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

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Received 27 July 1995/Accepted 7 November 1995

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 thermophilic and mesophilic enzymes. The chimeric enzyme is less thermostable than the thermophilic 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 thermophilic 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 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 mutant *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). 3-Isopropylmalyl-CoA (CoA-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 gcv496 thi hsdR17 supE44 relA1 Δlac-proAB] [F' traD36 proAB lacFZΔM15] and JA221 (F' hsdR trpE5 leuB8 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 SauI and SphI, and a fragment encoding the 3′ region of the *T.
thermotoga 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 MNGN 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 BamHI 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 B. subtilis leuB gene, covering 60% of the whole gene, which was also inserted into the pIT1 vector for the thermophile, pIT1 (35). The plasmidswere collected from all colonies. Thenthe1.2-kb BamHI 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 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.

Cloning of leuB genes. The genomic DNA was digested with HindIII; 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.

Construction of a randomly mutated DNA library. 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 SacI fragment of the T. thermophilus leuB gene, which was also inserted into the BamHI site of pUC19, 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. Enzyme activity was estimated from the increase in the rate of A405 in 100 mM potassium phosphate buffer (pH 7.6) containing 1 mM KCl, 0.2 mM MnCl2, 0.8 mM NAD, and 0.4 mM DL-IPM as described by Yamada et al. (37).

IPDH 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 NaHCO3 (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 MNGN and ligated with the rest of the gene. The ligates were used to transform E. coli JM109. About 10,000 colonies of the transformation were obtained, and the plasmids were collected from all colonies. Then a 1.2-kb BamHI 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 JM109.
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 ΔleuB 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 leu 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 substitution (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 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.
curves of the wild-type and its mutant enzymes. The peak temperature of the mutant enzyme (79.8°C) was slightly higher than that of the wild-type enzyme (78.8°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 ($K_m$) 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 semi-accessible 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.

**ACKNOWLEDGMENTS**

We are grateful to K. Yutani (Institute for Protein Research, Osaka University) for assisting with the DSC measurements. This work was supported by Grants-in-Aids for Scientific Research from the Ministry of Education, Science, and Culture of Japan (02403029 and 04044068). Support was also provided by the Mitsubishi Foundation.

**REFERENCES**


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

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
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<tbody>
<tr>
<td></td>
<td>0-3-IPM</td>
<td>NAD</td>
</tr>
<tr>
<td>Wild-type</td>
<td>31 ± 4</td>
<td>130 ± 23</td>
</tr>
<tr>
<td>A172V</td>
<td>22 ± 6</td>
<td>130 ± 23</td>
</tr>
</tbody>
</table>

*The initial rates were measured at 60°C in the presence of various concentrations of 0-3-IPM and NAD.

*Expressed as reaction per dimer.*