J. Microbiol. Biotechnol. (2009), **19**(12), 1536–1541
doi: 10.4014/jmb.0903.03017
First published online 22 August 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Thermostability of Chimeric Cytidine Deaminase Variants Produced by DNA Shuffling

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Received: March 20, 2009 / Revised: May 22, 2009 / Accepted: June 23, 2009

The DNA shuffling technique has been used to generate libraries of evolved enzymes in thermostability. We have shuffled two thermostable cytidine deaminases (CDAs) from *Bacillus caldolyticus* DSM405 (T53) and *B. stearothermophilus* IFO12550 (T101). The shuffled CDA library (SH1067 and SH1077 from the first round and SH2426 and SH2429 from the second round) showed various patterns in thermostability. The CDAs of SH1067 and SH1077 were more thermostable than that of T53. SH2426 showed 150% increased half-time than that of T53 at 70°C. The CDA of SH2429 showed about 200% decreased thermostability than that of T53 at 70°C. A single amino acid residue replacement that presented between SH1077 and SH2429 contributed to dramatic changes in specific activity and thermostability. On SDS–PAGE, the purified CDA of SH1077 tetramerized, whereas that of SH2429 denatured and became almost monomeric at 80°C. A simulated three-dimensional structure for the mutant CDA was used to interpret the mutational effect.

Keywords: Cytidine deaminase, directed evolution, DNA shuffling, thermostability

Cytidine deaminase (CDA, cytidine/2'-deoxycytidine aminohydrolase; E.C. 3.5.4.5) encoded by the *cdd* gene is a salvage pathway enzyme that catalyzes the hydrolytic deamination of cytidine and deoxycytidine to the corresponding uracil nucleosides. CDAs from various microorganisms such as *Escherichia coli*. *Bacillus* spp., and *Salmonella typhimurium* have been characterized and studied for nearly 4 decades [1, 2, 17, 19]. Secondary and tertiary structures of homotetrameric CDA from *Bacillus* species, namely, psychrophile *B. psychrophilus* and thermophile *B. caldolyticus*, were investigated by using CD spectrum analysis [2]. There was no strict corroboration for the stability of CDA from *B. caldolyticus* since the secondary structures of CDA from *B. psychrophilus* were very similar to those of the CDA of the former.

The CDA has been used for the production of lamivudine [(2'*R-cis*)-2'-deoxy-3'-thiacytidine; 3TC], an analog of cytidine, in the pharmaceutical industry. Lamivudine is the most well-known potent anti-human immunodeficiency virus (HIV) agent and antihepatitis (HBV) drug [10, 15]. The CDA deaminates 2'-deoxy-3'-thiacytidine enantioselectively to leave the 3TC with essential optical purity [12, 22]. Furthermore, utilization of thermostable CDA could curtail the expenses in manufacturing lamivudine [22]. In order to produce the highly qualified lamivudine at a low price, improvements in the thermostability and specific activity of CDA play a pivotal role in the enzymatic process.

DNA shuffling has been used in many aspects of biological systems, such as enhancement of thermostability [23], vaccine development [16], and biochemical production [13]. Our previous study on the first-round DNA shuffling of CDAs from *B. caldolyticus* DSM405 and *B. stearothermophilus* IFO12550 resulted in several chimeric CDAs. Two of these genes, SH1067 and SH1077, were chosen for the second-round shuffling of CDAs to change the specific activity and thermostability. In this study, the evolution of a second-generation pool of enzymes comprising CDA variants with changed thermostability was employed. In addition, the correlation between the positions of amino acid replacements and thermostability of chimeric CDAs based on their predicted 3D structures was analyzed and discussed.

Materials and Methods

Bacterial Strains, Plasmids, and Medium

Escherichia coli JF611 (*cdd1, pyrE60, thi1, argE3, his3, proA2, thr1, leu6, mdl1, xyl5, ana14, galK2, lacY, str31, λ-supE44*) was used for the expression and screening of chimeric CDA library. *E. coli* Rosetta(DE3)pLysS was used as the host strain for overexpression and purification of recombinant proteins [4]. The plasmids T-easy vector (Promega, Madison, U.S.A.) and pET-14b (Novagen, Madison,

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U.S.A.) were used as cloning vector and overexpression vector, respectively. The plasmids pSH1067 and pSH1077 harboring the products of the first-round shuffled *cdd* from *B. caldolyticus* DSM405 and *B. stearothermophilus* IFO2550 were provided by Hong [3, 8, 21].

The recombinants of *E. coli* JF611 were selected using AB medium supplemented with ampicillin (AP) and cytidine [8]. The recombinants of *E. coli* Rosetta(DE3)pLysS were cultivated in Luria–Bertani (LB) medium supplemented with AP (100 μg/ml) and chloramphenicol (CH, 25 μg/ml) [1].

Library Construction Using DNA Shuffling

Chimeric *cdd* genes from plasmids pSH1067 and pSH1077 were amplified by PCR using primers M13-F and M13-R (Table 1). The PCR amplification was performed for 35 cycles (1 min at 95°C, 1 min at 60°C, and 1 min at 72°C). Purified PCR products were digested with 0.6 U of DNase I in the presence of Mn^{2+} for 10 min at 15°C as previously described [11]. After inactivating DNase I, DNA fragments in the range of 50 to 200 bp were purified using the Gel Extraction kit (Qiagen, Hilden, Germany). The purified DNA fragments were then subjected to 60 cycles of the first PCR reaction without oligonucleotide primers (1 min at 95°C, 1 min at 60°C, and 1 min at 72°C). To generate the chimeric *cdd* libraries, a second PCR reaction was performed for 35 cycles as described above with primers P1 and P2 (Table 1). The shuffled products were cloned into the pGEM T-easy vector, and a chimeric library was constructed by transforming into *E. coli* JF611. CDA activity was compared with the parental source using the crude enzyme screening technique. Positive clones showing higher activity than the parental source at 70°C were selected.

Assay of Cytidine Deaminase Activity

Cytidine deaminase activity was assayed at various temperatures as described elsewhere [6]. One unit is defined as the amount of enzyme required to deaminate 1 mM of cytidine per minute. The protein concentration was measured using the Coomassie Protein Assay Kit (Pierce, Rockford, U.S.A.). Bovine serum albumin (BSA) was used as a standard. Standard error of the means were gained through JMP-IN software ver. 4 (SAS Institute Inc., Raleigh, U.S.A.).

Construction of the Overexpression Vector

The complete coding regions of recombinant *cdd* genes were amplified with C1 and C2 primers (Table 1). PCR products were digested with SmaI and BamHI, inserted into pET14b digested with the same restriction enzymes, and transformed into *E. coli* Rosetta(DE3)pLysS. Transformants were cultivated in LB medium supplemented with AP (100 μg/ml) and CH (25 μg/ml). Over–expression of recombinant proteins was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 24 h. Bacterial cells were harvested by centrifugation at 5,000 rpm for 10 min.

Table 1. Oligonucleotide primers used in this study.

Primer	Sequence
M13-F	5'-CAGGGTTTTCCCAGTCACGA-3'
M13-R	5'-CACAGGAAACAGCT ATGACCATG-3'
P1[a]	5'-ACA*GGATCC*AATTCTAATTTTTCTGTTA-CATTTTTG-3'
P2[b]	5'-ACA*CTGCAG*GATTTTCCTACGTTCG-GTCTT-3'
C1[a]	5'-*GGATCC*GTGGAGATTGAGCAGCTCAT-3'
C2[c]	5'-*CCCGGG*TTACGCATGCAAATCCTCCG-3'
S1[a]	5'-*GGATCC*ATGGAGATCGAACAGCTCAT-3'
S2[c]	5'-*CCCGGG*TTATTCATGCATATCCTCCG-3'

Underlined primer sequences indicate restriction enzyme sites; BamHI, PstI, and SmaI are indicated as a, b, and c, respectively.

Purification and Quaternary Structure Determination of Recombinant Cytidine Deaminase

The cells were washed with phosphate-buffered saline (PBS, pH 7.5) and sonicated. Cell-free extracts were obtained after centrifugation at 12,000 rpm for 10 min at 4°C. The recombinant proteins were purified by affinity chromatography with Ni–NTA chelating agarose CL-6B (Peptron, Daejoen, Korea). The protein solution was applied to the column, washed 4 times with a washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0), and eluted with an elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The purified proteins were treated with 5 units of thrombin protease (Amersham Biosciences, Uppsala, Sweden) at 25°C for 16 h to remove the His-tag region. To confirm the protein purity and determine the molecular weight of proteins, SDS–PAGE was performed. Acrylamide gel (15%) was used for electrophoresis and Precision Plus Protein Standards (Bio-RAD, Hercules, U.S.A.) were loaded on the gel as standard size markers. High temperature treatments of purified proteins were carried out using a TProfessional thermocycler (Biometra, Goettingen, Germany). The oligomerization state of SH1077 and SH2429 CDAs was ascertained by SDS–PAGE, were 1.75 mM SDS was applied for the gel [19]. A sample of CDA protein in loading buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 0.05% bromophenol blue, and 1.75 mM SDS) was heated for 2 min at a given temperature.

Thermostability Test of the Recombinant CDAs

The purified enzyme dissolved in 50 mM Tris-HCl, pH 7.5, was incubated at 70°C, 80°C, and 90°C under a mineral oil layer to prevent evaporation and then cooled at room temperature. Aliquots were collected at intervals, and the residual activity was measured at 70°C as a routine procedure. Each assay was performed in triplicate.

Prediction of the Three-Dimensional Structure

The three-dimensional structures of WT-CDA and mutant CDA were predicted using SwissModel (http://swissmodel.expasy.org//SWISS-MODEL.html). The structure of 1JTK (CDA of *B. subtilis* 168) previously solved by X-ray diffraction was used for simultaneously modeling CDA molecules. 1JTK shows 71% and 72% of homology in amino acid sequence with cytidine deaminase from *B. caldolyticus* DSM 405 and *B. stearothermophilus* IFO12550, respectively.

RESULTS

DNA Family Shuffling of *cdd* Genes

After the DNase I treatment and reassembly procedure, shuffled DNA products were cloned into the pGEM T-easy vector and transformed into *E. coli* JF611. Nearly 500 colonies of transformed *E. coli* JF611 appeared on AB agar plates without uridine, which is selective for strains showing

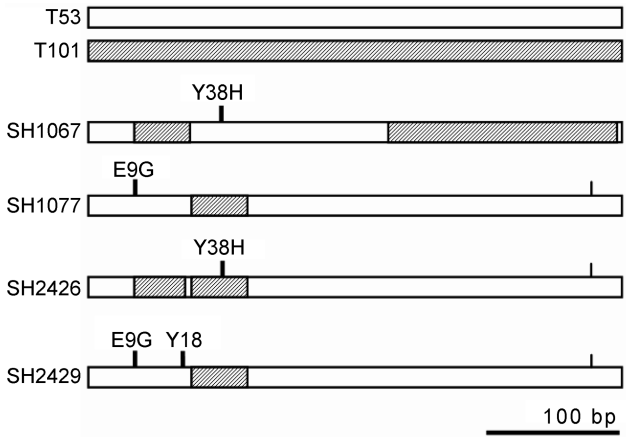

Fig. 1. Parental makeup of 4 chimeric *cdd* genes based on DNA sequence analysis.
The stick in bold indicates amino acid replacement; others mean nucleotide replacement.

CDA activity. One hundred and fifty colonies were selected to measure CDA activity at 70°C. In the process, the two *cdd* mutants (SH2426, SH2429) that showed rather high CDA activity were finally isolated in the mutant library.

Analysis of CDA Amino Acid Sequences

The DNA sequences of chimeric genes encoding variant CDAs were analyzed to establish the position and nature of the mutations. The DNA sequence of the resultant second-round shuffling products, SH2426 and SH2429, exhibited high homology with that of SH1077. The amino acid sequence of SH2429, in particular, was identical with that of SH1077 except for one replacement (Y18H) (Fig. 1). Amino acid replacement (Y38H) of SH2426 was inherited from SH1067. Three out of 4 chimeric CDAs had 98% amino acid sequence identity with T53 (Table 2). The amino acid sequence of SH1067 showed 92% and 93% homology with T53 and T101, respectively. The phylogenetic tree revealed that amino acid sequences of three variants were closely related to the parental source T53, whereas the sequence of SH1067 was homologous to that of T101 (Fig. 2).

Activity Bioassays of Recombinant CDAs

The specific CDA activities of recombinants at various temperatures are shown in Table 3. The optimum temperature

Table 2. Homology (%) of the deduced amino acid sequence.

	T53	T101	SH1067	SH1077	SH2426	SH2429
T53	100	86	92	98	98	98
T101	86	100	93	86	87	86
SH1067	92	93	100	90	92	89
SH1077	98	86	90	100	98	99
SH2426	98	87	92	98	100	97
SH2429	98	86	89	99	97	100

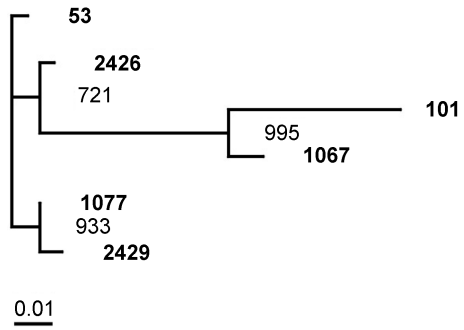

Fig. 2. Phylogenetic tree of all recombinant CDAs.
The tree was obtained using CLUSTAL-X and TreeView software. The lengths of branches indicate the divergence among the amino acid sequences.

of SH1067 and SH1077 was 80°C, which was 10°C higher than that of wild types (T53 and T101) and products of the second-round shuffling (SH2426 and SH2429) (Fig. 3.). SH2429 showed the highest activity at temperatures under 70°C, even when the CDA activity dropped suddenly at over 80°C.

Thermostability of CDA

The thermostability of recombinant CDA was determined at 70°C, 80°C, and 9°C. The CDA activity of SH1067 and SH1077 remained constant for 3 h at 70°C (Fig. 4). The half-time of residual activities of SH1067 and SH1077 increased up to 200% as compared with that of T53 at 80°C. SH1067 showed over 200% increased half-time than that of T53, although 120 min later, no activity remained at 90°C. SH2426 showed 150% increased half-time than that of T53 at 70°C (Table 4). SH2429 showed over 50–70% decreased thermostability than that of T53 at 70°C; residual activity disappeared after 10 min at over 80°C.

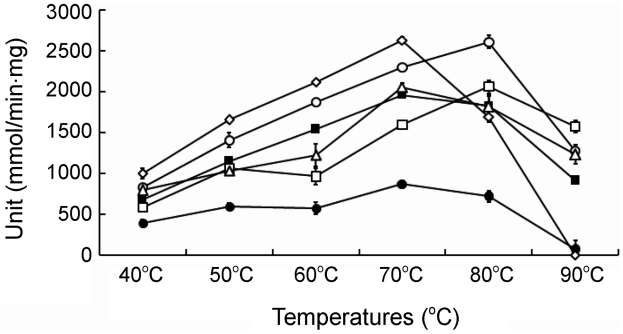

Fig. 3. Apparent temperature optimum profiles.
CDA activity of equimolar amounts of enzymes was measured in standard reaction mixtures (pH 7.5) at indicated temperature. Experiments were performed in triplicate. Values with error ranges within 10% were averaged. Error bars denote the standard deviation. T53, closed square; T101, closed circle; SH1067, open square; SH1077, open circle; SH2426, open triangle; SH2429, open diamond.

Table 3. Specific activity of chimeric CDA at various temperatures.

	Specific activity (mmol/mg·min)					
	T53	T101	SH1067	SH1077	SH2426	SH2429
40°C	682.9 ± 33.3	395.2±37.7	586.8±44.9	831.4±15.1	793.0±58.7	1001.9 ± 68.2
50°C	1152.1 ± 26.7	601.0 ± 42.2	1060.8 ± 57.5	1408.7 ± 91.5	1037.2 ± 22.2	1656.5 ± 32.7
60°C	1545.0 ± 33.0	573.6 ± 75.8	964.7 ± 99.6	1873.9 ± 17.1	1227.9 ± 140.1	2119.5 ± 24.9
70°C	1964.6 ± 40.4	870.0 ± 48.8	1601.2 ± 40.2	2306.1 ± 22.9	2058.1 ± 49.0	2634.4 ± 38.5
80°C	1827.3 ± 127.0	719.0 ± 64.9	2065.3 ± 78.4	2612.9 ± 80.0	1818.6 ± 152.4	1696.4 ± 67.1
90°C	915.5 ± 40.2	68.6 ± 115.3	1577.9 ± 78.4	1280.1 ± 14.4	1234.9 ± 114.7	0 ± 79.8

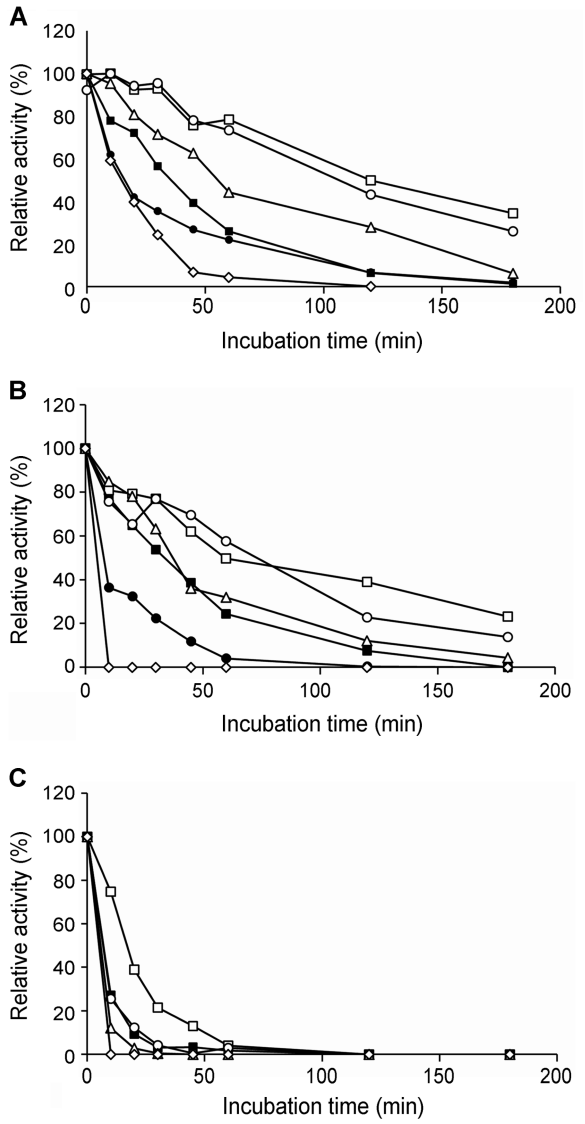

Fig. 4. Thermal stability of recombinant CDAs.
T53, closed square; T101, closed circle; SH1067, open square; SH1077, open circle; SH2426, open triangle; SH2429, open diamond. **A**. CDA activities measured after incubating for various times at 70°C. **B**. CDA activities measured after incubating for various times at 80°C. **C**. CDA activities measured after incubating for various times at 90°C. Relative activity is expressed as a percentage of original activity (before heat treatment).

Table 4. Thermostability of chimeric CDA at various temperatures.

	Half-time (min)					
	T53	T101	SH1067	SH1077	SH2426	SH2429
70°C	35.65	15.97	119.5	106.3	55.28	14.69
80°C	33.75	7.87	59.75	73.15	37.36	5
90°C	6.87	5.01	16.96	6.72	5.73	5

Purification and Quaternary Structure Determination of Recombinant Cytidine Deaminase

Most fusion His-tagged CDAs (MW 16 kDa) were expressed as soluble proteins on the SDS–PAGE gel (data not shown). After digestion of His-tag by thrombin cleavage, the His-tag-fusion proteins were purified to active CDAs of about 14 kDa in size, which was consistent with the predicted molecular mass of CDAs from wild-type strains.

In order to observe the difference in the quaternary structures between SH1077 and SH2429 under different temperatures, the protein samples treated at a given temperature were loaded on the SDS–PAGE gel. No difference in tetramer formation between them was observed at 70°C (Fig. 5). SH2429 was denatured and became almost monomeric at 80°C. In contrast, SH1077 was more stable and tetramerized at 80°C.

Prediction of Three-Dimensional Structure

According to the three-dimensional structures of recombinants predicted by the SwissModel program, the two parental CDAs were similar to 1JTK (*B. subtilis* 168 CDA). They have 5 alpha helixes and 3 beta sheets. The amino acid substitution exists on alpha1, beta2, and the loop region. The 3 replaced amino acid residues were toward the protein surface (Fig. 6).

Discussion

There are numerous techniques by which enzymes can be made to function under the desired conditions. DNA shuffling is a powerful molecular tool used for altering the phenotype. In many cases, the tool was applied for elevation of enzyme activity, high thermostability, and alteration of

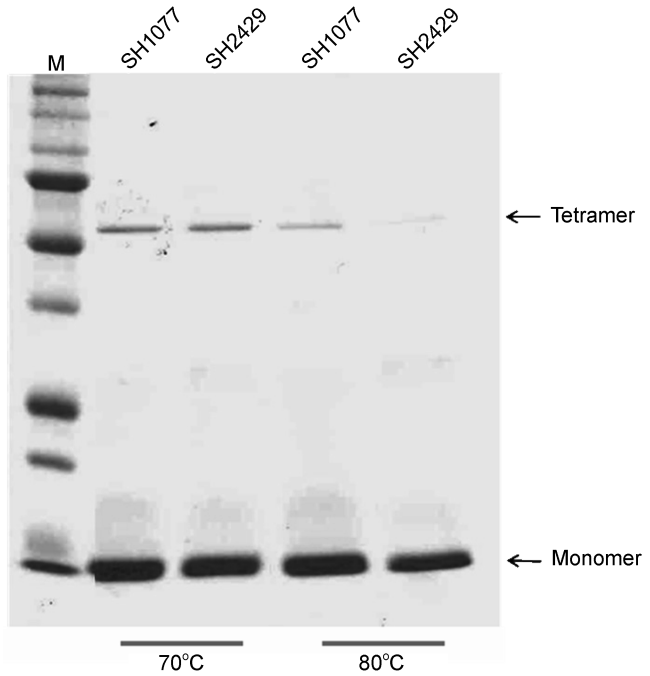

Fig. 5. Quaternary structures of CDA variants.
CDA variants in loading buffer were heated at a given temperature for 2 min and then developed on SDS–polyacrylamide gel. M, size marker; lanes 1 and 3, SH1077; lanes 2 and 4, SH2429.

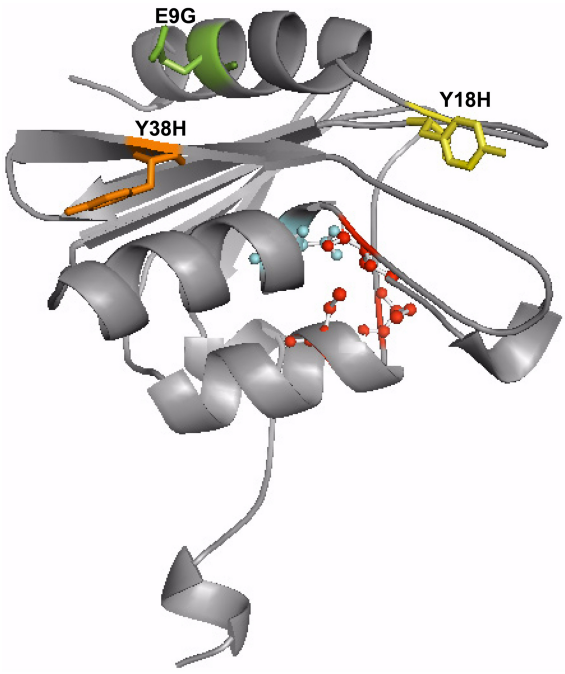

Fig. 6. Ribbon representation of the predicted *B. caldolyticus* CDA subunit structure.
The position of the three amino acid replacements that conferred the CDA activity or thermostability is shown by stick representation. The zinc ion ligands are shown as a ball-and-stick (red) model. The active site is shown as a ball-and-stick (cyan) model.

substrate specificity [7, 18, 24]. In this study, we have shuffled thermostable cytidine deaminases (CDAs) from thermophilic bacteria, resulting in the production of variants showing various patterns in thermostability.

Thus far, research groups studying thermal stability have found many factors that could affect thermostability such as increase in number of hydrogen bonds, better hydrophobic internal packing, enhanced secondary structure propensity, and optimization of salt bridge [20]. Through site-directed mutagenesis, a theory that improvements in thermostability can be equally efficient through mutations close and distant to the active site was verified [14].

In this case, Tyr38His is on the β2 strand in SH1067 and SH2426, but their phenotypes are different. The C terminal residues of SH2426 were derived from T53, and the N terminal regions were derived from T101. These results indicate that the thermostability of chimeras depends not only on single-site substitution, but also on the directed evolution of this change in concert with the appropriate context provided by the rest of the protein. For instance, the rather thermostable variant SH1077 has a single amino acid conversion, Glu9Gly. However, Gly had been found to cause instability of the protein structure [20]. A single amino acid residue replacement presented between SH1077 and SH2429 showed drastic change in specific activity and thermostability. Interestingly, Tyr18His caused SH2429 to be unstable at high temperatures. Tyr18 is known to be an amino acid residue on the loop region among conserved residues that constitute the interaction surfaces in tetrameric CDAs [9]. Flores and Ellington [5] observed a tight interaction between subunits, which contributed to increased thermal stability of an oligomeric protein, beta-glucuronidase. Based on quaternary structure determination, we presupposed that the replacement in SH2429 should result in greater structural flexibility that leads to highest activity under 70°C conditions. Otherwise, loose interaction between the subunits results in a drastic decrease in thermal stability over 80°C conditions.

The correlation between structure and function of enzymes cannot be definitely elucidated by directed evolution studies. Crystallographic studies of the tetrameric CDAs, in progress, will hopefully provide a clearer insight into the structural differences among the chimeras and parental CDAs.

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