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Further Stabilization of 3-Isopropylmalate Dehydrogenase of an Extreme Thermophile, *Thermus thermophilus*, by a Suppressor Mutation Method

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We succeeded in further improvement of the stability of 3-isopropylmalate dehydrogenase (IPMDH) from an extreme thermophile, *Thermus thermophilus*, by a suppressor mutation method. We previously constructed a chimeric IPMDH consisting of portions of thermophile and mesophile enzymes. The chimeric enzyme is less thermostable than the thermophile enzyme. The gene encoding the chimeric enzyme was subjected to random mutagenesis and integrated into the genome of a *leuB*-deficient mutant of *T. thermophilus*. The transformants were screened at 76°C in minimum medium, and three independent stabilized mutants were obtained. The *leuB* genes from these three mutants were cloned and analyzed. The sequence analyses revealed Ala-172→Val substitution in all of the mutants. The thermal stability of the thermophile IPMDH was improved by introducing the amino acid substitution.

Improvement of the thermal stability of proteins is one of the major concerns in protein engineering. Several principles have been proposed to increase the thermal stability of proteins (1, 17, 18, 20, 21, 25, 32, 38), but these principles are too preliminary to be generally applied to a variety of proteins. Moreover, the application of these principles is limited to the proteins whose three-dimensional structures are known. Intensive physicochemical analyses of thermal stability have been done mainly on a few small and monomeric proteins that unfold reversibly. Thus, establishment of the generality of the principles deduced with those model proteins is reserved for studies with other types of proteins such as those which unfold irreversibly, are oligomeric, and are much larger than such model proteins.

We have been analyzing the stability of 3-isopropylmalate dehydrogenase (IPMDH; EC 1.1.1.85) as a model enzyme. This enzyme is on the leucine biosynthetic pathway. The *leuB* gene encoding IPMDH has been cloned and sequenced from a variety of microorganisms such as yeasts (2, 6, 7, 26, 34), bacilli (11, 29, 30). *Salmonella typhimurium* (3), a cyanobacterium, *Spirulina platensis* (4), and two extreme thermophiles, *Thermus thermophilus* (12, 14) and *Thermus aquaticus* (15). The enzyme from *T. thermophilus* has been purified and characterized (37), and the crystal structure has been determined at 2.2-Å (1 Å = 0.1 nm) resolution (10). Crystal analysis revealed that the enzyme is a homodimer and each subunit consists of 345 amino acid residues.

We have recently constructed several chimeric IPMDHs consisting of portions of the enzymes from the extreme thermophile and a mesophile, *Bacillus subtilis* (22). One of such chimeric enzymes, 2T2M6T, was designed to have 20% of the sequence (residues 74 to 133) from the mesophile enzyme and the rest from the *T. thermophilus* enzyme (Fig. 1). Thermal stability of the chimeric enzyme was less than that of the thermophile enzyme (22). We have also developed an integration vector system that enables the expression of foreign genes in *T. thermophilus* (35). The gene encoding the chimeric enzyme was integrated and expressed in *T. thermophilus* by using this system. A *T. thermophilus* mutant harboring the chimeric enzyme gene was temperature sensitive with respect to leucine biosynthesis. By using the combinatorial technique, the gene encoding the stabilized enzyme was isolated (35).

In this study, we applied a suppressor mutation method to *T. thermophilus* IPMDH. The suppressor mutation method consists of two steps. First, a mutant enzyme which is less thermostable than the original enzyme is constructed and subjected to random mutation, and then the mutants with restored thermal stability are selected. Second, the stabilizing mutation which is independent of the first destabilizing mutation (suppressor mutation) is introduced into the wild-type enzyme so as to improve heat stability. This method has been applied to the stabilization of phage lambda repressor (9), staphylococcal nuclease (31), and yeast iso-1-cytochrome *c* (5). We also used this method in order to further stabilize the unusually stable enzyme from the extreme thermophile.

MATERIALS AND METHODS

Reagents. The mutagen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) was purchased from Nakarai Tesque Co., Ltd. (Kyoto, Japan). Restriction and DNA modification enzymes were obtained from Takara Shuzo Co., Ltd. (Kyoto, Japan), or Toyobo Co., Ltd. (Osaka, Japan). DL-3-Isopropylmalate (DL-3-IPM) and NAD were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and Oriental Yeast Co., Ltd. (Tokyo, Japan), respectively.

Bacterial strains and media. The wild-type strain, *T. thermophilus* HB27 (27), and the *leuB*-deficient strain, *T. thermophilus* MT106 (35), were cultured in *Thermus* nutrient medium (24) or *Thermus* minimum medium (36). *T. thermophilus* MT106 was used as a host for screening the genes encoding thermostabilized enzymes. *Escherichia coli* JM109 {*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ⁻ Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}*ZΔM15*]} and JA221 (F⁻ *hsdR trpE5 leuB6 lacY recA1* λ⁻) were used as hosts for plasmid amplification and for expression of the *leuB* genes, respectively. They were cultured in LB medium or 2YT medium.

Construction of a randomly mutated DNA library. Experimental procedures are illustrated in Fig. 2. Plasmid pNOBL2 harbors a chimeric *leuB* gene that consists of parts of *T. thermophilus* and *B. subtilis leuB* genes (22). The plasmid was digested with *Sac*I and *Sal*I, and a fragment encoding the 3′ region of the *T.*

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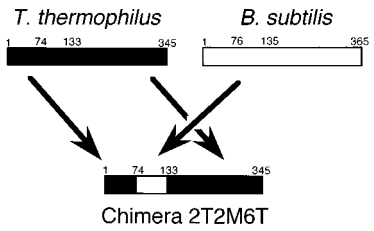

FIG. 1. Construction of the chimeric IPMDH. Filled boxes show the portion derived from *T. thermophilus* IPMDH; open boxes show the portion from *B. subtilis* IPMDH. The chimeric IPMDH, 2T2M6T, possesses *B. subtilis* residues 74 to 133; the rest of the sequence is from the *T. thermophilus* enzyme.

thermophilus leuB gene, covering 60% of the whole gene, was excised. This fragment (1 μg) was dissolved in 500 μl of phosphate buffer (200 mM sodium phosphate [pH 6.0]), and MNNG was added to a final concentration of 0.2 mg/ml. The solution was incubated at 37°C for 30 min and dialyzed twice against 500 ml of 10 mM Tris buffer (pH 7.6) containing 10 mM NaCl and 1 mM EDTA. The fragment was ligated with the rest of the gene and amplified in *E. coli* JM109. The 1.2-kb *Bam*HI fragment containing the mutated gene was excised and inserted into the integration vector for the thermophile, pIT1 (35). The ligate was amplified in *E. coli* JM109 and used as a randomly mutated *leuB* gene library.

Isolation of stabilized mutants. The *leuB*-deficient strain, *T. thermophilus* MT106, was transformed with the randomly mutated *leuB* gene library as described by Koyama et al. (16), with slight modifications (33). The transformed cells were incubated at the restrictive temperature of 76°C in 3 ml of minimum medium for 48 h. The culture was then plated on minimum medium, and strains with improved thermal stability were isolated after 2 days of incubation.

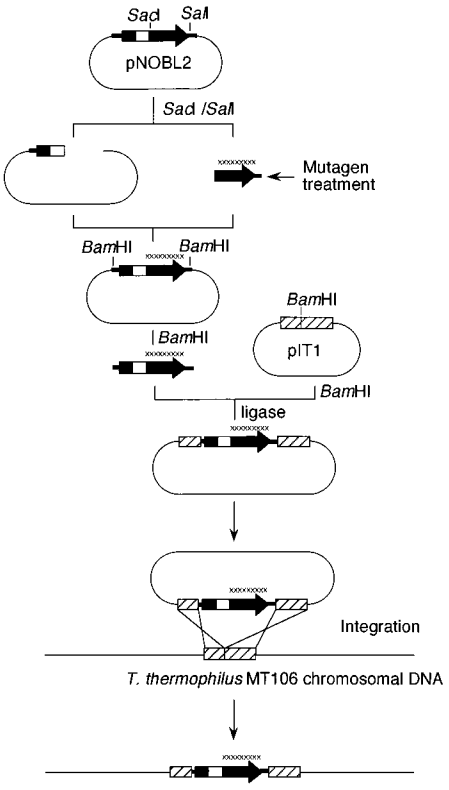

FIG. 2. Construction of a randomly mutated DNA library and integration into *T. thermophilus*. Plasmid pNOBL2 was digested with *Sac*I and *Sal*I; the smaller fragment containing the 3′ region of the *leuB* gene was treated with MNNG and ligated with the rest of the plasmid. The resulting plasmids were digested with *Bam*HI; the 1.2-kb fragments containing the whole gene were excised and inserted into the integration vector (pIT1) for *T. thermophilus*. The mutated genes were integrated into the chromosomal DNA of the *T. thermophilus* Δ*leuB* strain (MT106) by homologous recombination. Thick arrows with open boxes indicate the chimeric *leuB* genes; hatched lines show the flanking sequence of the *leuB* gene.

Genomic DNA preparation. The *T. thermophilus* strains were grown in *Thermus* nutrient medium. The cells were harvested by centrifugation at 4,000 × *g* for 10 min, and the pellets were resuspended in 500 μl of 100 mM Tris buffer (pH 8.0) containing 10 mM EDTA. They were treated with 10 mg of hen egg white lysozyme at 42°C for 20 min and then with 0.1 mg of RNase A per ml at 37°C for 30 min. One hundred microliters of 10% sodium lauryl sulfate solution and 1 mg of proteinase K were added, and the mixture was incubated at 50°C for another 30 min. The solution was successively extracted with phenol, phenol-chloroform, and chloroform, and the genomic DNA was wound around a glass rod after addition of 2 volumes of ethanol.

Cloning of *leuB* genes. The genomic DNA was digested with *Hin*dIII; the fragments were ligated with pUC19 and used for transformation of *E. coli* JA221. The transformants were spread on LB medium plates supplemented with ampicillin (150 μg/ml) and incubated for 16 h at 37°C. The replicas of the plates were made onto *Thermus* minimum medium plates coated with *T. thermophilus* MT106 in logarithmic growth phase. The plates were incubated at 70°C for 1 h and then at 60°C for 48 h. Since *E. coli* JA221 cells were killed at the high temperature, *T. thermophilus* MT106 cells were transformed by the DNA present in the *E. coli* cells. Accordingly, the *E. coli* colonies on the master plates that corresponded to colonies of the thermophilic bacteria returned to autotrophy on the replica plates were expected to harbor *leuB* genes encoding thermostabilized enzymes.

DNA sequencing. The *leuB* gene from a cloned *Hin*dIII fragment was subcloned into the *Bam*HI site of pUC119 to prepare sequencing templates. Sequence was determined by the dideoxy-chain termination method with an ABI Dye Primer Taq sequencing kit, ABI Taq Dye Primer Cycle sequencing kit, or ABI Oliglow Primer Taq sequencing kit. Synthetic oligonucleotide primers 5′GGT GCT TCT GGG AAG CGT3′, 5′GGC TCC CCG AAG TAG AT3′, 5′ATG GCG TCC ACA TAC TGG GT3′, 5′GGG GAT CTA CTT CGG GGA3′, 5′CTT CCC AGA AGC ACC GCC T3′, and 5′TCT CTA GGC CTC CTC CCC T3′ were used as the sequencing primers.

Construction of genes encoding mutant enzymes. For production of the mutant of the chimeric enzyme, the chimeric *leuB* gene containing the base substitution which results in an Ala-172→Val replacement in the amino acid sequence was cloned into pUC119 as described by Numata et al. (22). The base substitution was introduced into the *T. thermophilus leuB* gene by replacing the *Sac*I fragment of the *T. thermophilus leuB* gene, which was also inserted into the *Bam*HI site of pUC119, by the corresponding fragment of the chimeric gene with the substitution. The resulting plasmid was used for overexpression and preparation of the mutant enzyme.

Enzyme assay. Enzymate activity was estimated from the increase in the rate of A_{340} in 100 mM potassium phosphate buffer (pH 7.6) containing 1 M KCl, 0.2 mM $MnCl_2$, 0.8 mM NAD, and 0.4 mM DL-IPM as described by Yamada et al. (37).

IPMDH purification. For preparation of 2T2M6T, wild-type, and mutant enzymes, *E. coli* JA221 harboring the respective expression plasmid was cultivated in 2YT medium supplemented with ampicillin (150 μg/ml). Cells were harvested and disrupted by sonication, and soluble fractions were obtained by centrifugation at 60,000 × *g* for 20 min. The supernatant was treated at 60°C (chimera and its mutant) or 70°C (wild-type and its mutant) for 10 min and centrifuged at 60,000 × *g* for 20 min. Each enzyme was purified from the supernatant by using columns of butyl-Toyopearl (Tosho) and Mono Q (Pharmacia) successively (22). Each sample was homogeneous when analyzed by gel electrophoresis.

Thermal stability measurement. The enzymes were suspended in potassium phosphate buffer (20 mM potassium phosphate, 0.5 mM EDTA [pH 7.6]), heat treated at various temperatures, and immediately chilled on ice for 10 min. The chilled samples were centrifuged at 15,000 × *g* for 10 min, and the remaining activity of the supernatants was measured at 50°C for the chimeric IPMDH and its mutant or at 60°C for the wild-type IPMDH and its mutant.

Thermal denaturation was measured with a DASM-4 scanning microcalorimeter. To prevent aggregate formation after thermal denaturation, the differential scanning calorimetry (DSC) measurements were carried out in an alkaline buffer. The enzyme was dissolved in 20 mM $NaHCO_3$ (pH 10.2), and the concentration was 1 mg/ml. The scan rate was 0.5°C/min.

RESULTS AND DISCUSSION

Construction of a randomly mutated DNA library. The 700-bp DNA fragment containing the 3′ region of the chimeric *leuB* gene was treated with MNNG and ligated with the rest of the gene. The ligates were used to transform *E. coli* JM109. About 10,000 colonies of the transformant were obtained, and the plasmids were collected from all colonies. Then a 1.2-kb *Bam*HI fragment containing the randomly mutated gene was excised from the plasmids and ligated with the integration vector for *T. thermophilus*. The ligates were amplified in *E. coli*

TABLE 1. Growth of *T. thermophilus* strains in minimum medium without leucine[a]

T. thermophilus strain	Growth at:	
	70°C	76°C
HB27 (wild type)	+	+
MT106 (Δ*leuB*)	−	−
NB26 (chimeric *leuB*)	+	−
NK101 (heat resistant)	+	+
NK102 (heat resistant)	+	+
NK103 (heat resistant)	+	+

[a] Each strain was plated on minimum medium without leucine and incubated for 36 h at the indicated temperatures.

JM109 and used as a randomly mutated DNA library (Fig. 2). Because there are two possible directions of the *leuB* genes in the vector, the effective library size is about 5,000.

Screening and sequencing of *leuB* genes encoding stabilized enzymes. We have previously constructed the Δ*leuB* strain MT106 from *T. thermophilus* HB27 by gene disruption (35). We have also demonstrated the integration of the chimeric *leuB* gene into the chromosomal DNA of *T. thermophilus* MT106 by using the integration vector (35). The chimeric gene was expressed in the leucine operon of the transformed *T. thermophilus* NB26. Strain NB26 showed temperature sensitivity of leucine biosynthesis: the strain grew at 70°C but not at 76°C in minimum medium without leucine, while the wild-type strain of *T. thermophilus* grew at 76°C (Table 1). We transformed *T. thermophilus* MT106 with the randomly mutated DNA library (Fig. 2), screened the transformants at 76°C in medium lacking leucine, and isolated three independent mutants (NK101, NK102, and NK103) which grew under the conditions used (Table 1).

The *leuB* gene was cloned from NK101 and sequenced. The sequencing revealed that the gene had only a single base replacement, C-515→T. The mutated site was located in the *T. thermophilus* region of the chimeric gene, which had been treated with MNNG. This mutation corresponds to an Ala-172→Val amino acid replacement (Fig. 3).

The *leuB* genes were also cloned from the other two mutants (NK102 and NK103) and sequenced. The two genes had the same mutation as the first (C-515→T). The results suggest that the base point C-515 is a hot spot of the MNNG treatment. Alternatively, the mutation Ala-172→Val may be the only mutation that can improve the thermal stability of the chimeric enzyme. We are analyzing these possibilities, and the results will be published elsewhere.

Thermal stability of 2T2M6T and its mutant. To examine the thermal stability of the chimeric enzyme with the Ala-172→Val substitution, the mutant enzyme, 2T2M6T-A172V, was purified and the thermal properties were compared with those of the original enzyme, 2T2M6T. Figure 4A shows the

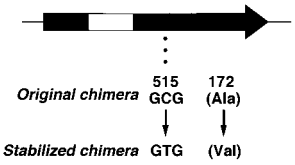

FIG. 3. Sequence of the *leuB* gene encoding the mutant of 2T2M6T. DNA sequencing showed that the chimeric *leuB* genes from the thermostabilized mutants had thymine at 515 instead of cytosine. This mutation causes an Ala-172→Val amino acid replacement.

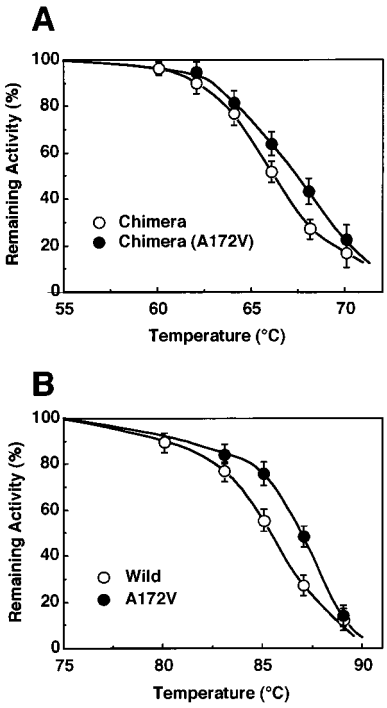

FIG. 4. Activities of IPMDHs after heat treatment. (A) Thermal stabilities of the chimeric enzymes; (B) thermal stabilities of the wild-type and mutant enzymes. Each enzyme was purified, diluted to 0.1 mg/ml with 20 mM potassium phosphate (pH 7.6) containing 0.5 mM EDTA, and treated at the indicated temperatures for 10 min. The remaining activity was expressed as a percentage of the original activity.

activities of the enzymes after heat treatment at various temperatures. The mutant enzyme was more resistant to heat than the original chimeric enzyme.

As thermal denaturation of proteins is an endothermic process, it is possible to monitor the denaturation process by DSC. The thermograms for the chimeric and mutant enzymes are shown in Fig. 5A. Two peaks can be seen in each curve. The enzyme has two structural domains in a subunit; one (domain 1) contains N and C termini, and the other (domain 2) contains the subunit interface (10). It has been suggested that the two separated peaks of the chimeric enzyme represent the separate denaturation of each domain, and the second endothermic peak is assigned to the thermal denaturation of domain 2, containing residue 172 (8). The two peak temperatures of the mutant enzyme (65 and 79°C) were higher than the corresponding peak temperatures of the original chimeric enzyme (62 and 72°C). These results clearly showed that Ala-172→Val substitution increased the thermal stability of the chimeric enzyme.

Improvement of the heat resistance of the thermophile enzyme. The amino acid substitution Ala-172→Val was introduced into the *T. thermophilus* wild-type IPMDH by gene recombination. The mutant of the wild-type enzyme was produced in *E. coli* and purified. The activities of the mutant and wild-type enzymes after heat treatment are compared in Fig. 4B. The mutant enzyme is more thermostable than the wild-type enzyme, by 1.5°C as judged by the half-inactivation temperature.

The thermal denaturation process was also analyzed by DSC at pH 10.2 (Fig. 5B). In contrast to the chimeric enzymes, which showed two peaks in the thermogram under the conditions used (Fig. 5A), only one peak was observed in the DSC

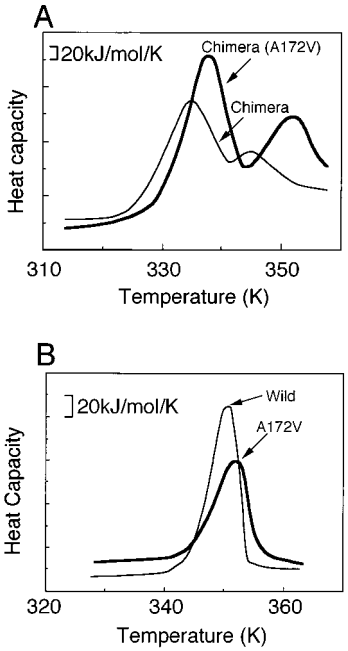

FIG. 5. DSC curves for the chimeric IPMDHs (A) and for *T. thermophilus* wild-type and mutant enzymes (B). The protein concentration was 1.0 mg/ml in 20 mM $NaHCO_3$ (pH 10.2). The rate of temperature increase was 0.5°C/min.

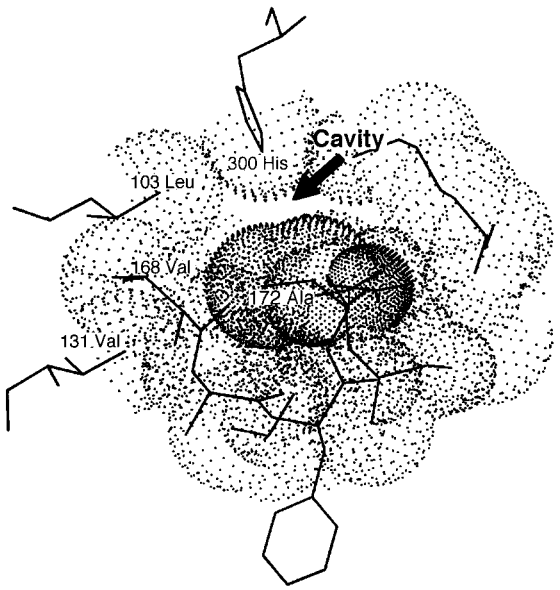

FIG. 6. The cavity around residue 172 of *T. thermophilus* IPMDH. The shadows represent the van der Waals radii of side chains of the enzyme. The drawing was rendered for the Silicon Graphics Indigo with the program QUANTA (Molecular Simulations Inc., Burlington, Mass.).

curves of the wild-type and its mutant enzymes. The peak temperature of the mutant enzyme (79°C) was slightly higher than that of the wild-type enzyme (78°C). Essentially the same results were obtained in repeated measurements. The results also showed that the A172V mutant is more thermostable than the wild-type enzyme from the structural standpoint.

Kinetic constants of the wild-type and mutant enzymes are listed in Table 2. While the Michaelis constants (K_ms) for NAD did not differ significantly between the two enzymes, the K_m for IPM and the catalytic constant (k_{cat}) were slightly improved by the Ala-172→Val substitution. These results showed that the Ala-172→Val substitution enhanced the thermal stability of the enzyme without significantly changing its catalytic properties.

Stabilization mechanism. According to the three-dimensional structure determined by X-ray crystallography (10), *T. thermophilus* IPMDH is a homodimer and each subunit consists of two domains. Ala-172 is in the domain interacting with the other subunit and in the locus forming an α helix. The crystal structure of the chimeric enzyme, 2T2M6T, has also been determined (23). In both enzymes, the methyl side chain of Ala-172 points to the hydrophobic core and is surrounded by hydrophobic residues such as Leu-103, Val-131, and Val-168. A small cavity is, however, detected around this side chain (Fig. 6). Computer analysis revealed that the cavity volume illustrated in Fig. 6 was reduced by 25 Å^3 upon the substitution of Ala-172 for Val. Therefore, the Ala-to-Val substitution may fill up this cavity without significant steric hindrance and increase the internal hydrophobic interaction. Several authors have reported improvement of the thermal stability of enzymes by increasing the hydrophobicity of side chains (13, 19, 38). A combination of hydrophobic interaction and packing effect has also been discussed (28).

The presence of a cavity inside the hydrophobic core has been searched for by computer-aided analyses (36a). However, the gap around Ala-172 shown in Fig. 6 was not detected. The vicinity around Ala-172 was involved in the hinge part which connects two domains in a subunit, and the cavity around the methyl group of Ala-172 was semiaccessible to the solute molecule and was recognized as the solute-accessible area (data not shown). Accordingly, our results show that filling the gap that is accessible to solute is also a way of improving the stability of proteins when the gap is surrounded by hydrophobic residues.

We attempted and succeeded in stabilization of the *T. thermophilus* wild-type enzyme, which is significantly stable before mutation. The result means that even the stability of the enzyme of this extreme thermophile can be improved.

TABLE 2. Kinetic constants of *T. thermophilus* wild-type and mutant enzymes[a]

Enzyme	K_m (μM)		k_{cat} (s^{-1})[b]
	D-3-IPM	NAD	
Wild-type	31 ± 4	130 ± 23	163 ± 22
A172V	22 ± 6	130 ± 23	220 ± 25

[a] The initial rates were measured at 60°C in the presence of various concentrations of D-3-IPM and NAD.
[b] Expressed as reaction per dimer.

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