Structural Insights into the Low pH Adaptation of a Unique Carboxylesterase from Ferroplasma

ALTERING THE pH OPTIMA OF TWO CARBOXYLESTERASES

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Background: Carboxylesterases typically have an alkaline pH optima, which limits their industrial application.

Results: An acidophilic carboxylesterase was crystallized and changed into an alkalophilic enzyme via quadruple mutations.

Conclusion: The extended hydrogen bonds in the active site were important for adaptation to a low pH.

Significance: The first structure of an acidophilic carboxylesterase revealed a novel strategy for the low pH adaptation of carboxylesterase.

To investigate the mechanism for low pH adaptation by a carboxylesterase, structural and biochemical analyses of EstFa_R (a recombinant, slightly acidophilic carboxylesterase from Ferroplasma acidiphilum) and SshEstI (an alkaliphilic carboxylesterase from Sulfolobus shibatae DSM5389) were performed. Although a previous proteomics study by another group showed that the enzyme purified from F. acidiphilum contained an iron atom, EstFa_R did not bind to iron as analyzed by inductively coupled plasma MS and isotothermal titration calorimetry. The crystal structures of EstFa_R and SshEstI were determined at 1.6- and 1.5-Å resolutions, respectively. EstFa_R had a catalytic triad with an extended hydrogen bond network that was not observed in SshEstI. Quadruple mutants of both proteins were created to remove or introduce the extended hydrogen bond network. The mutation on EstFa_R enhanced its catalytic efficiency and gave it an alkaline pH optimum, whereas the mutation on SshEstI resulted in opposite effects (i.e. a decrease in the catalytic efficiency and a downward shift in the optimum pH).

Our experimental results suggest that the low pH optimum of EstFa_R activity was a result of the unique extended hydrogen bond network in the catalytic triad and the highly negatively charged surface around the active site. The change in the pH optimum of EstFa_R happened simultaneously with a change in the catalytic efficiency, suggesting that the local flexibility of the active site in EstFa_R could be modified by quadruple mutation. These observations may provide a novel strategy to elucidate the low pH adaptation of serine hydrolases.

Serine hydrolases are an important class of hydrolytic enzymes that exist in all life and occupy ~1% of the human proteome (1). Carboxylesterases belong to one of the classes of serine hydrolases that catalyze the hydrolysis and transesterification of carboxyl esters (2–4). The mechanism of the hydrolysis of esters is analogous to that of peptide hydrolysis that is catalyzed by serine peptidases, which utilize catalytic mechanisms known as the catalytic triad. The canonical catalytic triad of carboxylesterases is a hydrogen bond network of Ser-His-Asp (5, 6) where the Ser residue acts as a nucleophile that attacks the carbonyl carbon of the ester linkage, whereas the His abstracts a proton from the catalytic Ser to enhance the nucleophilicity of the seryl O

Ka

value of 7.0 was from the titration of the catalytic His residue (9). Although an acidic pH is known to be severe for serine hydrolases, for a member of the sedolisin family, an exception has been discovered that allows it to function in acidic pHs (10). This acidophilic enzyme group has an overall fold that is similar to that of subtilisin, but it has a unique catalytic triad that consists of Ser-His-Asp (11). In a previous mutagenesis study, we substituted the Glu with His to show that the unique Glu residue is crucial for the acidophilic property of kumamolisin-As, which is a member of the sedolisin family (11).
higher. Surprisingly, however, the proteomics analyses of the hyperacidophilic archaeon *Ferroplasma acidophilum* have revealed that EstFa, a carboxylesterase from this organism, is a highly acidicophilic enzyme (12). Although the canonical catalytic triad is conserved in the primary amino acid sequences of EstFa, the reported optimum pH for catalytic activity of this enzyme is ~2.0–3.5, which is exceptionally low for the values of serine hydrolases (13). The authors reported that 86% of 186 *Ferroplasma* proteins, including EstFa, were shown to be iron-containing metalloproteins (12). However, the purity of the EstFa protein was not shown in the previous studies, and thus the importance of iron for EstFa catalysis remains unclear (12, 13).

In the present study, the recombinant EstFa (termed EstFa_R) was purified to homogeneity and enzymatically characterized. Unexpectedly, the recombinant protein displayed an activity without iron. The catalytic efficiency of EstFa_R was significantly lower than the value for SshEstI but even higher than the value reported previously for the enzyme purified from *F. acidophilum* (13). EstFa_R displayed a slightly acidophilic activity with a pH optimum of 5.0, which was still low for the values of carboxylesterases. The crystal structures of EstFa_R and SshEstI were determined and compared, and the structure of EstFa_R served as the first example of a protein from *Ferroplasma*. A detailed comparison of these two structures identified several structural features that were unique to EstFa_R. Based on these observations, detailed mutational, kinetic, and pH-dependent studies were carried out. The experimental data suggested that the extension of a hydrogen bond network around the active site of EstFa_R may have contributed to the lower catalytic efficiency and low pH adaptation of this enzyme. Possible contributions of other structural factors of EstFa_R for catalytic efficiency and low pH adaptation are also discussed.

**EXPERIMENTAL PROCEDURES**

*Archaeal Strains and Materials—* *F. acidophilum JCM10970* was obtained from the Japan Collection of Microorganisms, RIKEN BioResource Center (Tsukuba, Japan). p-Nitrophenyl (pNP) acetate and pNP butyrate were obtained from Sigma-Aldrich. pNP propionate and pNP caproate were obtained from Wako Pure Chemical Industries, Tokyo, Japan. pNP caprylate was obtained from Tokyo Chemical Industries, Tokyo, Japan. Plasmid pET22b was obtained from Novagen, Madison, WI. Isopropyl β-D-thiogalactoside was obtained from Nacalai Tesque, Kyoto, Japan. The restriction enzymes were obtained from Takara Bio, Kusatsu, Japan or New England Biolabs, Ipswich, MA. Polyethylene glycol (PEG) 3350 and PEG monomethyl ester 2000 were obtained from Hampton Research, Aliso Viejo, CA. Other reagents were of analytical grade and obtained from Sigma-Aldrich, Wako Pure Chemical Industries, and Nacalai Tesque. Construction of the plasmid pTrc-SshEstI was described previously (14).

Cloning and Mutagenesis—*F. acidophilum* (JCM10970) was cultured at 35 °C in a medium containing 0.04% MgSO₄·7H₂O, 0.02% (NH₄)₂SO₄, 0.01% KCl, 0.01% K₂HPO₄, 2.5%FeSO₄·7H₂O, and 0.016% yeast extract. The pH of the mixture without yeast extract was adjusted to 1.6–1.8 with H₂SO₄, and the mixture was then filter-sterilized. Yeast extract, prepared as an autoclaved 10% (w/v) solution, was added to the mixture immediately prior to use at the specified concentration. *F. acidophilum* genomic DNA was extracted according to the method of Bond et al. (15). The EstFa gene was amplified via a polymerase chain reaction (PCR) using genomic DNA as a template and the PCR primers EstFaAsel and EstFaNotI (see supplemental Table 1S for nucleotide sequences). The PCR was carried out as follows: 2 min at 94 °C followed by 30 thermal cycles where one thermal cycle consisted of 98 °C for 10 s, a linear gradient of 55–58 °C in 30 s, and then 68 °C for 1 min. The amplified fragment was digested with Asel and NotI, and the resultant 1-kbp fragment was ligated with the Ndel/NotI-digested pET22b to obtain pET22-EstFa. *Escherichia coli* Mach cells were transformed with pET22-EstFa, and the transformant cells were grown in an LB medium containing 60 μg of ampicillin/ml. The recombinant plasmid was extracted from the cells using a GenElute™ Plasmid Miniprep kit (Sigma-Aldrich) and sequenced using the primers EstFaseq185sense, EstFaseq299anti, and EstFaseq549 (supplemental Table 1S).

To create the N248A/P256Q/E257G/S283F (AQGF) mutant of EstFa_R, in vitro mutagenesis of the EstFa gene was carried out by recombinant PCR with plasmid pET22-EstFa (see above) as a template. First, N248A and S283F mutations were prepared. Then, three DNA fragments with overlapping ends were generated by PCR using each possible pair of the following primers: Ps/Pa248, Ps248/Pa283, and Ps283/Pa (supplemental Table 1S). The resultant three fragments were mixed and processed with PCR using primers Ps and Pa to produce the N248A/S283F mutant of EstFa_R. Then, P256Q and E257G mutations were introduced into the N248A/S283F mutant of EstFa_R. PCR was used to generate two DNA fragments with overlapping ends using the following primers: Ps/Pa256 and Ps256/Pa (supplemental Table 1S). The fragments were mixed and subjected to PCR using primers Ps and Pa to produce the N248A/P256Q/E257G/S283F mutant. The amplified fragment was digested with Asel and NotI, and the resultant 1-kbp fragment was ligated with the Ndel/NotI-digested pET22b. *E. coli* Mach cells were transformed with the resultant plasmid, pET22-AQGF, and *E. coli* transformant cells harboring the plasmid were grown in an LB medium containing 60 μg of ampicillin/ml.

Mutants of SshEstI (A241N, F276S, Q249P/G250E (termed PE), A241N/F276S (termed NS), Q249P/G250E/F276S (termed PES), A241N/Q249P/G250E (termed NPE), A241N/Q249P/G250E/F276S, NPE, A241N/Q249P/G250E, NPE, A241N/Q249P/G250E/F276S).

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The abbreviations used are: EstFa_R, recombinant EstFa; ITC, isothermal titration calorimetry; pNP, p-nitrophenyl; AQGF, N248A/P256Q/E257G/S283F; PE, Q249P/G250E; NS, A241N/F276S; PES, Q249P/G250E/F276S; NPE, A241N/Q249P/G250E; NPE, A241N/Q249P/G250E/F276S.
mids pTrc-PES and pTrc-NPE expressing the PES and NPE mutants, respectively, were derived from the plasmid pTrc-PE. The plasmids pTrc-NES, pTrc-NPS, and pTrc-NPES expressing the NES, NPS, and NPE mutants, respectively, were derived from the plasmid pTrc-NS. Sequences of mutagenesis primers are listed in supplemental Table 15.

**Heterologous Expression and Purification**—For the expression of EstFa_R and its AQGF mutants, E. coli BL21(DE3) transformant cells harboring the plasmids pET22-EstFa and pET22-AQGF, respectively, were grown at 37 °C overnight by shaking in a liquid LB medium containing 60 μg of ampicillin/ml. The overnight culture was diluted 100-fold with the same medium, and the cells were further grown under the same conditions. When optical turbidity of the culture at 600 nm reached 0.3–0.5, isopropyl β-D-thiogalactoside was added to the broth at a final concentration of 1.0 mM, and the cells were grown at 30 °C for 7 h for induction.

All purification steps were performed at 4 °C unless otherwise stated. The standard buffer that was used was 20 mM potassium phosphate, pH 7.2. For the EstFa_R and its AQGF mutant, harvested cells were resuspended in the standard buffer and disrupted by ultrasonication followed by centrifugation. The supernatant solution was filtered through a Minisart filtration on a Centricon YM-10 membrane (Millipore, Bil数字位置, 48242, Germany) and processed with an ÄKTA purifier system equipped with a Macro-Prep ceramic hydroxyapatite column as described above.

The sample was then processed with an ÄKTA purifier system equipped with a HiTrap Q HP column (5 ml; GE Healthcare) equilibrated with the standard buffer. After washing the column extensively with the standard buffer, the enzyme was eluted with a linear gradient of NaCl (0–0.4 M; total gradient volume, 40 ml) in the same buffer at a flow rate of 1.5 ml/min. Active fractions were then processed with an ÄKTA purifier system equipped with a Macro-Prep ceramic hydroxyapatite column (1.0 × 10 cm, Type I, 40 mm; Bio-Rad) equilibrated with the standard buffer. After washing the column with the standard buffer, EstFa_R was eluted with a linear gradient of potassium phosphate (0.02–0.5 M; total volume, 100 ml) in the same buffer at a flow rate of 1.5 ml/min. Active fractions were collected and dialyzed against the standard buffer. The sample was then processed with an ÄKTA purifier system equipped with a Mono Q HR 10/10 column (GE Healthcare), which was previously equilibrated with the standard buffer. After washing the column with 5 bed volumes of this buffer, the carboxylesterase activity was eluted by a linear gradient of NaCl (0–0.4 M; total gradient volume, 80 ml) in the same buffer at a flow rate of 1.0 ml/min. The purified enzyme was dialyzed against 10 mM HEPES and concentrated to 10 mg/ml by ultrafiltration on a Centricon YM-10 membrane (Millipore, Billerica, MA).

Wild-type SshEstI and its mutants were heterologously expressed in E. coli cells and purified to homogeneity essentially as described previously (14). However, for the purification of enzymes other than the NPES and PES mutants, HiTrap Q HP chromatography was omitted. Instead, the crude extracts of these enzymes were subjected to heat treatment at 60 °C for 1 h followed by centrifugation. The supernatant solution was subjected to chromatography on a Macro-Prep ceramic hydroxyapatite column as described above.

**Quantitative and Thermodynamic Analyses**—Quantitative determination of iron in the purified EstFa_R was performed by inductively coupled plasma mass spectrometry (MS) using an 8800 Triple Quadrupole ICP-MS (Agilent Technologies). Thermodynamic analyses for the interactions between EstFa_R and iron were performed by ITC using a MicroCal iTC200 system (GE Healthcare). The purified EstFa_R (10 μM) was dialyzed against buffers with either neutral pH (20 mM potassium phosphate, 1 mM Na2S2O4, pH 7.0) or acidic pH (20 mM sodium acetate, 1 mM Na2S2O4, pH 4.9), then placed in a calorimeter cell, and titrated with either FeSO4 or FeCl3 (1 mM each in the same buffers) under anaerobic conditions. The ligand solutions were injected 20 times in 2-μl portions over a period of 60 s. As a positive control, 10 μM apotransferrin (bovine; Sigma-Aldrich) was titrated with 1 mM FeSO4 under the same conditions at pH 7.0. Data acquisition and analysis were done using ORIGIN 7.0 software (GE Healthcare).

**Enzyme Assays and Kinetics**—The progress curves of the carboxylesterase-catalyzed hydrolysis of the fatty acid pNP esters were followed by monitoring p-nitrophenol production at 348 nm (ε348 = 5539 M⁻¹ cm⁻¹) in 1-cm-path length cells with a Hitachi double beam spectrophotometer (Model U-2000). Unless otherwise specified, the reaction mixture consisted of 50 mM KH2PO4-CH3COOH-NaOH buffer, pH 7.0, 1 mM pNP butyrate, 0.01% Triton X-100 (final concentrations), and enzyme in a final volume of 0.4 ml. The mixture without enzyme was preincubated at a specified temperature for 2 min, and the reaction was started by addition of the enzyme. Stock solutions of substrates were prepared by dissolving the substrates in acetonitrile. All initial velocity data were obtained in duplicate or triplicate and corrected for non-enzymatic hydrolysis of the substrate. Data for initial velocity versus substrate concentration were fitted to the Michaelis-Menten equation by non-linear regression analysis using a computer program (SigmaPlot 12 Enzyme Kinetics module, Systat Software, Inc.) (16).

**pH-Activity Profiles**—The pH-activity profiles of EstFa_R and its AQGF mutant were examined using 2 mM pNP butyrate as a substrate at 35 °C over a pH range of 2.5–8.5. The buffers used were as follows: pH 2.5–3.5, 50 mM glycine HCl or 100 mM sodium citrate; pH 3.0–5.0, 100 mM sodium acetate; pH 5.0–7.0, 100 mM MES-HCl; and pH 7.0–8.5, 100 mM HEPES-NaOH. The pH-activity profiles of ShsEstI and its mutants were examined using 2 mM pNP butyrate as a substrate at 40 °C with the following buffers: pH 2.5–3.0, 50 mM glycine HCl; and pH 3.5–9.0, 50 mM KH2PO4-CH3COOH-NaOH.

**Thermal Stability Studies**—For stability studies of EstFa_R and its AQGF mutant, the enzyme was incubated at 35, 40, 45, and 50 °C (for the wild-type enzyme) or at 40, 50, and 60 °C (for AQGF) in 20 mM HEPES-NaOH buffer, pH 7.5. At appropriate time intervals (1, 15, 30, and 45 min after incubation for EstFa_R and 1, 15, 30, 45, 60, and 120 min after incubation for AQGF mutant), an aliquot of the incubation mixture was withdrawn and immediately assayed for remaining activity as described above.

For stability studies of the NPES mutant of ShsEstI, the enzyme was incubated at 40, 50, and 60 °C in 20 mM HEPES-NaOH buffer, pH 7.5. At 10, 20, and 30 min after incubation, an
aliquot of the incubation mixture was withdrawn and immediately assayed for remaining activity as described with the noted exception of 50 mM KH2PO4-CH3COOH-NaOH buffer, pH 6.0 used as the buffer component.

Crystalization, Data Collection, and Structure Determination—Protein crystals were obtained at 20 °C by vapor diffusion, mixing 2 μl of protein solution with 2 μl of reservoir solution. The reservoir solutions for each protein were as follows: EstFa_R, 15% PEG 3350, 100 mM sodium acetate, pH 8.0; SshEstI, 13% PEG monomethyl ester 2000, 100 mM Tris-HCl, pH 7.0; the PE mutant of SshEstI, 12% PEG 3000, 200 mM NaCl, 100 mM phosphate-citrate, pH 4.2. Crystals were frozen at 90 K using 20% PEG 400 or 20% glycerol as cryoprotectants. X-ray diffraction data sets of the PE mutant of SshEstI, SshEstI, and EstFa_R were collected using an in-house CuKα source (Rigaku MicroMax HF007) and synchrotron radiation on beamline BL44XU at SPring-8 (Hyogo, Japan) and beamline BL-17A at the photon factory (High Energy Accelerator Research Organization, Tsukuba, Japan), respectively. The diffraction data were processed and scaled using the HKL2000 program (17). The crystal structure of SshEstI was solved by the molecular replacement method using EST2 (Protein Data Bank code 1EVQ), a thermostable carboxylesterase from *Alicyclobacillus acidocaldarius* as a search model. The sequence identity of EST2 to SshEstI is 41.7%. The crystal structure of EstFa_R was solved using SshEstI (Protein Data Bank code 3WJ1) as a search model. The sequence identity of SshEstI to EstFa is 35.6%. Molecular replacement was performed using the MOLREP CCP4 suite (18). The structure of the PE mutant of SshEstI was solved by direct Fourier synthesis of an sHHsEstI model. The percentages of reflections used for the calculation of R_free values were as follows: EstFa_R, 5%; SshEstI, 5.1%. The R_free column in the data set of the PE mutant of SshEstI was extended from that of SshEstI. The Coot program (19) was used for manual model building, and the resultant model was refined in conjunction with model rebuilding using the Refmac program (20). Omit maps on all residues in the models were prepared to remove initial model biases that may have been introduced. Molecular images were created using the PyMOL program (21). The Cα atoms of SshEstI and EstFa_R structures were superimposed using the DaliLite program (22). Electrostatic potentials of protein structures were calculated by the Adaptive Poisson-Boltzmann Solver (APBS) program within the PyMOL program (23).

RESULTS

*EstFa_R* Was Active without Iron—The enzymatic study of LigFa, a DNA ligase from *F. acidiphilum*, revealed its unusual iron requirement for activity and stability (24). Because EstFa was also reported to contain one iron per protein monomer, we attempted to obtain an iron-containing form of EstFa_R. The EstFa gene was amplified from *F. acidiphilum* genomic DNA by PCR with primers based on its reported nucleotide sequence (DDBJ/GenBank™/EBI Data Bank accession number AA850914). The amplified DNA fragment was cloned into a pET22b vector, and several different *E. coli* host strains were transformed with the resultant plasmid termed pET22-EstFa. The transformant cells were grown in an LB medium supplemented with 100 μM FeCl2, and crude extracts were prepared and then dialyzed at 4 °C against 10 mM sodium citrate buffer, pH 2.0. However, in all cases examined, the crude extracts displayed no carboxylesterase activity. By contrast, crude extract of *E. coli* BL21(DE3) harboring pET22-EstFa without supplemental FeCl2 displayed a carboxylesterase activity at pH 5.0, although EstFa has been reported to show almost no activity at that pH value (12). To obtain a homogeneous preparation of EstFa_R, three successive chromatographies were performed (for details, see “Experimental Procedures”). Inductively coupled plasma MS analysis of the purified EstFa_R confirmed the absence of iron, indicating that iron is not necessary for carboxylesterase activity of EstFa_R, unlike the activity of LigF, which is completely dependent on iron (24). To examine whether the purified EstFa_R binds iron, we performed thermodynamic analyses by ITC. As a result, EstFa_R showed no significant change in enthalpy by titrations of either the ferrous or ferric form of iron under acidic and neutral pH conditions (Fig. 1).

Table 1 shows the kinetic parameters of the purified EstFa_R during the hydrolysis of various fatty acid pNP esters at pH 6.0 and 35 °C. kcat values increased with increasing alky1 chain lengths (up to C4) of substrates. Likewise, Km values decreased with increasing alky1 chain lengths (up to C4) of substrates. The highest kcat/Km value was obtained with pNP butyrate (C4; 56 ± 3 s⁻¹ and 173 s⁻¹ mM⁻¹, respectively). These values were significantly lower than the corresponding values of SshEstI (859 ± 32 s⁻¹ and 6850 s⁻¹ mM⁻¹, respectively, at pH 7.0; see Table 2). It must be noted that the observed substrate specificity of EstFa_R contrasted with the reported specificity of EstFa where pNP propionate (C3) served as the best substrate (13). When kcat and kcat/Km values were compared for the best substrates of EstFa_R and native EstFa, the value of EstFa_R (56 s⁻¹ and 173 s⁻¹ mM⁻¹, respectively, at pH 6.0 and 35 °C for pNP butyrate) was higher even at a lower temperature than the value of native EstFa (29 s⁻¹ and 64 s⁻¹ mM⁻¹, respectively, at pH 2.0 and 50 °C for pNP propionate) (13).

EstFa_R was a slightly acidophilic enzyme. The pH dependence of pNP butyrate hydrolysis catalyzed by EstFa_R showed a bell-shaped profile with an optimum pH of 5.0 (Fig. 2A), which was higher than the value reported previously for EstFa (pH 2.0–3.5) (12, 13) but still low for a carboxylesterase. The optimum temperature of EstFa_R for pNP butyrate (1 mM) as a substrate was near 50 °C (Fig. 2B). This value was similar to the value reported for acidophilic carboxylesterase activity of EstFa. Stability studies revealed that EstFa_R was inactivated at 50 °C in biphasic kinetics where the t½ value of the fast phase of inactivation was less than 1 min (Fig. 2C). Thus, EstFa_R was less stable than the enzyme purified from *Ferroplasma* with a t½ value of inactivation for EstFa at 50 °C and pH 2.0 that was reported to be 48 min (12).

Crystal Structures of EstFa_R and SshEstI—EstFa_R is still considered to be acidophilic in contrast with other known carboxylesterases. Although *S. shibatae* inhabits the same type of environment as *F. acidiphilum*, SshEstI shows an alkaliphilic pH optimum of 8.0 (Ref. 14; see also Fig. 2A). To better understand the mechanism of a unique pH dependence of EstFa_R, we attempted to crystallize the purified enzyme, which was sub-
jected to x-ray crystallographic analysis. SshEstI (sequence identity to EstFa_R, 35.6%) was also crystallized and subjected to crystallographic analysis to compare the structures of acido-philic and alkaliphilic types. Despite the instability of EstFa_R (see above), we were able to obtain crystals of EstFa_R that diffracted to a resolution of 1.6 Å. Crystals of SshEstI were also successfully obtained and diffracted to a resolution of 1.5 Å. The crystal structures of EstFa_R and SshEstI were solved by means of a molecular replacement method by using the coordinates of EST2 (17, 18), a carboxylesterase from A. acidocaldarius (for SshEstI), and EstFa_R (for EstFa_R) as search models. Data collection statistics for the structures of EstFa_R and SshEstI are summarized in Table 3. The monomer structure of EstFa_R exhibited a significant structural similarity to that of SshEstI (Fig. 3, A and B) with a root mean square deviation of 1.24 Å (292 residues). The overall structures of EstFa_R and SshEstI contain eight-stranded β/α- sheets and nine β/α-helices and consist of three subdomains (Fig. 3 C) where the fold type of the largest subdomain corresponded to the β/α- hydrolase fold. In both structures, two remaining subdomains clustered on the carboxyl edge of the β-sheet and formed a cap over the putative active site. These remaining subdomains were helical with the first incorporating the N-terminal residues (45 residues in EstFa_R and 39 residues in SshEstI) and the second constituting

![FIGURE 1. Purification and isothermal titration calorimetry of EstFa_R. A, purified EstFa_R analyzed by SDS-PAGE. Calorimetric titrations of EstFa_R either at pH 7 (B) or at pH 5 (C) with Fe²⁺ or Fe³⁺ ions under anaerobic conditions were performed. The top panels contain raw binding data, and the bottom panels show the binding isotherms created by integrations of heat peaks against the molar ratio of the ligand. D, calorimetric titrations of apotransferrin (bovine) with Fe²⁺ ion.](image)

### Table 1: Kinetic parameters of EstFa_R and its AQGF mutant

|   |   |   |
|---|---|---|
|   | kcat |   | km |
|   | s⁻¹ | mM | s⁻¹ mM⁻¹ |
| EstFa_R |   |   |   |
| pNP acetate | 13 ± 1 | 0.73 ± 0.12 | 17.8 |
| pNP propionate | 28 ± 2 | 0.37 ± 0.07 | 74.4 |
| pNP butyrate | 56 ± 3 | 0.32 ± 0.05 | 173 |
| pNP caproate | 11 ± 1 | 0.13 ± 0.04 | 79.5 |
| AQGF |   |   |   |
| pNP acetate | 31 ± 2 | 1.20 ± 0.17 | 258 |
| pNP propionate | 59 ± 4 | 0.70 ± 0.14 | 84.6 |
| pNP butyrate | 101 ± 7 | 0.48 ± 0.11 | 211 |
| pNP caproate | 159 ± 4 | 0.13 ± 0.02 | 1275 |

### Table 2: Kinetic parameters of SshEstI and its mutants for pNP butyrate hydrolysis

|   |   |   |
|---|---|---|
|   | kcat |   | km |
|   | s⁻¹ | mM | s⁻¹ mM⁻¹ |
| SshEstI |   |   |   |
| NPES | 6 ± 0.2 | 0.05 ± 0.01 | 119 |
| PE | 872 ± 23 | 0.18 ± 0.01 | 4807 |
| NPES | 859 ± 32 | 0.13 ± 0.02 | 6850 |
| PE | 872 ± 23 | 0.18 ± 0.01 | 4807 |

Reactions were conducted at pH 6.0 and 35 °C due to the instability of the wild-type EstFa_R.

Reactions were conducted at pH 7.0 and 40 °C due to the instability of the NPES mutant.
an insert (33 residues in length in EstFa_R and SshEstI) between strands 6 and 7. There were no electron densities of metal ions such as irons observed in either structure.

In contrast to the structural conservation of subdomains, the active sites of EstFa_R and SshEstI showed a significant difference in the atomic level detail. Both structures contained a catalytic triad, a hydrogen bond network consisting of serine, histidine, and aspartic acid residues (i.e. Ser-151, His-274, and Asp-244 in SshEstI and Ser-156, His-281, and Asp-251 in EstFa_R (Fig. 4A)), all of which were located at the canonical positions in the α/β-hydrolase fold. In the SshEstI structure, Asp-244, a member of the catalytic triad, also formed hydrogen bonds with the main-chain carbonyl oxygen of Leu-246 and one water molecule, H2O-1, which in turn created a hydrogen bond with another water molecule, H2O-2. The H2O-2 further formed hydrogen bonds with the main-chain oxygen atoms of Pro-178 and Leu-246 (Fig. 4A and B). The corresponding hydrogen bond network was conserved in all crystal structures of carboxylesterases determined thus far. In striking contrast, however, in EstFa_R, the catalytic triad was uniquely extended from Asp-251 via Asn-248 to Ser-283 (Fig. 4A). The Oκ2 atom of Asp-251 was also hydrogen-bonded to a water molecule (H2O-1; distance, 2.80 Å), which formed another hydrogen bond with the main-chain NH of Asn-248 (2.84 Å) and the Oκ1 atom of Glu-257 (2.71 Å). Glu-257 of EstFa_R was replaced by glycine (Gly-250) in SshEstI, and the side chain of Glu-257 of EstFa_R was located at the position equivalent to the H2O-2 site in the SshEstI structure (Fig. 4A). Because of the hydrophobic environment around Glu-257, the Oκ2 atom of the Glu-257 from EstFa_R should likely be protonated and form hydrogen bonds with main-chain oxygen atoms of Pro-185 (distance, 2.65 Å) and Leu-253 (3.33 Å) while under alkaline pH at which EstFa_R was crystallized (pH 8.0). There were no significant differences between EstFa_R and SshEstI around putative oxyanion-binding sites that are formed by the main-chain NH groups of two contiguous glycine residues (i.e. Gly-80 and Gly-81 in SshEstI and Gly-85 and Gly-86 in EstFa) in the conserved His-Gly-Gly-Gly sequence as well as the main-chain NH group of alanine immediately downstream from the catalytic serine (i.e. Ala-152 in SshEstI and Ala-157 in EstFa).

**Exchangeing Hydrogen Bond Networks Mediated by the Catalytic Triads**—Alignment of the amino acid sequence of EstFa with those of SshEstI and other carboxylesterases (Fig. 4C)
showed that the unique structure near the catalytic triad of EstFa_R arose from the specific occurrences of asparagine, proline, glutamic acid, and serine residues at positions 241, 249, 250, 256, 257, and 283 of EstFa_R. Likewise, amino acid residues of EstFa_R at positions 248, 256, 257, and 283 of EstFa_R arose from the specific occurrences of asparagine, proline, glutamic acid, and serine residues at positions 241, 249, 250, 256, 257, and 283 of EstFa_R. These unique substitutions of conserved residues among carboxylesterases occurring in EstFa suggested that some of the unique properties (e.g., a low catalytic efficiency and a slightly acidophilic property) of EstFa_R might arise from its extended hydrogen bond network near the catalytic triad. To address this issue, the amino acid residues of SshEstI at positions 241, 249, 250, 256, 257, and 283 were replaced by those found in the EstFa_R sequence to obtain the quadruple mutant (termed AQGF) of SshEstI; the half-life at 60 °C was 30 min for the NPES mutant (Fig. 5A), which was in striking contrast to a half-life of greater than 60 min at 90 °C for the wild type (9). This mutant unexpectedly showed activations by heat treatment at either 40 or 50 °C (Fig. 5A). For the pH-activity profile, the NPES and NPS mutants showed pH-activity profiles below pH 5.0 and above pH 8.0 (Fig. 2A). For the pH-activity profile of the NPES mutant of SshEstI (pH 8.0) was higher than that of the wild-type EstFa_R (pH 5.0) and similar to the value of SshEstI.

The NPES mutant of SshEstI showed $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ values for pNP butyrate hydrolysis of $6 \pm 2$ s$^{-1}$ and $119$ s$^{-1}$ mm$^{-1}$, respectively, as determined by initial velocity analysis at pH 7.0 and 40 °C (Table 2). These values were significantly lower than the values of the wild-type SshEstI ($859 \pm 32$ s$^{-1}$ and $6580$ s$^{-1}$ mm$^{-1}$, respectively, at pH 7.0 and 40 °C; Table 2). The NPES mutant was more labile than the wild-type SshEstI; the half-life at 60 °C was 30 min for the NPES mutant (Fig. 5A), which was in striking contrast to a half-life of greater than 60 min at 90 °C for the wild type (9). This mutant unexpectedly showed activations by heat treatment at either 40 or 50 °C (Fig. 5A). For the pH-activity profile, the NPES and NPS mutants showed pH-activity profiles below pH 5.0 and above pH 8.0 (Fig. 2A).

To further identify the important residues that result in the sharp pH-activity profile of the NPES mutant of SshEstI, the triple and double mutants of SshEstI, NES, NPS, PNE, and PE were also prepared and purified, and their pH-activity profiles were examined. None of these mutants displayed a pH-activity profile that was identical to that of NPES (Fig. 5B). However, the PE mutant (Fig. 5B, shown in closed triangles) displayed a pH-activity profile that was more similar to that of NPES, showing larger decreases in activities under acidic and alkaline conditions. The pH-activity profile of the PE mutant (closed circles) was also distinct from that of the NPES mutant and rather similar to the profile of PNE (shown in open squares). The $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ values of the PE mutant were significantly higher than the values of NPES (Table 2). NES and NPS mutants showed pH-activity profiles that were the most similar to that of the wild-type enzyme. Based on these observations, it could be possible that the

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**Table 3** Crystallographic data collection and refinement statistics

| Parameters                  | SshEstI | EstFa_R | The PE mutant of SshEstI |
|-----------------------------|---------|---------|-------------------------|
| Data collection             |         |         |                         |
| Space group                 | $I222$  | $P2_1$  | $I222$                  |
| Unit cell parameters        |         |         |                         |
| $a$ (Å)                     | 58.4    | 62.8    | 59.0                    |
| $b$ (Å)                     | 71.9    | 137.4   | 71.7                    |
| $c$ (Å)                     | 137.3   | 76.9    | 137.9                   |
| $\beta$ (°)                 | 90.0    | 91.5    | 90.0                    |
| Wavelength (Å)              | 0.900   | 1.000   | 1.542                   |
| Resolution (Å)              | 24.8–1.50 (1.58–1.50) | 76.9–1.61 (1.67–1.61) | 50.0–1.80 (1.86–1.80) |
| Measured reflections       | 45,391  | 855,298 | 181,396                 |
| Redundancy                  | 3.7 (3.7) | 5.1 (4.2) | 6.6 (5.3) |
| Completeness (%)            | 14.7 (3.6) | 27.5 (4.8) | 30.3 (7.9) |
| $R_{merge}$ (%)             | 8.8 (29.8) | 9.90 (93.1) | 9.99 (99.9) |
| Refinement                  |         |         |                         |
| Resolution                  | 24.8–1.5 | 46.4–1.61 | 29.5–1.80 |
| Protein atoms               | 2,395   | 9,444   | 2,381                   |
| Octyl glucose               | 20      |         |                         |
| Water molecules             | 273     | 983     | 241                     |
| $R_{merge}/R_{free}$ (%)    | 15.7/18.0 | 18.8/22.5 | 15.2/18.7 |
| Root mean square deviations | 0.012   | 0.029   | 0.018                   |
| Bond angle (°)              | 1.455   | 1.790   |                         |
| Protein Data code           | 3WJ1    | 3WJ2    | 4P0N                    |

* $R_{merge} = 100[I - I_{merge}]/I$ where $I$ is the observed intensity and $I_{merge}$ is the average intensity of multiple observations of symmetry-related reflections.
A241N substitution had the least effect on the pH profile, and the F276S substitution had the largest effect.

Among these mutants of SshEstI, the PE mutant was successfully crystallized, and the structure was determined. Data collection statistics for this mutant structure are summarized in Table 3. The overall structure of the PE mutant was similar to that of wild-type SshEstI, but in the active site of the PE mutant, one of the water molecules observed in the wild-type structure was replaced by Glu-250 (Fig. 4A). The O\(^{\ominus}\) atom of Glu-250 potentially formed hydrogen bonds with main-chain oxygen atoms of Pro-178 (distance, 2.57 Å) and Leu-246 (3.44 Å) (Fig. 4B).

**DISCUSSION**

EstFa was reported previously to be an iron-containing protein (12, 13) that displayed a highly acidophilic enzyme activity with a maximum at pH 2.0–3.5. In the present study, the EstFa gene was heterologously expressed in *E. coli* cells, and the expressed product (EstFa_R) was purified to homogeneity. Unexpectedly, EstFa_R did not bind iron atoms as shown by inductively coupled plasma MS analysis and ITC studies. However, it displayed a considerable level of catalytic activity, although its *k*\(_{cat}\) and *k*\(_{cat}/K_m\) values were significantly lower than the values of SshEstI and other carboxylesterases. Moreover, the activity of EstFa_R was slightly acidophilic with a pH optimum of 5.0, which was still exceptionally low for serine carboxylesterase enzymes. The differences in the metal binding, catalytic efficiency, and pH dependence between EstFa_R and the enzyme purified from *Ferroplasma* (13) might arise from a lack of homogeneity in the native EstFa sample. Because the pH-activity profiles of EstFa_R were different from those of its alkaliphilic counterpart (SshEstI), comparative analyses of structures, catalytic activities, and their pH-dependences using these two enzymes were carried out to pursue mechanistic insights into the catalytic competence and the low pH adaptation of serine carboxylesterase.

Although the overall structures and the lengths of polypeptide chains of EstFa_R and SshEstI were similar to each other, the total accessible surface area calculated for the monomer structure of SshEstI (12,149 Å\(^2\)) was consistently smaller than that of EstFa_R (13,130 Å\(^2\)), indicating that SshEstI was folded more compactly than EstFa_R, which had a
catalytic triad with an extended hydrogen bond network that was not observed in SshEstI and other structurally characterized carboxylesterases. EstFa_R had a B-factor distribution with lower values around the active site compared with SshEstI (Fig. 6A), indicating that the active site of EstFa_R was basically less flexible than that of SshEstI probably because of the presence of the extended hydrogen bond network. It is generally accepted that a high local flexibility around the active site can be responsible for high catalytic activity of enzymes (25). It is noteworthy in this regard that the activity of EstFa_R was enhanced by site-directed mutations mimicking the active site of SshEstI, and conversely, the activity of SshEstI was diminished by those mimicking the active site of EstFa. Specifically, the replacement of the unique residues of EstFa_R in the vicinity of the extended hydrogen bond network (Asn-248, Pro-256, Glu-257, and Ser-283) with the corresponding residues of SshEstI (Ala-241, Gln-249, Gly-250, and Phe-276) (i.e. AQDF substitution) resulted in higher \( k_{cat} \) and \( k_{cat}/K_m \) values (14- and 16-fold, respectively, for pNP caproate hydrolysis) than those of the wild-type EstFa_R (Table 1), although the values were still lower than those of the wild-type SshEstI. Likewise, the \( k_{cat} \) and \( k_{cat}/K_m \) values (for pNP butyrate hydrolysis) of the NPES mutant of SshEstI, which was predicted to have a hydrogen bond network mimicking that of EstFa_R, were much lower than those of the wild-type SshEstI (Table 2).

These observations could be consistently explained in terms of the local flexibility of the active site of the enzymes. The \( k_{cat} \) and \( k_{cat}/K_m \) values of the PE mutant were comparable with those of the wild-type SshEstI (Table 2), and consistently, there was no additional hydrogen bond extended from Asp-244 to the protein structure in the PE mutant (Fig. 4A). In this context, a possible explanation for the heat activations of the NPES mutant of SshEstI might be that the putative extended hydrogen bond network in this mutant was partially disrupted, resulting in higher activities at either 40 or 50°C.

Another important issue of the present study was to clarify the structural consequence of the acidophilic behavior of EstFa_R activity. A comparison of electrostatic surfaces between EstFa_R and other archaeal carboxylesterases (Fig. 6, A and B) showed that a large area of negative charge existed specifically around the active site of EstFa_R. It is possible that the \( pK_a \) values of catalytic residues of EstFa_R were perturbed by electrostatic interactions between ionizable groups around the active site. Moreover, the quadruple (NPES and AQGF) mutations of EstFa_R and SshEstI, respectively, altered the pH dependence of their catalytic activities. The NPES substitution of SshEstI (mimicking the active site of EstFa_R) resulted in a downward shift of the optimum pH of SshEstI from pH 8 to pH 6. The reversal (the AQGF substitution of EstFa_R to mimic the active site of SshEstI) resulted in an upward shift of the opti-
mum pH of EstFa_R from pH 5 to pH 8. These observations strongly suggested that the unique extensions of the hydrogen bond network in EstFa_R at least in part had a role in its unique slightly acidophilic pH dependence. It must also be mentioned that the changes of the pH optima of SshEstI and EstFa_R upon the quadruple substitutions were usually bound to the changes of their catalytic efficiencies (see Tables 1 and 3 and Fig. 2). Because the catalytic efficiencies of these carboxylesterases are partly related to the active site flexibility according to the hydrogen bond network types, it would be tempting to speculate that pH dependence of carboxylesterase catalysis might be in part governed by the pH-dependent changes in local flexibility of the active site, although additional data (e.g. a series of crystal structures in different pH values) would be needed to support this hypothesis.

In summary, crystallographic and mutational studies of two different carboxylesterases resulted in the following conclusions. 1) The catalytic efficiencies of EstFa_R and SshEstI could be altered by modifying the local flexibility of their active site, which was mediated by the formation of different hydrogen bond networks around the catalytic triad. 2) A large area of negative charge uniquely exists around the active site of EstFa_R and may be responsible for the slightly acidophilic activity of the enzyme. Mutational studies also revealed that the extended hydrogen bond network around the active site of EstFa_R was also related to the low pH adaptation of the enzyme. The present work provides novel ideas to elucidate the low pH adaptation of carboxylesterases and help with the conversion of alkaliphilic carboxyesterase into an acidophilic enzyme, which is useful for industrial applications.

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REFERENCES

1. Simon, G. M., and Cravatt, B. F. (2010) Activity-based proteomics of enzyme superfamilies: serine hydrolases as a case study. J. Biol. Chem. 285, 11051–11055
2. Arpigny, J. L., and Jaeger, K. E. (1999) Bacterial lipolytic enzymes: classification and properties. Biochim. J. 343, 177–183
3. Jaeger, K. E., and Reetz, M. T. (1998) Microbial lipases form versatile tools for biotechnology. Trends Biotechnol. 16, 396–403
4. Jaeger, K. E., Dijkstra, B. W., and Reetz, M. T. (1999) New families of carboxyl peptidases: serine-carboxyl peptidase with collagenase activity. J. Biol. Chem. 274, 15100–15103
5. Liu, P., Wang, Y. F., Ewis, H. E., Abdelal, A. T., Lu, C. D., Harrison, R. W., Oyama, H., Oda, K., Nishino, T., and Nakayama, T. (2004) Crystal structure of brefeldin A esterase, a bacterial homolog of the mammalian hormone-sensitive lipase. Nat. Struct. Biol. 6, 340–345
6. De Simone, G., Menchise, V., Manco, G., Mandrich, L., Sorrentino, N., Lang, D., Rossi, M., and Pedone, C. (2001) The crystal structure of a hyperthermophilic carboxylesterase from the archaeon Archaeoglobus fulgidus. J. Mol. Biol. 314, 507–518
7. Liu, P., Wang, Y. F., Ewis, H. E., Abdelal, A. T., Lu, C. D., Harrison, R. W., and Weber, I. T. (2004) Covalent reaction intermediate revealed in crystal structure of the Geobacillus stearothermophilus carboxylesterase Est30. J. Mol. Biol. 342, 551–561
8. Mandrich, L., Menchise, V., Alterio, V., De Simone, G., Pedone, C., Rossi, M., and Manco, G. (2008) Functional and structural features of the oxygen hole in a thermophilic esterase from Alicyclobacillus acidocaldarius. Proteins 71, 1721–1731
9. Zhong, S., Haghjoo, K., Kettner, C., and Jordan, F. (1995) Proton magnetic resonance studies of the active center histidine of chymotrypsin complexed to peptidboronic acids: solvent accessibility to the Nα and Nε sites can differentiate slow-binding and rapidly reversible inhibitors. J. Am. Chem. Soc. 117, 7048–7055
10. Oda, K. (2012) New families of carboxyl peptidases: serine-carboxylic acid peptidases and glutamic peptidases. J. Biochem. 151, 13–25
11. Whodawer, A., Li, M., Gustchina, A., Tsuruoka, N., Ashida, M., Minakata, H., Oyama, H., Oda, K., Nishino, T., and Nakayama, T. (2004) Crystallographic and biochemical investigations of kumamolisin-As, a serine-carboxyl peptidase with collagenase activity. J. Biol. Chem. 279, 21500–21510
12. Golyshina, O. V., Golyshin, P. N., Timmis, K. N., and Ferrer, M. (2006) The ‘pH optimum anomaly’ of intracellular enzymes of Ferroplasma acidiphilum. Environ. Microbiol. 8, 416–425
13. Ferrer, M., Golyshina, O. V., Beloukhi, A., Golyshin, P. N., and Timmis, K. N. (2007) The cellular machinery of Ferroplasma acidiphilum is iron-protein-dominated. Nature 445, 91–94
14. Ejima, K., Liu, J., Oshima, Y., Hirooka, K., Shimano, S., Yokota, Y., Hemmi, H., Nakayama, T., and Nishino, T. (2004) Molecular cloning and characterization of a thermostable carboxylesterase from an archaeon, Sulfolobus shibatae DSM5389: non-linear kinetic behavior of a hormone-sensitive lipase family enzyme. J. Biosci. Bioeng. 98, 445–451
15. Bond, P. L., Sriga, S. P., and Banfield, J. F. (2000) Phylogeny of microorganisms populating a thick, subaerial, predominantly lithotrophic biofilm at an extreme acid mine drainage site. Appl. Environ. Microbiol. 66, 3842–3849
16. Leatherbarrow, R. J. (1990) Using linear and non-linear regression to fit biochemical data. Trends Biochem. Sci. 15, 455–458
17. Otwinowski, Z., and Minor, W. (1997) Processing of x-ray diffraction data collected in oscillation mode. Methods Enzymol. 276, 307–326
18. Vagin, A., and Teplyakov, A. (2010) Molecular replacement with MOLREP. Acta Crystallogr. D Biol. Crystallogr. 66, 22–25
19. Emsley, P., and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132
20. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Refinement of macromolecular structures by the maximum-likelihood method. Acta Crystallogr. D Biol. Crystallogr. 53, 240–255
21. Delano, W. L. (2002) The PyMOL Molecular Graphics Systems, Schrödinger, LLC, New York
22. Holm, L., and Park, J. (2000) Dalilite workbench for protein structure comparison. Bioinformatics 16, 566–567
23. Baker, N. A., Sept, D., Joseph, S., Holst, M. J., and McCammon, J. A. (2001) Electrostatics of nanosystems: application to microtubules and the ribosome. Proc. Natl. Acad. Sci. U.S.A. 98, 10037–10041
24. Ferrer, M., Golyshina, O. V., Beloukhi, A., Böttger, L. H., Andreu, J. M., Polaina, J., De Lacey, A. L., Trautwein, A. X., Timmis, K. N., and Golyshin, P. N. (2008) A purple acidophilic di-ferric DNA ligase from Ferroplasma. Proc. Natl. Acad. Sci. U.S.A. 105, 8878–8883
25. Siddiqui, K. S., and Cavicchioli, R. (2006) Cold-adapted enzymes. Annu. Rev. Biochem. 75, 403–433