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Evolvability of Thermophilic Proteins from Archaea and Bacteria

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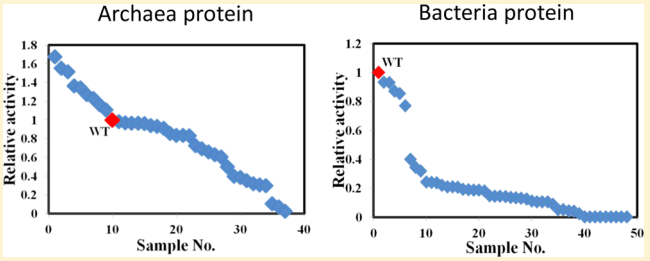
Supporting Information

ABSTRACT: Proteins from thermophiles possess high thermostability. The stabilization mechanisms differ between archaeal and bacterial proteins, whereby archaeal proteins are mainly stabilized via hydrophobic interactions and bacterial proteins by ion pairs. High stability is an important factor in promoting protein evolution, but the precise means by which different stabilization mechanisms affect the evolution process remain unclear. In this study, we investigated a random mutational drift of esterases from thermophilic archaea and bacteria at high temperatures. Our results indicate that mutations in archaeal proteins lead to improved function with no loss of stability, while mutant bacterial proteins are largely destabilized with decreased activity at high temperatures. On the basis of these findings, we suggest that archaeal proteins possess higher "evolvability" than bacterial proteins under temperature selection and are additionally able to evolve into eukaryotic proteins.



Proteins from thermophiles and hyperthermophiles generally exhibit higher stability than their mesostable counterparts.[1−4] Thermophiles and hyperthermophiles are found in both archaea and bacteria that have divided at an early stage of evolution.[5] In archaea, thermophiles and hyperthermophiles originated in a hot environment, while those in bacteria recolonized at a later stage under extreme conditions.[6−9] Berezovsky and Shakhnovich[10] showed that proteins from archaea are more compact and hydrophobic than their mesophilic homologues. In contrast, proteins from some bacteria are stabilized by specific interactions, such as ion pairs. Mizuguchi et al.[11] further reported differences in the amino acid compositions of archaeal and bacterial proteins. The group concluded that thermal adaptation is achieved in distinct ways in the archaeal and bacterial kingdoms. Furthermore, comparison of ribonucleases H (RNases H) from hyperthermophilic archaea and bacteria revealed different unfolding rates. Specifically, RNase H2 from the hyperthermophilic archaeon *Thermococcus kodakarensis* and RNase H1 from the hyperthermophilic archaeon *Sulfolobus tokodaii* unfold more slowly than RNases H2 from the hyperthermophilic bacteria *Thermotoga maritima* and *Aquifex aeolicus*.[12] The unfolding characteristics of hyperstable proteins are ascribed to differences in the evolutionary history of thermophilic archaea and bacteria.

In protein evolution, stability is a major constraint, whereby generation of a protein with improved activity or novel function is often accompanied by a loss of stability.[13−15] This may be attributed to a protein stability−function trade-off.[16,17] Thus, extra stability provides evolvability of proteins, signifying that mutants of thermophilic proteins are more likely to exhibit new or improved functions.[17,18] Here, we hypothesize that different stabilization mechanisms of thermophilic proteins between archaea and bacteria affect their evolution.

The technique of directed evolution mimics natural selection, allowing the evolution and adaptation of proteins under controlled conditions.[19−21] Directed evolution via random mutagenesis using error-prone polymerase chain reaction (PCR) has been shown to be effective in modifying and improving protein functions.[22,23] An experimental system for estimating protein evolvability using the directed evolution technique has been reported.[24] Randomly mutated target genes are transformed into *Escherichia coli* for overexpression, and colonies of variants are grown, followed by lysis of bacteria and assay of activity. On the basis of the levels of activity of the mutant library, the capacity to evolve for each protein is evaluated.

In this study, we investigated a random mutational drift of esterases from thermophilic archaea and bacteria at high temperatures. Esterases from *S. tokodaii* (Sto-Est) and *T. kodakarensis* (Tk-Est) were examined as the archaeal proteins.[25] For bacterial proteins, we used esterases from *Alicyclobacillus acidocaldarius* (Aac-Est) and *Th. maritima* (Tm-Est).[26−28] The known characteristics of hosts and esterases are summarized in Table 1. The crystal structures of Sto-Est and Aac-Est have been determined, as presented in Figure 1. On the basis of the known structures, we found that both archaeal and bacterial esterases display a typical stabilization feature. Directed evolution experiments for these esterases disclosed distinct characteristics of archaeal and bacterial proteins. Our results

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Table 1. Characteristics of Hosts and the Respective Esterases

host			esterase	
		optimal growth temperature (°C)	optimal temperature (°C)	T_m (°C)
S. tokodaii	archaea	80[a]	75[b]	81[b]
T. kodakarensis	archaea	85[c]	–	–
A. acidocaldarius	bacteria	60[d]	70[e]	91[f]
Th. maritima	bacteria	80[g]	95[h]	

[a]From ref 41. [b]From ref 25. [c]From ref 42. [d]From ref 43. [e]From ref 26. [f]From ref 27. [g]From ref 44. [h]From ref 28.

collectively support a crucial link between protein evolution and stabilization mechanisms based on the evolutionary history of organisms.

METHODS

Cells, Genome, and Plasmids. *A. acidocaldarius* subsp. *acidocaldarius* ATCC 27009 was from ATCC. Genomic DNA of *A. acidocaldarius* was prepared from a Sarkosyl lysate. *E. coli* BL21(DE3) codon plus was from Stratagene. Genomic DNA of *Th. maritima* JCM 10099T was obtained from RIKEN BioResource Center. The pET28a plasmid was from Novagen. *E. coli* transformants were grown in Luria-Bertani medium containing 50 mg L^{-1} kanamycin and 30 mg L^{-1} chloramphenicol.

Construction of Plasmids. A plasmid for Sto-Est overexpression, designated pET28a/Sto-Est, was constructed, as described previously.[25] Plasmids for overexpression of Tk-Est, Aac-Est, and Tm-Est (pET28a/Tk-Est, pET28a/Aac-Est, and pET28a/Tm-Est, respectively) were constructed by ligating part of each esterase gene amplified using PCR into the pET28a vector. Genomic DNA samples of *T. kodakarensis*, *A. acidocaldarius*, and *Th. maritima* were used as templates. Nucleotide sequences were confirmed with an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer). All DNA oligomers for PCR were synthesized by Hokkaido System Science.

Random Mutagenesis. The pET28a/Sto-Est, pET28a/Tk-Est, pET28a/Aac-Est, and pET28a/Tm-Est plasmids were used as templates at the first generation. Following the second generation, the gene encoding the protein with the highest activity was employed. Random mutations were introduced via error-prone PCR using rTaq polymerase (Toyobo). The reaction mixture contained 0.12 mM (Tk) or 0.15 mM (Sto, Aac, and Tm) $MnCl_2$, optimized to generate an error frequency of one to three substitutions per gene per generation. Mutated genes were ligated into the plasmid and transformed into *E. coli* BL21(DE3) codon plus. Transformants were selected using plasmid antibiotic resistance markers.

Overproduction and Partial Purification. Sto-Est, Tk-Est, Aac-Est, Tm-Est, and their derivatives were overproduced in *E. coli*. When the absorbance of the culture at 600 nm reached 0.5, 0.25 mM (Sto, Tk, and Aac) or 0.50 mM (Tm) isopropyl β-D-thiogalactopyranoside (IPTG) was added to the medium and cultivation continued for an additional 4 h (Tm) or overnight (Sto, Tk, and Aac) at 15 °C (Sto and Aac), 25 °C (Tk), or 37 °C (Tm). *E. coli* cells were collected by centrifugation and resuspended in 50 mM Tris-HCl (pH 8.0) containing 10 mM EDTA and 150 mM NaCl. Cells were lysed with 1 mg mL^{-1} lysozyme for 30 min on ice and 0.1% Triton X-100 for 1 h on ice. In the case of Tk-Est, the supernatant was heated at 80 °C for 30 min and collected after centrifugation. The expression level of each protein was analyzed using 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with CS Analyzer 3 (ATTO) standardized with several concentrations of BSA (Figure S1 of the Supporting Information).

Activity Assay. The esterase activity of each wild-type protein was examined under optimal conditions[25,26,28] using 0.5 mM *p*-nitrophenyl butyrate (Sto), 0.5 mM *p*-nitrophenyl caproate (Tk), 0.2 mM *p*-nitrophenyl caproate (Aac), and 0.5 mM *p*-nitrophenyl caprylate (Tm) as the substrate in a reaction

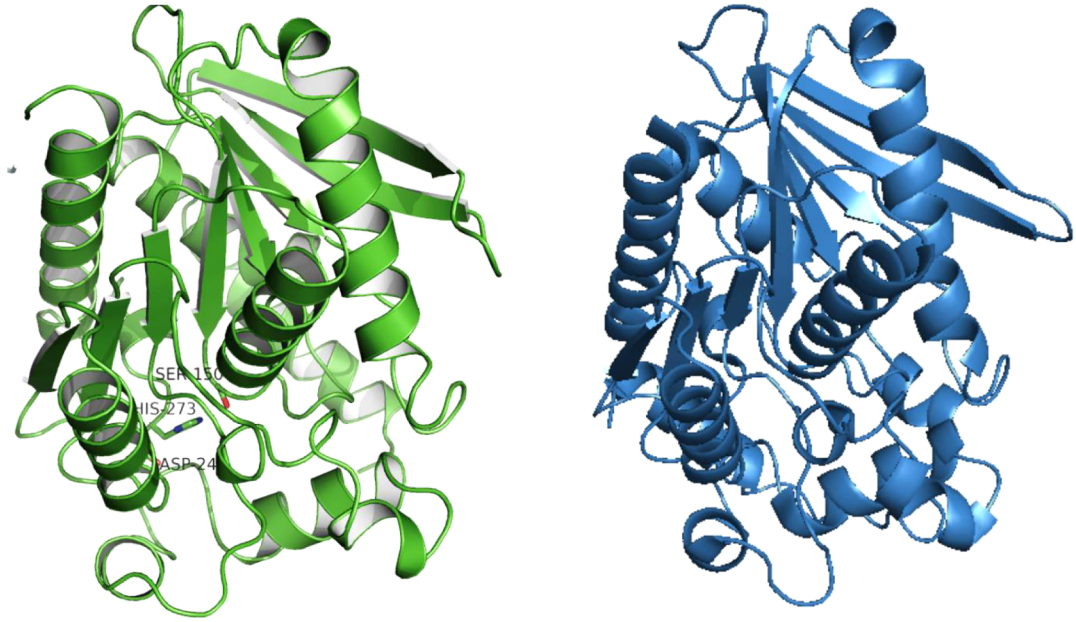

Figure 1. Crystal structures of Sto-Est (left, PDB entry 3aik) and Aac-Est (right, PDB entry 1evq). Side chains of active site residues in Sto-Est, Ser150, Asp243, and His273, are shown. This figure was created with PyMOL.[29]

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mixture containing 10 mM phosphate buffer (pH 6.0) and 10% acetonitrile (Sto), 50 mM Tris-HCl (pH 8.0) and 1% (v/v) 2-propanol (Tk and Tm), and 40 mM phosphate buffer (pH 7.1), 0.09% (v/v) gum arabic, 0.36% (v/v) Triton X-100, and 2% (v/v) 2-propanol (Aac) at 75 °C (Sto), 70 °C (Tk), 65 and 40 °C (Aac), and 85 °C (Tm). We used ∼10 ng of protein, as estimated from SDS−PAGE data. The reaction was terminated by the addition of 1% SDS. The amount of product was measured using a spectrophotometer at 405 nm.

Thermal Stability. The residual activity of each wild-type protein was measured under the conditions described above after incubation at 80 °C at appropriate intervals.

To analyze the stability of Sto-Est and Aac-Est variants against heat treatment, we measured the residual activity at 75 °C (Sto) and 40 °C (Aac) after incubation at 90 °C (Sto) and 80 °C (Aac) for 10 min.

Structural Characteristics. The ASA and number of ion pairs were calculated with PyMOL.[29] The amino acid distribution on the surface and burial within the structure were assessed using ASA-View.[30] Residues with relative solvent accessibility of >25% were regarded as being exposed to solvent. The total molecular surface area was calculated using the ASA of the unfolded state.[31,32] Hydrogen bonds were estimated with HBOND.[33]

RESULTS

Structural Comparison between Sto-Est and Aac-Est. The amino acids of Sto-Est are 44% identical with those of Aac-Est. When the structures of Sto-Est and Aac-Est are superimposed, they closely resemble each other with a 1.6 Å root-mean-square deviation of Cα atoms. The structural features of Sto-Est and Aac-Est are summarized in Table 2.

Table 2. Structural Characteristics of Sto-Est and Aac-Est

	Sto-Est	Aac-Est
amino acid distribution		
total (no./%)	283/100	304/100
surface (no./%)	95/34	133/44
buried (no./%)	188/66	171/56
molecular surface area		
total (Å^2)	55938	53366
accessible (exposed/ASA) (Å^2)	11905	13558
buried (Å^2)	44033	39808
buried/total	0.79	0.75
ASA		
total (Å^2/%)	11905/100	13558/100
charge (Å^2/%)	4699/39	6170/46
polar (Å^2/%)	3214/27	2375/18
hydrophobic (Å^2/%)	2870/24	2132/16
other (Å^2/%)	1121/10	2881/20
hydrogen bonds (no.)	199	209
ion pairs (no.)	15	24

In terms of amino acid distribution, Sto-Est abounds in buried residues more than Aac-Est. Analysis of the molecular surface area additionally revealed a larger buried area of Sto-Est, compared to that of Aac-Est. Furthermore, Sto-Est has a larger total surface area but a smaller accessible surface area (ASA), suggesting a more compact structure. It has been reported that a hydrophobic environment is a key factor in the stability of thermophilic proteins.[34] In contrast, more charged ASA and ion pairs have been identified in Aac-Est, indicative of ubiquitous ion pairs on the surface. The number of hydrogen bonds also differs between Sto-Est and Aac-Est. These structural features indicate that Sto-Est is stabilized by hydrophobic interactions and compactness, whereas specific interactions, such as ion pairs and hydrogen bonds, maintain the conformation of Aac-Est.[35] Packing of external residues may also contribute to the stability of Aac-Est.[36] The respective stabilization mechanisms are typical for archaeal and bacterial proteins,[10] and therefore, both proteins were considered suitable for our study.

Stability of Wild-Type Esterases. To compare protein stabilities, wild-type esterases were subjected to heat treatment at 80 °C. At the appropriate intervals, an aliquot of solution was withdrawn and analyzed for residual activity at 75 °C (Sto), 70 °C (Tk), 65 °C (Aac), and 85 °C (Tm), representing the optimal temperatures for each protein. As shown in Figure 2,

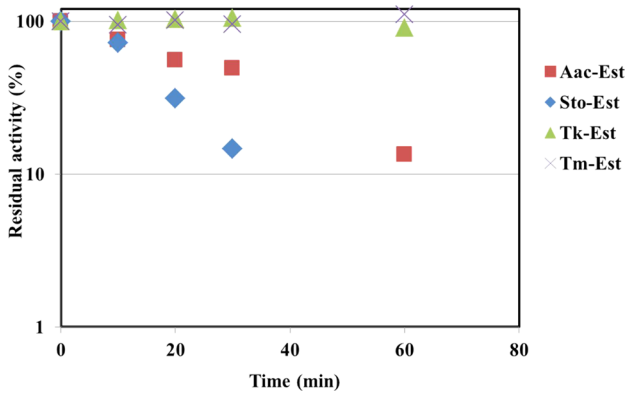

Figure 2. Residual activities of wild-type Sto-Est, Aac-Est, Tk-Est, and Tm-Est. Residual activities of Sto-Est (diamonds), Aac-Est (squares), Tk-Est (triangles), and Tm-Est (crosses) were measured at 75, 65, 70, and 85 °C, respectively, after incubation at 80 °C for 10 min.

Tm-Est did not lose activity, because the heat treatment temperature (80 °C) was lower than its optimal temperature (85 °C). Additionally, Tk-Est was stable at 80 °C for at least 2 h. In contrast, Sto-Est and Aac-Est lost activity, with Sto-Est displaying a lower stability than Aac-Est.

Directed Evolution of Sto-Est. In the first round, more than 90% of randomly selected Sto-Est variants showed activity that was >20% of the wild-type activity at 75 °C, as shown in Figure 3a. However, only a quarter of the variants displayed activity higher than that of the wild type. Similar results were obtained with another set of variants from a different directed evolution experiment (data not shown). The highest activity recorded among variants was 1.7-fold that of the wild type. Using this variant as a template, a second round of experiments was performed (Figure 3b). In the second generation, the percentage of variants with activity higher than that of the template (13%) decreased. The highest activity was 1.7 times greater than that of the template and 2.2 times greater than that of the wild type. In the third round, variants with increasing activity still appeared, but the proportion was significantly smaller (6%) (Figure 3c). However, interestingly, a small proportion of the variants (12%) displayed improved function in the fourth generation (Figure 3d). Thus, the four rounds of direct evolution experiments with Sto-Est produced variants with activity up to 2.7-fold higher than that of the wild type. Our results indicate that random mutations promote increased enzymatic activity of Sto-Est at high temperatures.

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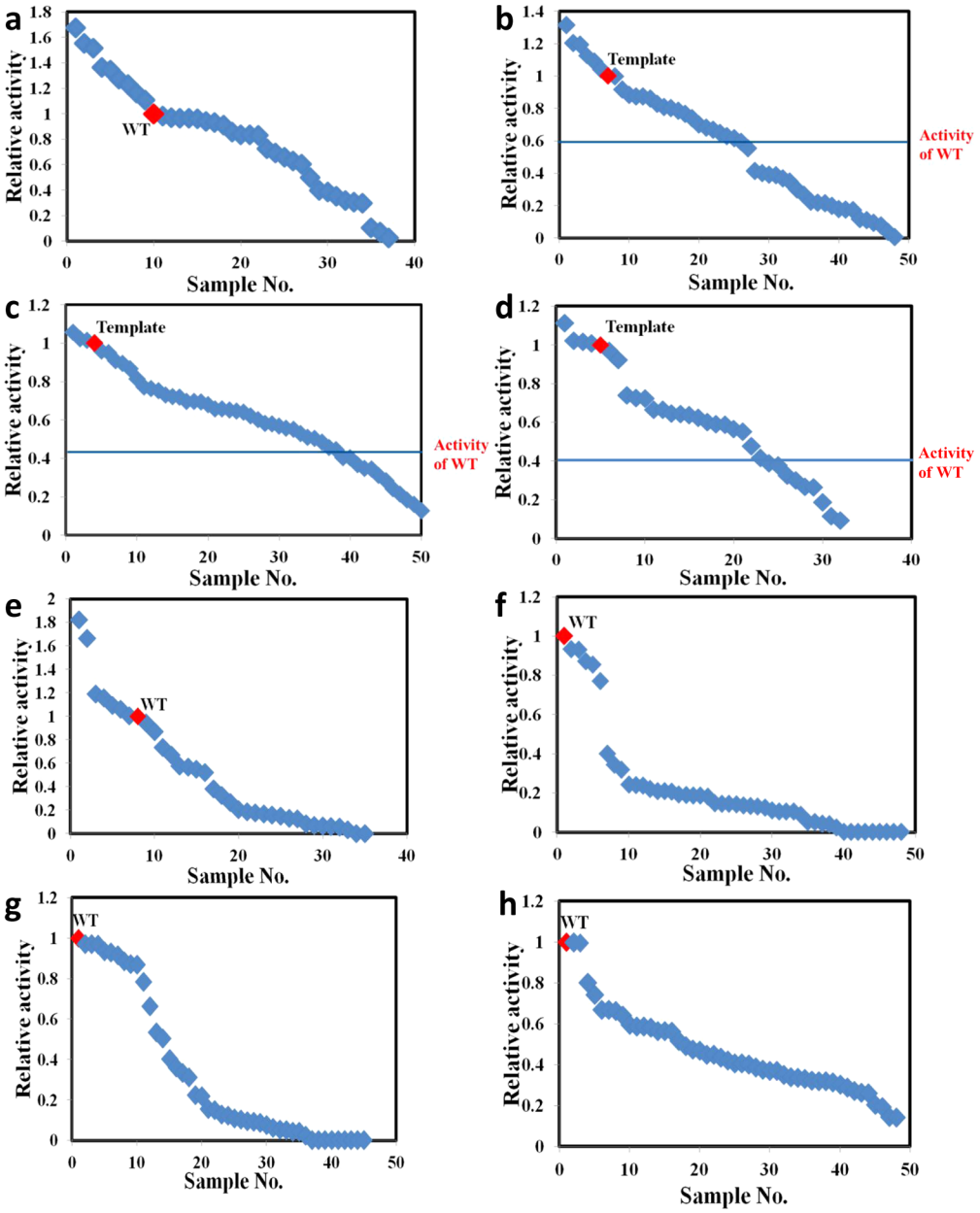

Figure 3. Relative activities of variants from directed evolution by random mutation experiments: (a) Sto-Est at the first generation, (b) Sto-Est at the second generation, (c) Sto-Est at the third generation, (d) Sto-Est at the fourth generation, (e) Tk-Est at the first generation, (f) Aac-Est at the first generation, (g) Aac-Est at the first generation from a different experiment, and (h) Tm-Est at the first generation.

Directed Evolution of Tk-Est. Similar to Sto-Est, 20% of Tk-Est variants displayed enhanced activity at 70 °C, as shown in Figure 3e. The experiment yielded variants with an 1.8-fold improvement in activity relative to that of the wild type. Our data suggest the theory that Tk-Est activity continues to increase with further rounds of directed evolution experiments.

Directed Evolution of Aac-Est. As expected, all variant proteins exhibited decreased activity at 65 °C (Figure 3f). Similar results were obtained with another set of variants from a different directed evolution experiment (Figure 3g), supporting the reproducibility of directed evolution of Aac-Est. Furthermore, the activities of more than 50% of variants were less than 20% of the wild-type activity in both experiments. Aac-Est readily lost activity at 65 °C following mutation, indicating that the protein lacks the capacity to improve function at its optimal temperature.

Directed Evolution of Tm-Est. The majority of variants had activity that was >20% of the wild-type activity at 85 °C (Figure 3h). However, we detected no variants with activity higher than that of the wild type, as well as Aac-Est. Tm-Est and Aac-Est are bacterial proteins, which may have limitations in terms of activity, stability, and conformation at high temperatures upon random mutation.

Amino Acid Substitutions in Directed Evolution. Amino acid substitutions of some Sto-Est and Aac-Est variants were further identified (summarized in Table 3). Sto-Est variants with higher activity contained substitutions near the active site. For example, the highest-activity variant at the first generation possesses two mutations, P234S and I272V. Here, Ile272 is the neighbor residue of the active site residue, His273 (Figure S2 of the Supporting Information). Furthermore, in the highest-activity variant at the second generation, the I282K

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Table 3. Amino Acid Substitutions of Variants

variant	activity relative to that of the wild type	substitution	location
Sto-Est			
highest activity at the first generation	1.7	P234S	far from the active site
		I272V	near the active site
highest activity at the second generation	2.2	P234S	far from the active site
		I272V	near the active site
		I282K	near the active site
highest activity at the first generation[a]	1.6	I14T	far from the active site
		N159K	near the active site
lowest activity at the first generation	0.02	P224A	far from the active site
		L323S	far from the active site
		R246G	near the active site
Aac-Est			
lower activity at the first generation	0.24	F30S	far from the active site
		Y218H	far from the active site
		L260P	far from the active site
lower activity at the first generation	0.21	V272D	far from the active site
lower activity at the first generation	0.18	I182N	near the active site

[a]From a different experiment.

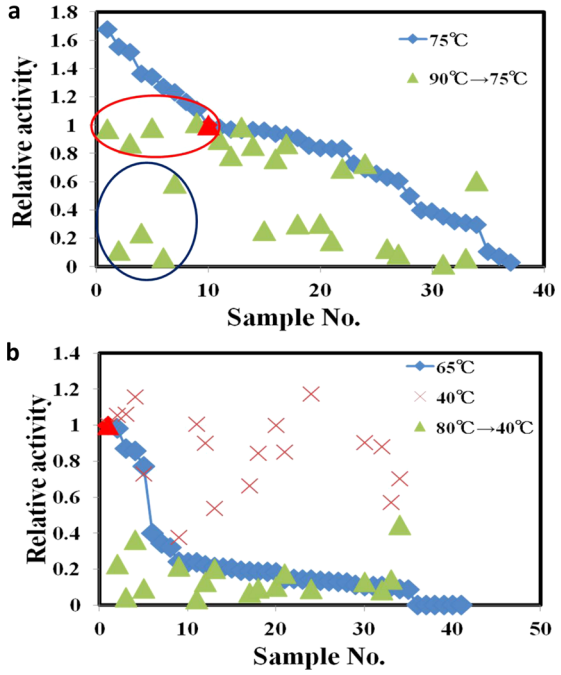

Figure 4. Relative residual activities of variants from random mutation experiments. (a) Sto-Est at the first generation. The residual activity of Sto-Est variants (triangles) was measured at 75 °C after incubation for 10 min at 90 °C. Relative activities at 75 °C (diamonds) are additionally presented. Wild-type data are colored red. Red and blue ovals indicate variants without and with stability loss, respectively (see the text). (b) Aac-Est at the first generation. The residual activity of Aac-Est variants (triangles) was measured at 40 °C after incubation for 10 min at 80 °C. Relative activities at 40 and 75 °C (crosses and diamonds, respectively) are additionally presented. Wild-type data are colored red.

substitution was added. Ile282 is located near the active site (Figure S2 of the Supporting Information). This finding suggests that the mutations directly alter the active site conformation, resulting in the improvement of activity. However, the variant displaying the lowest activity also possessed substitutions near the active site. Thus, it appears that modifications around the active site induce both increases and decreases in activity.

In contrast, randomly selected variants of Aac-Est displaying ~20% of the wild-type activity were not always mutated near the active site, suggesting that long-range interactions affecting enzymatic activity or inducing loss of activity include other effects due to mutation, such as protein denaturation at high temperatures. For example, Ile182 and Tyr218 are evolutionarily conserved residues, so that the substitutions of these residues may cause destruction of the esterase structure. For Val272, the site prefers hydrophobic amino acid residues among esterase homologues. The substitution with Asp probably destabilizes the protein.

Stability of Variant Esterases. To analyze the stability of variants, residual activity was measured after heat treatment. Sto-Est proteins were incubated at 90 °C for 10 min, and residual activity was assayed at 75 °C (Figure 4a). Half of the variants displayed decreased residual activity, resulting from destabilization because of the mutation. For variants with higher activity, some variants had little residual activity (blue circle), possibly as a result of protein a stability−function trade-off.[37,38] On the other hand, some variants maintained residual activity comparable to that of the wild type (red circle). Mutation of Sto-Est improved activity with no stability loss.

For Aac-Est, all variants exhibited decreased activity at 65 °C, as described above (Figure 3f,g). Surprisingly, at 40 °C, activity was recovered in many variants (Figure 4b). Moreover, the activities of some variants were higher than that of the wild type. The residual activity relative to that of the wild type after heat treatment at 80 °C for 10 min was significantly lower. These results indicate that Aac-Est activity is reduced at 65 °C upon mutation, accompanied by inadequate stability.

DISCUSSION

Data from this study indicate that the activities of thermophilic archaeal esterases, Sto-Est and Tk-Est, are improved at high temperatures by random mutations, whereas the bacterial enzymes, Aac-Est and Tm-Est, fail to adapt. The reasons underlying these differences in adaptability remain to be established. One general possibility is the intrinsic stability of parent (wild-type) proteins, because protein evolvability depends on stability.[39] However, among the esterases examined, Tm-Est (bacteria) and Tk-Est (archaea) are more stable than Aac-Est (bacteria) and Sto-Est (archaea), as shown in Figure 2, indicating that stability of the wild-type protein is not the underlying reason. Our results suggest differences in the sensitivity of stability to mutations, originating from differences in the stabilization mechanisms between archaea and bacteria.

Random mutations in Aac-Est produced several variants with activity at 40 °C higher than that of the wild type (Figure 4b),

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similar to that of Sto-Est at 75 °C. However, these "improved" variants were not selected at 65 °C, because they could not adapt to high temperatures. This finding signifies that mutations seriously affect the stability of Aac-Est (stability−sensitivity mutants). On the other hand, Sto-Est variants displayed altered activity to various extents at high temperatures, but some maintained stability (stability−insensitivity mutants). The structure of Sto-Est may thus be sufficiently flexible to buffer mutation-induced damage.

We unintentionally sampled 20 amino acid substitutions from Sto-Est and Aac-Est variants by random mutation experiments. Half of the selected Sto-Est substitutions were from variants with residual activity that was >70% of that of the wild type (with no stability loss), while others, including Aac-Est substitutions, were from those with <70% of wild-type activity (with stability loss). Substitutions were classified into two groups, specifically, located in the interior or on the surface of molecules (summarized in Table 4). Sto-Est variants that maintained stability had substitutions both in the interior and on the surface, whereas in destabilized variants, buried residues were mainly replaced. Thus, surface substitutions do not appear to affect stability. This can probably be ascribed to the characteristic of thermophilic archaeal proteins that are chiefly stabilized by the buried residues, not by the surface residues.[10] In contrast to Sto-Est, both substitutions in the interior and on the surface destabilized Aac-Est. Although changes in stability due to mutation at buried sites are generally larger than that at exposed sites,[40] it is possible that substitutions at the surface of thermophilic bacterial proteins lead to major destabilization, because residues are stabilized by surface charges.[10]

Table 4. Locations of Substituted Residues

	no. of substitutions	
	interior	surface
Sto-Est		
variants with stability loss	9	1
variants without stability loss	3	7
Aac-Est		
variants with stability loss	9	11

In summary, disparate stabilization mechanisms between archaeal and bacterial thermostable proteins originating from the evolutionary history of organisms induce differential effects on stability due to mutations, leading to alterations in evolvability at high temperatures. This is an important finding in explaining the evolution of organisms and may be of significant use in biotechnology. According to the three-domain system of Woese et al.,[5] the tree of life consists of three domains: archaea, bacteria, and eukarya. A phylogenetic tree of life shows that eukarya are derived from archaea. This may be related to the evolvability of archaeal proteins. Interestingly, archaeal, but not bacterial, proteins are able to evolve into eukaryal proteins. Directed evolution is an effective protein engineering method used to generate proteins with desirable properties. Here, parent proteins from archaea or bacteria have been used for various purposes in biotechnology.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

SDS−PAGE analysis of crude lysates for wild-type esterases (Figure S1) and structure around the active site residues (S150, D243, and H273) of Sto-Est (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

Aac-Est, esterase from *A. acidocaldarius*; ASA, accessible surface area; PCR, polymerase chain reaction; PDB, Protein Data Bank; RNase H, ribonuclease H; Sto-Est, esterase from *S. tokodaii*; Tk-Est, esterase from *T. kodakaraensis*; T_m, melting temperature; Tm-Est, esterase from *Th. maritima*.

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