Identification of catalytically essential residues in *Escherichia coli* esterase by site-directed mutagenesis

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Abstract ***Escherichia coli*** **esterase (EcE) is a member of the hormone-sensitive lipase family. We have analyzed the roles of the conserved residues in this enzyme (His^{103}, Glu^{128}, Gly^{163}, Asp^{164}, Ser^{165}, Gly^{167}, Asp^{262}, Asp^{266} and His^{292}) by site-directed mutagenesis. Among them, Gly^{163}, Asp^{164}, Ser^{165}, and Gly^{167} are the components of a G-D/E-S-A-G motif. We showed that Ser^{165}, Asp^{262}, and His^{292} are the active-site residues of the enzyme. We also showed that none of the other residues, except for Asp^{164}, is critical for the enzymatic activity. The mutation of Asp^{164} to Ala dramatically reduced the catalytic efficiency of the enzyme by the factor of 10^4 without seriously affecting the substrate binding. This residue is probably structurally important to make the conformation of the active-site functional.**

Key words: Site-directed mutagenesis; Active site; Hormone-sensitive lipase; Esterase; *Escherichia coli*

1. Introduction

Escherichia coli esterase (EcE), which is a member of the hormone-sensitive lipase (HSL) family, is composed of 319 amino acid residues and functions in a monomeric form [1]. The enzyme hydrolyzes fatty acid esters with the acyl chain length of less than 8 and tributyrin, but does not effectively hydrolyze fatty acid esters with an acyl chain length of more than 10, triolein, and cholesterol oleate. An esterase with similar enzymatic activities but with a higher thermostability has been obtained from *Bacillus acidocaldarius* as well [2]. These enzymes are functionally different from mammalian HSLs, which hydrolyze triacylglycerols stored in adipose tissue under acute hormonal control and are therefore responsible for the liberation of fatty acids that serve as an energy source (for a review see [3]). Physiological functions of these bacterial enzymes remain to be determined.

Since the three-dimensional structure of HSL has not been determined, detailed analyses for the structures and functions of these bacterial HSL homologs are expected to facilitate understanding of the functional structure of HSL. HSL is a multifunctional protein and is composed of an N-terminal domain with unknown function, the catalytic domain, and the regulatory domain [4,5]. The bacterial proteins in the HSL family so far identified are only homologous to this catalytic domain of HSL. Recently, the X-ray crystal structure of brefeldin A esterase (BFAE) from *B. subtilis*, which is also a member of the HSL family, has been determined at 2.5 Å resolution [6]. BFAE has a typical α/β hydrolase fold with a unique extended N-terminal subdomain, and is composed of a eight-stranded β-sheet and 11 α-helices. The high resemblance of this structure to a model for the three-dimensional structure of the catalytic domain of HSL [7] strongly suggests that all proteins in the HSL family share a common structure.

Lipases and esterases usually have a catalytic triad consisting of Ser, Asp/Glu, and His [8]. Based on the crystal structure of BFAE, Ser^{202}, Asp^{308}, and His^{338} have been proposed to form a catalytic triad [6]. Previous data that the individual mutations of the corresponding residues inactivated rat HSL [9,10] support this proposal. However, the enzymatic activities of the mutant proteins of rat HSL were not determined by using purified proteins, but determined by using homogenized COS cells, in which mutated cDNAs for rat HSL were expressed. Therefore, the possibility cannot be ruled out that the enzymatic activity of a mutant protein is below the background level but is substantially higher than the negligible level. To get more accurate and reliable data, we constructed EcE variants, in which the conserved residues were individually altered by site-directed mutagenesis, purified them, and analyzed their enzymatic activities.

2. Materials and methods

2.1. Cells and plasmids

Plasmid pDR319, which contains the *ybaC* gene encoding EcE under the control of the *tac* promoter, was previously constructed [1]. Competent cells of *E. coli* JM109 (recA1, endA1, gyrA96, thi, hsdR17, SupE44, relA1, λ⁻, Δ(lac-ProAB)/F′, traD36, $ProA^+B^+$, $lacI^qZ\Delta M15$) were obtained from Toyobo Co., Ltd. Cells were grown in Luria-Bertani (LB) medium [11] containing 100 mg/l ampicillin.

2.2. Preparation of mutant enzymes

The mutant *ybaC* genes were constructed by site-directed mutagenesis by the PCR overlap extension method [12] using a 5′ primer with the *Bam*HI site, a 3′ primer with the *Sal*I site, and 5′ and 3′ mutagenic primers containing the desired mutations. All primers were synthesized by Sawady Technology Co., Ltd. PCR was performed in 25 cycles with a Perkin Elmer DNA Thermal Cycler (model PJ2000) using KOD polymerase from Toyobo Co., Ltd. After the digestion by *Bam*HI and *Sal*I, the PCR fragments were ligated to the *Bam*HI-*Sal*I site of plasmid pDR319 to construct expression vectors for the mutant proteins. The nucleotide sequences of the genes encoding mutant proteins were confirmed by the dideoxy chain termination method [13].

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Abbreviations: EcE, *Escherichia coli* esterase; BFAE, brefeldin A esterase from *Bacillus subtilis*; HSL, hormone-sensitive lipase; CD, circular dichroism

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The overproducing strains were constructed by transforming *E. coli* JM109 with the resultant pDR319 derivatives. Cultivation of the *E. coli* JM109 transformants with these plasmids, overproduction of the mutant proteins, and purification of the mutant proteins from cells were carried out as described previously for the wild-type protein [1]. The purity of the mutant proteins was analyzed by SDS-PAGE [14].

2.3. Assay for enzymatic activity

The esterase activity was determined by using *p*-nitrophenyl butyrate as a substrate. The hydrolysis of the substrate was carried out at 25°C for 15 min in 100 μl of 20 mM phosphate buffer (pH 7.1) containing 5% acetonitrile. The reaction was terminated by the addition of SDS to a final concentration of 0.2%. The amount of *p*-nitrophenol produced by the reaction was determined from the molar absorption coefficient value of 14200 M^{-1} cm^{-1} at 412 nm. One unit of enzymatic activity was defined as the amount of the enzyme that produced 1 μmol of *p*-nitrophenol per min at 25°C. The specific activity was defined as the enzymatic activity per mg of protein. For the determination of the kinetic parameters, the substrate concentration spanned the K_m values. The hydrolysis of the substrate with the enzyme followed Michaelis-Menten kinetics, and the kinetic parameters, K_m and V_{max}, were determined from the Lineweaver-Burk plot.

2.4. Protein concentration

Protein concentration was determined from UV absorption on the assumption that all mutant proteins have the same absorption coefficient as that of the wild-type protein. We use an $A_{280}^{0.1\%}$ value of 1.60, which has previously been determined by amino acid analysis for the wild-type protein [1].

2.5. Circular dichroism (CD)

The far-UV (200–260 nm) and near-UV (250–320 nm) CD spectra were measured in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA at 25°C on a J-720 automatic spectropolarimeter (Japan Spectroscopic Co., Ltd.). The protein concentration and optical path length were 0.2 mg/ml and 2 mm for far-UV CD spectra, and 1.0 mg/ml and 10 mm for near-UV CD spectra, respectively. The mean residue ellipticity, [θ], which has the units of deg cm^2 $dmol^{-1}$, was calculated by using an average amino acid molecular weight of 110.

2.6. Structural prediction

Secondary structure of the protein was predicted by the PredictProtein PHD program [15] from the European Molecular Biology Laboratory. The amino acid sequences of proteins were aligned with Clustal W [16], with manual modifications. The atomic coordinates of BFAE (1JKM) was obtained from the Brookhaven protein data bank (PDB) and visualized on a Power Macintosh with RasMol [17].

Table 1
Kinetic parameters of EcE and its mutants for the hydrolysis of *p*-nitrophenyl butyrate

Protein	K_m (mM)	k_{cat} (s^{-1})	Relative k_{cat} (%)
Wild-type	0.50	30.9	100
H103N	0.25	12.1	39
E128Q	0.35	7.4	24
G163A	0.50	2.9	9.4
D164A	1.0	0.007	0.024
S165A	–	< 0.003	< 0.01
G167A	2.0	49.4	160
D262A	0.40	0.034	0.11
D266A	0.50	3.4	11
H292A	–	< 0.003	< 0.01

The hydrolysis of *p*-nitrophenyl butyrate with the enzyme was carried out at 25°C for 15 min in 100 μl of 20 mM phosphate buffer (pH 7.1) containing 5% acetonitrile. Kinetic parameters were determined by a least-squares fit of the data obtained from Lineweaver-Burk plots. Errors, which represent the 67% confidence limits, are within 10% of the values reported.

3. Results and discussion

3.1. Mutant design

When the amino acid sequences of various proteins in the HSL family are compared with one another, they are well conserved only in limited regions. The sequences of these regions for various proteins in the HSL family are shown in Fig. 1. In addition to them, the sequences which contain a HGGG motif (His^{91}–Gly^{94} in EcE) are well conserved. The main-chain nitrogen atoms of the second and third glycine residues in this motif have been suggested to be involved in the formation of an oxyanion hole [18]. Among the well conserved sequences shown in Fig. 1, three sequences (Gly^{163}–Gly^{167}, Glu^{260}–Ser^{268}, and Gly^{289}–Ala^{293} in EcE) contain invariant residues (Gly^{163}, Ser^{165}, Gly^{167}, Asp^{262}, Asp^{266}, and His^{292} in EcE). Of them, a Gly-Asp/Glu-Ser-X-Gly sequence, in which X represents any amino acid, has been suggested to be characteristic of the HSL family [19]. The serine residue within this motif forms a catalytic triad according to the crystal structure of BFAE [6]. Therefore, we constructed the mutant proteins G163A, D164A, S165A, and G167A, in which Gly^{163}, Asp^{164}, Ser^{165}, and Gly^{167} are individually replaced by Ala, to analyze the role of this motif. In addition, we constructed the mutant

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                  *                  *               *** *            *    *                 *
EcE   102 THDRI 106   124 TLSPE 128   163 GDSAG 167   260 EFDPLLDDS 268   289 GTLHA 293
BaE    92 THDPV 96    114 RLAPE 118   153 GDSAG 157   250 QYDPLRDVG 258   279 DLIHG 283
BFAE  138 VHRRW 142   161 AWTAE 165   200 GESGG 204   306 ELDPLRDEG 314   335 GLVHG 339
AcE   138 THDEA 142   160 PLAPE 164   199 GDSAG 203   293 GHDVLHDEG 301   322 DQTHG 326
REE    97 SSDPF 101   119 RLAPE 123   158 GESAG 162   258 ELDPLRDEG 266   287 GTFHG 291
AfE    97 SHDAL 101   119 PLAPE 123   158 GDSAG 162   253 EYDPLRDEG 261   282 GVLHG 286
NtE   100 MYYTV 104   122 RLAPE 126   169 GDSSG 173   270 EKDLIKDTE 278   299 GVGHS 303
PBL    92 THDNL 96    114 RLAPE 118   153 GDSAG 157   248 EFDPLRDEG 256   277 GMIHG 281
AeL   141 SHDPL 145   163 RLGPQ 167   202 GDSAG 206   300 GYDPLHDEG 308   329 GMIHD 333
MtLH   99 THDPV 103   121 RLAPE 125   160 GDSAG 164   258 EHDPLRDDG 266   287 TMVHG 291
ML2   176 THHEF 180   198 RMAPE 202   237 GDSAG 241   359 ELDILRDEG 367   388 GAPHG 392
hHSL  361 SHEPY 365   383 SLAPE 387   422 GDSAG 426   691 ALDPMLDDS 699   720 DLPHG 724
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Fig. 1. Alignment of the sequences of the proteins involved in the HSL family. Only the sequences at the mutated sites in EcE and corresponding ones in other proteins are shown. Fully conserved residues are shown in boldface and the mutated residues in the EcE sequence are marked by asterisks. The sequences have been deposited in the following databases. GenBank: X62835 for *Bacillus acidocaldarius* esterase (BaE), AF056081 for *B. subtilis* brefeldin A esterase (BFAE), Z1507210A for *Acinetobacter calcoaceticus* esterase (AcE). GI: AAC45283 for *Rhodococcus* sp. heroin esterase (REE), AAB89533 for *Archaeoglobus fulgidus* esterase A (AfE), and AAC38151 for *Pseudomonas* sp. B11-1 lipase (PBL). PIR: S42807 for *N. tabacum* HSR203J protein (NtE) and I39567 for *Alcaligenes eutrophus* lipase-like enzyme (AeL). EMBL: CAB02180 for *Mycobacterium tuberculosis* lipH (MtLH). Swiss-Prot: YBAC ECOLI for EcE, LIP2 MORSP for *Moraxella* TA144 lipase 2 (ML2), and LIPS HUMAN for human HSL (hHSL).

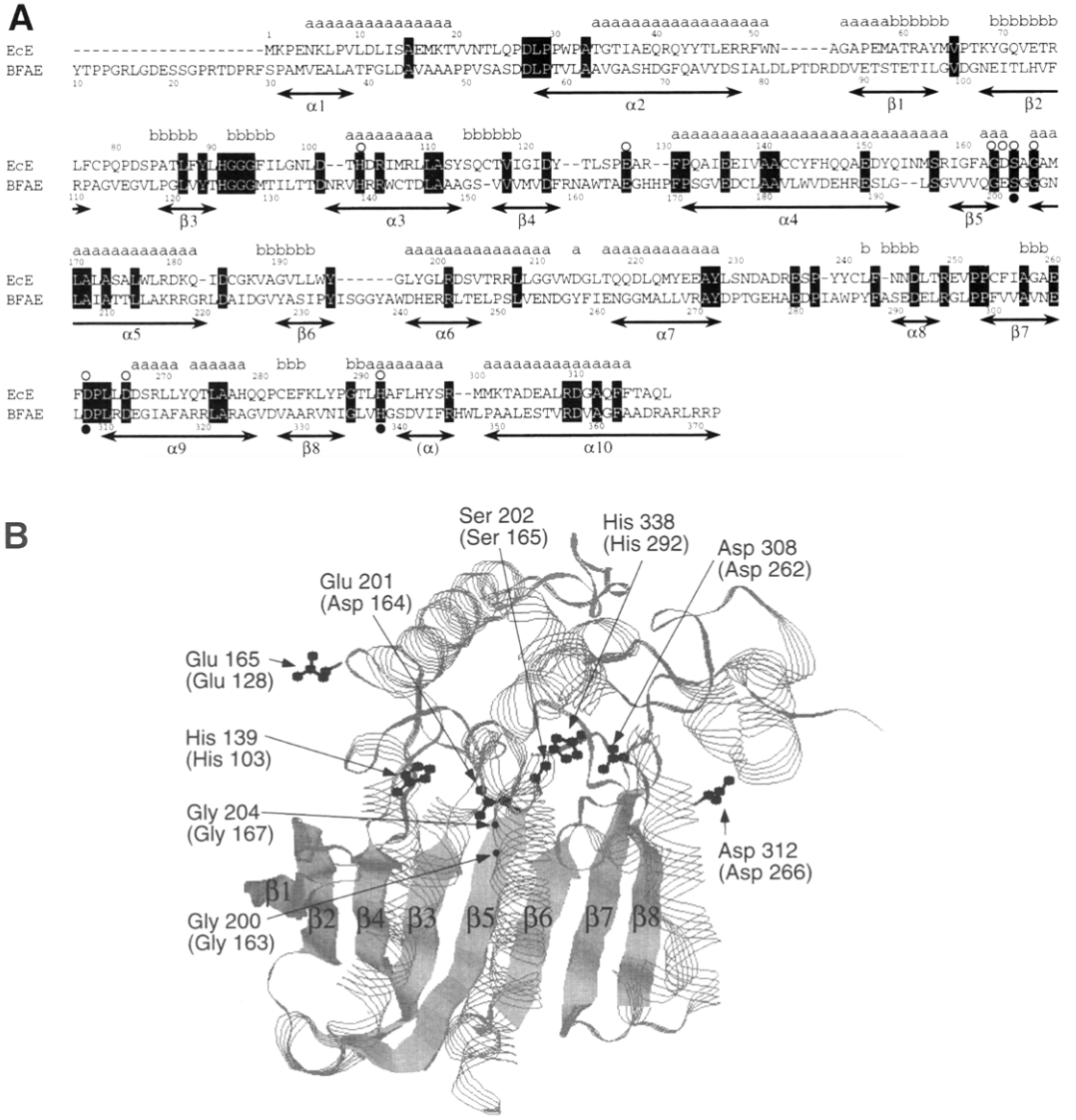

Fig. 2. Localizations of the amino acid residues corresponding to those mutated in the EcE sequence in the BFAE structure. A: Alignment of EcE and BFAE sequences based on secondary structure elements. Secondary structure elements predicted by PHD for EcE are shown above the amino acid sequences. 'a' and 'b' represent α-helix and β-strand, respectively. The ranges of the 11 α-helices and eight β-strands of BFAE are shown below the amino acid sequences. These secondary structures are designated according to Wei et al. [6]. Because an α-helix located between β8 and α10 (residues 340–345) has not been designated, it is shown in parentheses. Numbers represent the positions of the amino acid residues, which start from the initiator methionine for each protein. Gaps are denoted by dashes. The conserved residues between these two sequences are highlighted in black. The amino acid residues that were mutated in this study are marked by open circles above the sequences and those that are presumed to form a catalytic triad are marked by closed circles below the sequences. B: Backbone structure of BFAE. This structure was drawn with RasMol. The amino acid residues corresponding to those mutated in the EcE sequence, which are shown in parentheses, are displayed in ball-and-stick representation. This crystal structure is deposited in PDB with the accession number 1JKM.

proteins D262A, D266A, and H292A, in which Asp^{262}, Asp^{266}, and His^{292} are individually replaced by Ala, to analyze the role of these invariant residues. Two other sequences (Thr^{102}–Ile^{106} and Thr^{124}–Glu^{128} in EcE) do not contain invariant residues. However, a histidine (His^{103} in EcE) and a glutamic acid (Glu^{128} in EcE) residues are relatively well conserved. Therefore, we constructed the mutant proteins H103N and E128Q, in which His^{103} and Glu^{128} are replaced by Asn and Gln, respectively, to analyze the role of these well conserved residues as well.

To understand the role of the amino acid residues mentioned above, it would be worth knowing the localizations of the corresponding residues in the crystal structure of BFAE. The sequence identity between EcE and BFAE is 19.4%. This value may not be high enough to consider that the three-dimensional structures of these two proteins are highly similar to each other. However, when the secondary structures of EcE were predicted from its sequence and compared with those in the crystal structure of BFAE, all secondary structure elements predicted for EcE, except for that corresponding to β5, are arranged equally with those present in BFAE (Fig. 2A). This result strongly suggests that the three-dimensional structure of EcE is basically identical to that of BFAE. The amino acid residues corresponding to His^{103}, Glu^{128}, Gly^{163}, Asp^{164}, Ser^{165}, Gly^{167}, Asp^{262}, Asp^{266}, and His^{292} in EcE are His^{139}, Glu^{165}, Gly^{200}, Glu^{201}, Ser^{202}, Gly^{204}, Asp^{308}, Asp^{312}, and His^{338} in BFAE, respectively. The localizations of these residues in the BFAE structure are

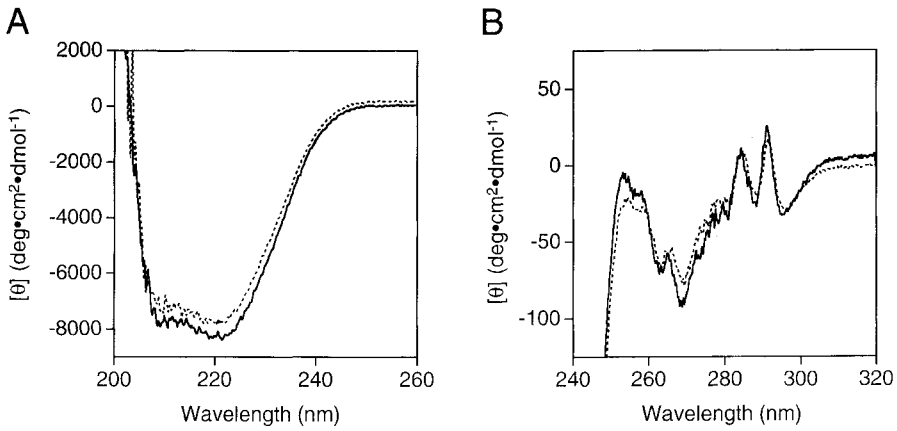

Fig. 3. CD spectra of the wild-type and mutant proteins. The far-UV (A) and near-UV (B) CD spectra of all mutant proteins were examined, and those of the mutant protein S165A (broken line), which showed the largest difference from those of the wild-type protein (solid line), are shown as a representative. All spectra were measured as described in Section 2.

shown in Fig. 2B. It is noted that all of these residues are located relatively close to the catalytic site, which is presumed to be formed by Ser^{202}, Asp^{308}, and His^{338}.

3.2. Properties of the mutant proteins

All the mutant proteins overproduced in *E. coli* were purified to give a single band on SDS-PAGE (data not shown). The far- and near-UV CD spectra of all mutant proteins were nearly identical to those of the wild-type protein (Fig. 3), indicating that none of the mutant proteins is markedly changed in its secondary and tertiary structures. The enzymatic activities of the mutant proteins were determined by using *p*-nitrophenyl butyrate (C_4) as a substrate. The kinetic parameters of the mutant proteins are summarized in Table 1. Based on these results, we discuss the roles of individual amino acid residues or motifs in the following sections.

3.3. Identification of the catalytic triad

As expected, the mutant proteins S165A and H292A showed little enzymatic activity (less than 0.01% of that of the wild-type protein). On the other hand, the mutant protein D262A showed a weak enzymatic activity. However, determination of the kinetic parameters revealed that the k_{cat} value was dramatically decreased, whereas the K_m value was unchanged, as compared to those of the wild-type protein. The k_{cat} value of this mutant protein was 0.11% of that of the wild-type protein. These results suggest that Asp^{262} is involved in the catalytic function, rather than the substrate binding. It has been proposed that the aspartic acid residue in a catalytic triad of serine proteases is required to stabilize a tautomer of the catalytic histidine residue [20,21] and a transition state [22]. Such a rather auxiliary role of the aspartic acid residue in catalysis may be the reason why EcE was not completely inactivated by the mutation of Asp^{262}. In fact, it has previously been reported for subtilisin that the mutation of Ser or His which form a catalytic triad reduced the k_{cat} value of the enzyme by 10^6 times, whereas the mutation of Asp, which is another component of a catalytic triad, reduced it only by 10^4 times [23]. Therefore, there seems no doubt that Ser^{165}, Asp^{262}, and His^{292} form a catalytic triad in EcE. Because the geometry of the catalytic triad in BFAE is similar to those in serine proteases [6], the catalytic mechanism of EcE is probably similar to those of serine proteases.

3.4. Role of a G-D/E-S-A-G motif

Gly^{163} and Gly^{167} are the first and the last residues in the G-D/E-S-A-G motif in EcE. The mutation of Gly^{163} to Ala reduced the k_{cat} value by 90% without affecting the K_m value, whereas the mutation of Gly^{167} to Ala resulted in a 60% increase in the k_{cat} value along with a 4-fold increase in the K_m value (Table 1). These results suggest that the former mutation affects the catalytic efficiency, whereas the latter mutation mainly affects the substrate binding.

A G-X-S-X-G motif which contains the active-site serine residue is conserved in most esterases and lipases [24]. This motif is usually located between a β-strand and an α-helix (β5 and α5 in BFAE and EcE) and assumes an extremely sharp turn [25]. In this so-called *nucleophile elbow*, the side chains of the first and the last residues are located very close to each other and therefore glycine residues are preferred at these positions to avoid a potential steric problem. In addition, according to the crystal structure of BFAE, these glycine residues are located close to the main-chain carbonyl oxygen of Thr^{124} (Leu^{90} in EcE). Thr^{124} is located at the C-terminal end of the β3 strand and is immediately followed by a HGGG motif (His^{125}–Gly^{128}), which is involved in the formation of the oxyanion hole [18]. Therefore, introduction of a methyl group into the first or the last position of the nucleophile elbow must create a steric hindrance and thereby alters the conformation of the nucleophile elbow and/or the oxyanion hole. This may be the reason why the $Gly^{163} \rightarrow$ Ala and the $Gly^{167} \rightarrow$ Ala mutations considerably affect the enzymatic activity. However, the reason why the former only affects the catalytic efficiency and the latter mainly affects the substrate binding remains to be solved.

Our results indicate that the presence of the glycine residues at the first and the last positions of the nucleophile elbow are not critical to make the conformation of the active site functional, because the mutations at these positions did not abolish the enzymatic activity. These results are consistent with the observations that mutations at the equivalent positions do not seriously affect the enzymatic activity, unless the size of the amino acid side chain is seriously altered by the mutation [26–28], and the lipases from *Bacillus* species often have an A-X-S-X-G motif, instead of a G-X-S-X-G motif [29–31].

In contrast to the mutations of glycine residues, the mutation of Asp^{164} to Ala resulted in a drastic decrease in the k_{cat}

value by a factor of 10^4, along with a slight increase in the K_m value (Table 1). It has been proposed that Glu[199] at the equivalent position in acetylcholinesterase from *Torpedo californica* is required to maintain the active-site serine residue in a precise orientation [32]. Therefore, Asp[164] may also be required to make the conformation of the active site functional. Further structural and mutational studies will be necessary to understand the role of the acidic amino acid residue which is fully conserved in the nucleophile elbow of the HSL members.

3.5. *His[103], Glu[128], and Asp[266]*

The mutations of His[103] to Asn, Glu[128] to Gln, and Asp[266] to Ala reduced the k_{cat} value by 61, 76, and 89%, respectively, without seriously affecting the K_m value (Table 1). Because the corresponding residues in BFAE (His[139], Glu[165], and Asp[312]) are located relatively close to the active site, alteration in the interactions between these residues and the surrounding residues may affect the conformation of the active site. However, none of the residues that are located in the vicinity of either one of these residues is conserved in the sequences of the HSL members. Therefore, it still remains to be solved why His[103], Glu[128], and Asp[266] in EcE are well conserved and the mutations of these residues considerably reduce the enzymatic activity.

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