$^b$</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$ Results are means ± SD of three separate experiments.

$^b$ ND, not determined.

**Table 3**

Kinetic constants for wildtype rat carboxylesterase 6.1 and S247A mutant inhibition at pH 8.0

<table>
<thead>
<tr>
<th>Carbamate</th>
<th>$K_d$ (µM)</th>
<th>$k_2$ (s$^{-1}$)</th>
<th>$k_3$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype Carbaryl</td>
<td>35 ± 4$^a$</td>
<td>0.030 ± 0.004</td>
<td>0.011 ± 0.001</td>
</tr>
<tr>
<td>Ethyl carbaryl$^b$</td>
<td>4.0 ± 1.1</td>
<td>0.058 ± 0.010</td>
<td>ND$^c$</td>
</tr>
<tr>
<td>S247A Carbaryl</td>
<td>24 ± 9</td>
<td>0.012 ± 0.002</td>
<td>0.010 ± 0.001</td>
</tr>
</tbody>
</table>

$^a$ Results are means ± SD of three separate experiments.

$^b$ Ethyl carbaryl, naphthalen-5-yl ethylcarbamate.

$^c$ ND, not determined.

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**Fig. 3.** pH profile of both the wildtype and S247A mutant. Specific activity was measured by the hydrolysis of pNPA as described in Materials and methods. Maximal rates of hydrolysis were set to 100% at pH 8.2 to facilitate comparison of the wildtype and S247A mutant.

**Table 4**

Comparison of kinetic constants $K_d$ and $k_2$ from wildtype rat carboxylesterase 6.1 and the S247A mutant at different pH units in 20mM Tris–HCl

<table>
<thead>
<tr>
<th>pH</th>
<th>$K_d$ (µM)</th>
<th>$k_2$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wildtype</td>
<td>S247A</td>
</tr>
<tr>
<td>7.0</td>
<td>42 ± 6</td>
<td>42 ± 9</td>
</tr>
<tr>
<td>7.5</td>
<td>45 ± 9</td>
<td>34 ± 1</td>
</tr>
<tr>
<td>8.0</td>
<td>35 ± 4</td>
<td>24 ± 9</td>
</tr>
<tr>
<td>8.5</td>
<td>33 ± 1</td>
<td>20 ± 4</td>
</tr>
</tbody>
</table>

$^a$ Results are means ± SD of three separate experiments.
family of enzymes called the serine proteases [26,27]. Serine proteases are enzymes that have a catalytic site similar to that of esterases except the catalytic acid and base are towards the N-terminal of the nucleophilic serine rather than towards the C-terminal [28]. In almost all chymotrypsin-like proteases, the acid (Asp in chymotrypsin) is hydrogen-bonded to this second serine [29].

Investigation into the role of this serine in serine proteases has produced a number of contradictory observations. Mutagenesis of at least two different serine proteases revealed that although the rate of catalysis ($k_{\text{cat}}$) is affected by the substitution of this second serine it may not be essential for catalysis [26,30]. From these two studies it was proposed that the serine was most likely to be a structural feature, stabilizing the spatial orientation of the catalytic triad enabling it to perform the hydrolysis. In contrast, a more recent study concluded from mutagenesis of this serine in human $\alpha$-thrombin that the catalytic function ($k_{\text{cat}}$) is not affected but rather the serine is involved in the substrate binding to enzyme ($K_m$) [31]. It has been additionally elucidated that the main chain carbonyl of this serine is hydrogen-bonded to the catalytic histidine [32,33]. This interaction is also thought to stabilize the histidine and thus facilitate catalysis. Overall, these investigations conclude that this second serine is not involved directly in catalysis but is important for either, the stability and correct orientation of the catalytic triad or, the correct spatial environment for optimal substrate binding.

In a study analyzing a number of esterase and lipase sequences, it was observed that this conserved second serine could also be found in these enzymes, including AchE [1]. It was proposed in this study that this second serine could be involved in the positioning of the water that is required for the deacylation of the acyl–enzyme complex (Fig. 1A, 2). Although this second serine is often depicted as being hydrogen-bonded to the glutamic acid of the catalytic triad [34], there has been no direct work in carboxylesterases that has explored the role of this amino acid. Hence, we were interested in exploring the ramifications of removing this serine and replacing it with either, a glycine, an alanine or a cysteine. Because it was hypothesized that this serine could be important in positioning a water molecule during deacylation of the acyl–enzyme intermediate [1], we wanted to compare the kinetic parameters of both the wildtype esterase and its mutants. We anticipated that the nucleophilic attack of the acyl–enzyme complex (Fig. 1A, 3) by the water would be slower in the mutants than with the wildtype. Hence, we would expect a decrease in $k_2$, the rate of deacylation.

Initial kinetic analysis revealed as expected that $k_{\text{cat}}$ rather than $K_m$ was suppressed in the S247A and S247G mutants in comparison to the wildtype enzyme (Table 2). These observations support work done with $\alpha$-lytic protease where the introduction of the mutation altered the $k_{\text{cat}}$ with very little affect on the $K_m$ [30].

Employing the classical two-step carbamate cholinesterase inhibition model outlined by Main [25] (Fig. 1B), carbamate inhibitors were then employed to measure the kinetic parameters $K_d$, $k_2$, and $k_3$ for both the wildtype and the S247A mutant (Table 3). Some carbamates, including carbaryl, are used as insecticidal inhibitors of AchE. The ethyl-homolog is used as the nontoxic inhibitor of general esterase activity. These compounds form a carbamylated-enzyme (Fig. 1B, 2) analogous to the acyl–enzyme complex (Fig. 1A, 2). Because no difference in $k_3$ was observed between these two proteins, it is unlikely that this serine is involved in positioning the water for the final step of the hydrolysis. Instead, an unexpected 2.5-fold decrease in $k_2$ was observed in the S247A mutant as compared to the wildtype (Table 3). Because $k_{\text{cat}}$ is really a measure of both $k_2$ and $k_3$, the observed decrease in $k_{\text{cat}}$ appears to be a direct reflection of the decrease in $k_2$. The stability of the ethyl carbaryl was too high to be of value in this experimental approach (Table 3).

The rate of formation of the acyl–enzyme complex ($k_2$) can also dictate the relative value of $K_m$ [35]. When $k_2$ is fast, $K_m$ may not reflect the true affinity for the substrate due to the large impact of $k_2$ on its value. Hence, in this case, as there is no significant change in the in $K_m$ even after a change in $k_2$ induced by the S247A mutant, $K_m$ would appear to be a good approximation for substrate affinity. Therefore, for this enzyme, it does not appear that this second serine is involved directly in substrate binding. Although these findings are supported by work performed with $\alpha$-lytic protease [30], these observations are slightly different to the analysis of thrombin [31]. The large impact on the $K_m$ after the substitution of alanine for the second serine in thrombin implies a significant role for this amino acid in substrate–enzyme binding (10-fold increase in $K_m$). Comparison of $k_{\text{cat}}/K_m$ values from this study reveals that $\alpha$-thrombin alanine mutant is approximately 18-fold less than the wildtype which is similar to that observed in the S247A mutant (28-fold decrease in $k_{\text{cat}}/K_m$; Table 2). Detailed kinetic information revealed a smaller 2-fold decrease in $k_2$ in this same thrombin mutant. Comparison of these two enzymes suggests that this serine plays a minor role in both substrate-binding and stabilizing the spatial orientation of the catalytic triad for optimal hydrolysis. The small changes in $k_2$ or $k_{\text{cat}}/K_m$ in S→A mutants (either 2-fold decrease or increase as seen in trypsin [26]) appear to be the only factor that remains consistent in both serine proteases and this esterase. As substrates may vary widely in both these two families of enzymes perhaps this spatial orientation provided by this second serine is the predominant reason for its conservation.

The S247G mutant is slightly more active than the S247A mutant (Table 2), although both glycine and alanine are incapable of hydrogen-bonding to the catalytic
glutamic acid. Crystal structures of esterases, including a similar esterase, human carboxylesterase 1 (71% identity; 81% similarity with the rat carboxylesterase pI 6.1), indicate that the second serine is in the correct orientation and within hydrogen-bonding distance (2.49 Å) to the catalytic glutamic acid (Fig. 4) [7,36]. The serine may move during catalysis and is capable of hydrogen-bonding to the peptide backbone. In this case, its steric properties more closely resemble glycine than alanine. For this reason, the S247A mutant was used for all the detailed kinetic analysis. In addition, the decrease in $k_2$ suggests that the potential interaction between this serine and the catalytic glutamic acid appears to be more important during the initial part of the reaction in which the acyl–enzyme complex (Fig. 1A, 2) is generated rather than its hydrolysis. The glutamic acid itself stabilizes the histidine in order that it in turn can activate the catalytic serine in the formation of the acyl-intermediate. During the hydrolysis of the acyl-intermediate, the histidine activates a water molecule rather than the catalytic serine. It is not known why it appears to be important that the glutamic acid is in the correct orientation for the formation of the acyl–enzyme intermediate (Fig. 1A, 2) rather than its degradation. The activation of the water molecule may be less dependent on the correct orientation of the Glu–His ‘charge relay’ system.

The $pK_a$ of the active site of the S247A mutant did not seem to change from the wildtype enzyme (Fig. 3 and Table 4) between pH 7.0 and 8.5. We were limited to this pH range because the enzyme is not active below pH 7, and the background extremely high above pH 8.5. The $pK_a$ of the histidine in the active site of serine proteases is approximately 7 [37], and likely to be similar in carboxylesterases. Because the histidine has a large impact on the active site $pK_a$ it may mask any minor change introduced by the mutant.

In summary, these observations suggest that, as seen with the serine protease family [26,30], this second serine in carboxylesterases is most likely to be a structural support employed to stabilize the orientation of the catalytic triad via hydrogen-bonding rather than an essential catalytic amino acid.

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