

論文 / 著書情報
Article / Book Information

題目(和文)	進化分子工学によるThermus thermophilusおよびBacillus subtilis由来3-イソプロピルリンゴ酸脱水素酵素の耐熱化
Title(English)	Improvement of protein thermostability : evolutionary molecular engineering studies on 3-isoprophylmalate dehydrogenases from thermus thermophilus and bacillus subtilis
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出典(和文)	学位:博士(理学), 学位授与機関:東京工業大学, 報告番号:甲第3755号, 授与年月日:1998年3月26日, 学位の種別:課程博士, 審査員:
Citation(English)	Degree:Doctor (Science), Conferring organization: Tokyo Institute of Technology, Report number:甲第3755号, Conferred date:1998/3/26, Degree Type:Course doctor, Examiner:
学位種別(和文)	博士論文
Type(English)	Doctoral Thesis

Improvement of Protein Thermostability

**Evolutionary Molecular Engineering Studies on
3-Isopropylmalate Dehydrogenases from *Thermus thermophilus*
and *Bacillus subtilis***

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1998

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Chapter 1: General Introduction

Proteins

Proteins play a predominant part in most biological processes. Almost chemical reactions in biological systems are catalyzed by enzymes, which are usually proteins or proteins associated with non-protein components essential for catalytic activity. Most enzymes show enormous catalytic power: they usually increase reaction rates by at least a million fold.

The basic structural units of proteins are the 20 kinds of L-amino acids. The side chains of these amino acid differ in size, shape, charge, hydrogen-bonding capacity and chemical reactivity. A number of amino acid monomers are linked by peptide bond to form a polypeptide chain. A particular protein may comprise one or more linear polypeptide chains, which may be identical or different. The critical determinant of the biological function of proteins is its conformation, which is specified on the basis of the amino acid sequence.

Protein engineering

Protein engineering is concerned with the construction, analysis, and use of proteins with modified properties including changing the substrate specificity or increasing stability (Ulmer, 1983). Proteins have unique amino acid sequences that are specified by genes. Recent advances in molecular biology and chemical synthesis of DNA have given us the ability to modify the gene of a protein. It is now possible to introduce any desired mutation into a cloned and sequenced segment of DNA using the technique of oligonucleotide-directed mutagenesis. Advances in the techniques for structure determination of proteins, including X-ray crystallography and computer modeling, also offer the prospect for developing protein engineering technology to make novel proteins not found in nature. By using a known protein structure, we can attempt to alter some functional properties and stability of the protein by directly modifying the gene.

Several successful examples of altering substrate specificity by protein engineering have been reported. Subtilisin, a serine protease, has been engineered to alter substrate specificity (Wells et al., 1987) and has been converted to a cysteine protease displaying novel peptide ligase activity (Abrahmsen et al., 1991; Jackson et al., 1994). Glutathione reductase has been converted to a trypanothione reductase (Bradley et al., 1991; Henderson et al., 1991) and has had its coenzyme specificity converted from NADPH to NADH (Scrutton et al., 1990). Similarly, lactate dehydrogenase has been engineered to alter coenzyme specificity from NADH to NADPH (Feeney et al., 1990), and additionally has been designed to produce malate dehydrogenase with higher activity than the naturally occurring malate dehydrogenase enzyme from the same species (Wilks et al., 1988). Lactate dehydrogenase has also been converted to a phenylpyruvate dehydrogenase and to an L-hydroxyisocaproate dehydrogenase (Dunn et al., 1991). Protein engineering have also been used to study the basic properties of proteins by replacing essential amino acid residues for function. These studies include probing the active site, mapping the ligand-binding site, and tracing the pathway of protein folding.

Improvement of protein thermostability by protein engineering

Improvement of protein thermostability by rational design is one of the important subjects of protein engineering. Thermostable enzymes used in industrial applications have advantages of increase rate of reaction, higher substrate solubility, longer shelf-lives at usual storage temperatures and lowered risk of microbial contamination when reactions are done at high temperatures.

In the past decade, many workers have successfully increased thermal stability of proteins, including T4 lysozyme (Matthews et al., 1987; Matsumura et al., 1988; Nicholson et al., 1988; Kuroki et al., 1989; Matsumura et al., 1989), α -amylase (Suzuki et al., 1989), cytochrome c (Linske-O'Connell et al., 1995), triosephosphate isomerase (Ahern et al., 1987), ribonuclease HI (Ishikawa et al., 1993), subtilisin (Pantoliano et al., 1987; Pantoliano et al., 1988), tryptophan synthase α subunit (Yutani et al., 1987) and so on. From these studies, several principles to stabilize native proteins have been proposed, for example: (i) introducing disulfide bonds or metal binding sites to reduce the difference in entropy between native and denatured states (Matthews et al., 1987; Pantoliano et al., 1988; Kuroki et al., 1989; Matsumura et al., 1989), (ii) changing specific residues to stabilize the dipoles of α -helices (Nicholson et al., 1988), (iii) improving hydrophobic interactions and packing ratio in the interior core (Yutani et al., 1987), and (iv) reducing the area of solvent-accessible hydrophobic surface (Wigley et al., 1987). However, these principles are too preliminary to be generally applied to a variety of proteins. In other words, general methods to increase protein thermostability have not been established and we are still far from a comprehensive understanding of protein stability. To establish the general principles of molecular design for protein stabilization, more information is required with respect to the structure-stability relationship of proteins.

Evolutionary molecular engineering

The evolutionary molecular engineering is an alternative way to produce modified proteins. The concept of the evolutionary modification technique was first proposed by Eigen and Gardiner (1984). The evolutionary technique requires introduction of mutations, selection of variants and reproduction of desired products. An advantage of this technique is that we can feasibly obtain modified proteins with desired properties in the absence of information on their three-dimensional structures. The technique has been used to improve the binding and catalytic properties of proteins, and has also been applied to other biopolymers such as RNA. So far, the well-studied examples of evolutionary engineering with RNA have been the improvement of catalytic activity of ribozymes and the development of aptamers that bind specifically to target molecules. Very recently, Wright and Joyce (1997) reported an *in vitro* evolution experiment where the template-directed ligation activity of the catalytic RNA was improved by continuous evolution in test tubes.

A pioneer evolutionary experiment of a protein was reported by Hall (1981). He cultured a *lacZ*-defective strain of *E. coli* with lactose as a sole carbon source. The wild-type evolved β -galactosidase (*ebg*) enzyme does not hydrolyze the β -galactoside bond and therefore does not allow the *lacZ*-deleted *E. coli* to grow in the lactose medium. However, several spontaneous mutants which could grow in the lactose medium were isolated, and the *ebg* enzyme prepared from the

spontaneous mutant hydrolyzed lactose effectively.

The first example of *in vivo* selection of proteins with enhanced thermostability was reported by Liao et al. (1986) with kanamycin nucleotidyltransferase. In that study, a strain of a moderate thermophile, *B. stearothermophilus*, which was transformed with the plasmid carrying kanamycin nucleotidyltransferase gene was cultured at 63°C. Since the enzyme is not fully stable at 63°C, the transformant could not grow at 63°C in the presence of kanamycin. However, a mutant strain which could grow at 63°C in the medium supplemented with kanamycin was isolated. Thermal stability of the enzyme from the mutant strain was higher than that of the original enzyme. A more thermostable mutant was derived from the first mutant by selecting a kanamycin-resistant strain at 70°C.

Integration vector system for *Thermus thermophilus*

An extremely thermophilic bacterium, *Thermus thermophilus* (ATCC 27634), was isolated from a hot spring at Mine, Shizuoka, Japan, by Oshima and Imahori (1974). The bacterium is gram-negative, non-sporing, aerobic rod containing yellow pig. The optimum temperature for growth is 70-75°C, the minimum being 48°C and maximum being 85°C. The GC content of the DNA is 69%. A remarkable characteristic of the genetics of *T. thermophilus* is the high homologous recombination efficiency (Koyama et al., 1986). This characteristic can be used to construct a genetic manipulation system for screening of modified proteins *in vivo*. However, the high efficiency of genetic recombination with a foreign DNA may cause difficulty in genetic handling with plasmid DNA (Takada et al., 1993); an introduced sequence may be integrated into the homologous sequence within the chromosomal DNA. Therefore, Tamakoshi et al. (1995) have developed a strategy for using an integration vector system. By this system, a foreign gene can be integrated into the homologous gene of *Thermus* cells by homologous recombination and be maintained in the genome.

3-Isopropylmalate dehydrogenases

3-Isopropylmalate dehydrogenase (IPMDH, EC 1. 1. 1. 85) catalyzes the oxidative decarboxylation of (2R,3S)-3-isopropylmalate to 2-oxoisocaproate in the third step of the leucine biosynthetic pathway in microorganisms and plants. The gene encoding the enzyme is designated as *leuB* and usually used as a genetic marker of the leucine auxotrophs. The gene has been cloned from a variety of microorganisms including yeast (Andreadis et al., 1984; Davidow et al., 1987; Hamasawa et al., 1987; Takagi et al., 1987; Sakai and Tani, 1992), bacilli (Imai et al., 1987; Sekiguchi et al., 1987), *Salmonella typhimurium* (Andreadis and Rosenthal, 1992), a cyanobacterium, *Spirulina platensis* (Bini et al., 1992), extreme thermophiles, *T. thermophilus* (Tanaka et al., 1981) and *T. aquaticus* (Kirino and Oshima, 1991), and a thermoacidophilic archaeon, *Sulfolobus* sp. strain 7 (Suzuki et al., 1997). These *leuB* genes encode highly similar amino acid sequences.

The enzyme from *T. thermophilus* has been purified and characterized (Yamada et al., 1990), and shown to be unusually thermostable. The three-dimensional structure of the enzyme has been determined at 2.2 Å (1 Å = 0.1 nm) resolution by X-ray diffraction (Imada et al., 1991), showing

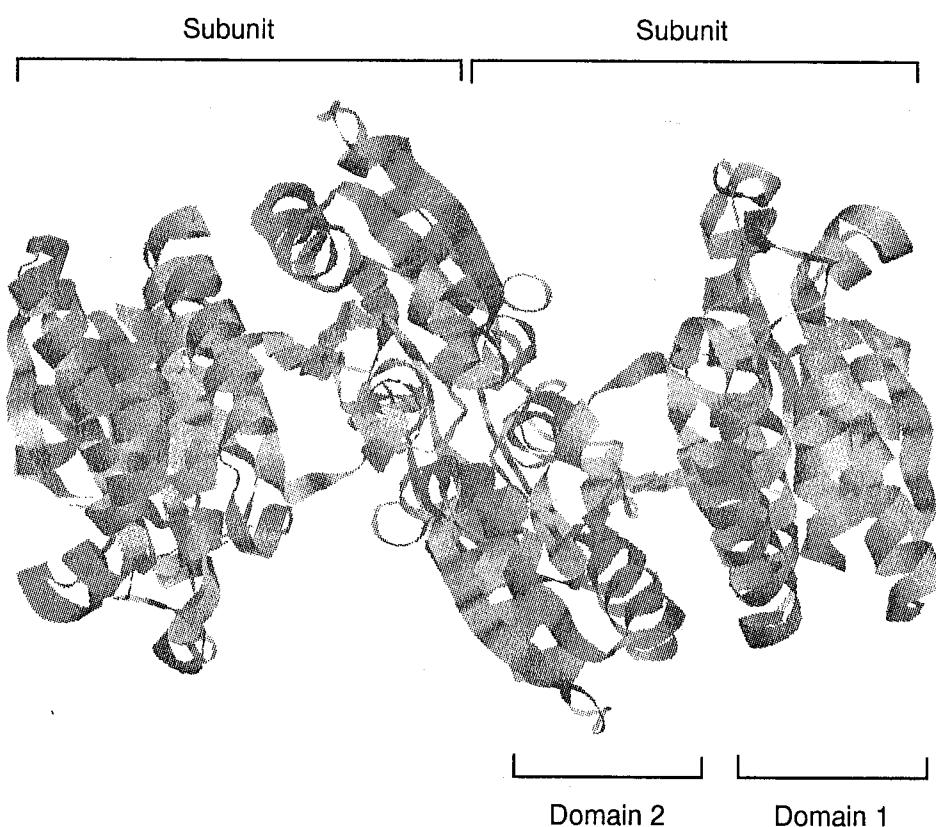


Figure 1-1. Ribbon representation of the dimer structure of *T. thermophilus* IPMDH.

that the enzyme is a dimeric protein composed of two identical subunits, each consisting of 345 amino acid residues (Fig. 1-1). The subunit can be divided into two structural domains; domain 1 consists of residues 1-99 and 255-345, thus including N- and C- termini, and domain 2 is involved in the subunit interaction.

The sequence of the *leuB* gene cloned from *B. subtilis* has also been determined (Imai et al., 1987). The amino acid sequence of *B. subtilis* IPMDH shows 56% identity with that of *T. thermophilus* IPMDH. The enzyme from *B. subtilis* has been purified and characterized (Numata et al., 1995), showing that the enzyme is less thermostable than the *T. thermophilus* enzyme by 40°C (Fig. 1-2). The crystallization experiment of the *B. subtilis* enzyme has been unsuccessful so far, precluding any crystallographic analyses. However, the high sequence identity between *B. subtilis* and *T. thermophilus* IPMDHs suggests that both enzymes have highly similar secondary and tertiary structures.

Stabilization of chimeric IPMDH 2T2M6T

Several chimeric IPMDHs have been constructed by combining portions of the enzymes from *T. thermophilus* and *B. subtilis* (Numata et al., 1995). One of such chimeric enzymes, designated as 2T2M6T, was designed to have about 20% of the sequence (residues 74 to 133) from the mesophile enzyme and the rest from the *T. thermophilus* enzyme (Fig. 1-3). The chimeric enzyme has

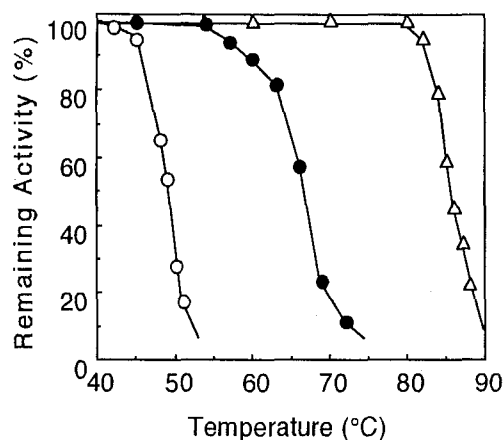


Figure 1-2. Thermal stabilities of IPMDHs. Activity of each enzyme was measured after heat treatment for 10 min at indicated temperatures. The remaining activities are expressed as percentages of the original activities. Open circles, *B. subtilis* IPMDH; closed circles, chimera 2T2M6T; open triangles, *T. thermophilus* IPMDH.

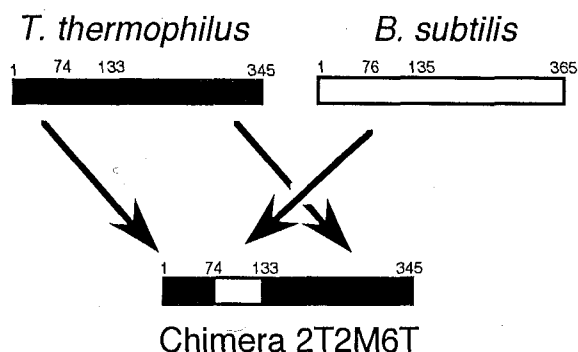


Figure 1-3. Construction of the chimeric IPMDH 2T2M6T. Filled boxes show the portion derived from *T. thermophilus* IPMDH; open boxes show the portion from *B. subtilis* IPMDH. The chimeric IPMDH, 2T2M6T, possesses *B. subtilis* residues 74 to 133; the rest of the sequence is from *T. thermophilus* enzyme.

been well characterized in terms of its catalytic properties, thermal stability and tertiary structure (Onodera et al., 1994; Numata et al., 1995). The chimeric enzymes have the very similar backbone structure with the *T. thermophilus* enzyme, whereas the thermal stability of the chimeric enzyme is less than that of the *T. thermophilus* enzyme by 20°C (Fig. 1-2). The gene encoding the chimeric enzyme has been used for a thermostabilization experiment (Tamakoshi et al., 1995).

A *leuB*-deficient strain of *T. thermophilus* MT106 was constructed from *T. thermophilus* wild-type strain HB27 by gene disruption. The chimeric *leuB* gene was integrated into the chromosomal DNA of *T. thermophilus* MT106 by using the integration vector system (Tamakoshi et al., 1995). The gene was expressed in the leucine operon of transformed *T. thermophilus* NB26. Because the chimeric enzyme is less thermostable than the thermophile enzyme, strain NB26 shows temperature sensitivity of leucine biosynthesis: the strain grew at 70 but not at 76°C in minimum medium without leucine while the wild-type strain of *T. thermophilus* grew at 79°C without leucine. A phenotypically thermostabilized strain evolved from the temperature-sensitive strain by selecting a leucine-autotrophic mutant at 79°C. DNA sequence of the chimeric *leuB* gene from the thermostabilized strain showed a single amino acid replacement, isoleucine to leucine at 93. It was confirmed that the mutant chimeric IPMDH from the thermostabilized strain was more thermostable than the original chimeric enzyme (Tamakoshi et al., 1995).

In this thesis, thermal stabilities of the thermophile and mesophile IPMDHs are improved based on the evolutionary molecular engineering technique and several principles for making the enzymes more stable are proposed. In Chapters 2 and 3, the inherent thermostable enzyme of *T. thermophilus* IPMDH were further stabilized by a suppressor mutation method. In Chapter 4, effects of polar residues introduced into a hydrophobic core of the thermophile enzyme were investigated with respect to thermal stability, catalytic properties and structure by site-directed mutagenesis and mo-

lecular modeling studies. In Chapter 5, a tandem sequence duplication is shown as a genetic-based mechanism by which the thermal stability of a carboxyl-terminal modified enzyme was restored. In Chapters 6 and 7, the thermal stability of *B. subtilis* IPMDH was improved upon stepwise mutations that were generated either by the evolutionary engineering using *T. thermophilus* or by site-directed mutagenesis.

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Chapter 2: Further Stabilization of 3-Isopropylmalate Dehydrogenase of an Extreme Thermophile, *Thermus thermophilus*, by a Suppressor Mutation Method

ABSTRACT

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 Ala172→Val substitution in all of the mutants. The thermal stability of the thermophile IPMDH was improved by introducing the amino acid substitution.

INTRODUCTION

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 (Ahern et al., 1987; Matthews et al., 1987; Yutani et al., 1987; Matsumura et al., 1988; Nicholson et al., 1988; Pantoliano et al., 1988; Kuroki et al., 1989; Suzuki et al., 1989), 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) 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 yeast (Andreadis et al., 1984; Davidow et al., 1987; Hamasawa et al., 1987; Takagi et al., 1987; Sakai and Tani, 1992), bacilli (Imai et al., 1987; Sekiguchi et al., 1987), *Salmonella typhimurium* (Andreadis and Rosenthal, 1992), a cyanobacterium, *Spirulina platensis* (Bini et al., 1992), and two extreme thermophiles, *T. thermophilus* (Kagawa et al., 1984) and *T. aquaticus* (Kirino and Oshima, 1991). The enzyme from *T. thermophilus* has been purified and characterized (Yamada et al., 1990), and the crystal structure has been determined at 2.2 Å resolution (Imada et al., 1991). 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* (Numata et al., 1995). 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. Thermal stability of the chimeric enzyme was less than that of the thermophile enzyme (Numata et al., 1995). We have also developed an integration vector system that enables the expression of foreign genes in *T. thermophilus* (Tamakoshi et al., 1995). 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 (Tamakoshi et al., 1995).

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 (Hecht et al., 1985), staphylococcal nuclease (Shortle and Lin, 1985), and yeast iso-1-cytochrome C (Das et al., 1989). We also used this method in order to further stabilize the unusually stable enzyme from the extreme thermophile.

EXPERIMENTAL PROCEDURES

Reagents

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

Bacterial strains and media

The wild-type strain, *T. thermophilus* HB27 (Sakaki and Oshima, 1975), and the *leuB*-deficient strain, *T. thermophilus* MT106 (Tamakoshi et al., 1995), were cultured in *Thermus* nutrient medium (Oshima and Imahori, 1974) or *Thermus* minimum medium (Tanaka et al., 1981). *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^qZ Δ M15*)) 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 (Sambrook et al., 1989).

Construction of a randomly mutated DNA library

Experimental procedures are illustrated in Fig. 2-1. Plasmid pNOBL2 harbors a chimeric *leuB* gene that consists of parts of *T. thermophilus* and *B. subtilis leuB* genes (Numata et al., 1995). The plasmid was digested with *SacI* and *SaII*, and a fragment encoding the 3' region of the *T. thermophilus leuB* gene, covering 60% of the whole gene, was excised. This fragment (1 μ g) was dissolved in 500 μ l 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, pITI (Tamakoshi et al., 1995). 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 Koyame et al. (1986), with slight modifications (Takada et al., 1993). 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.

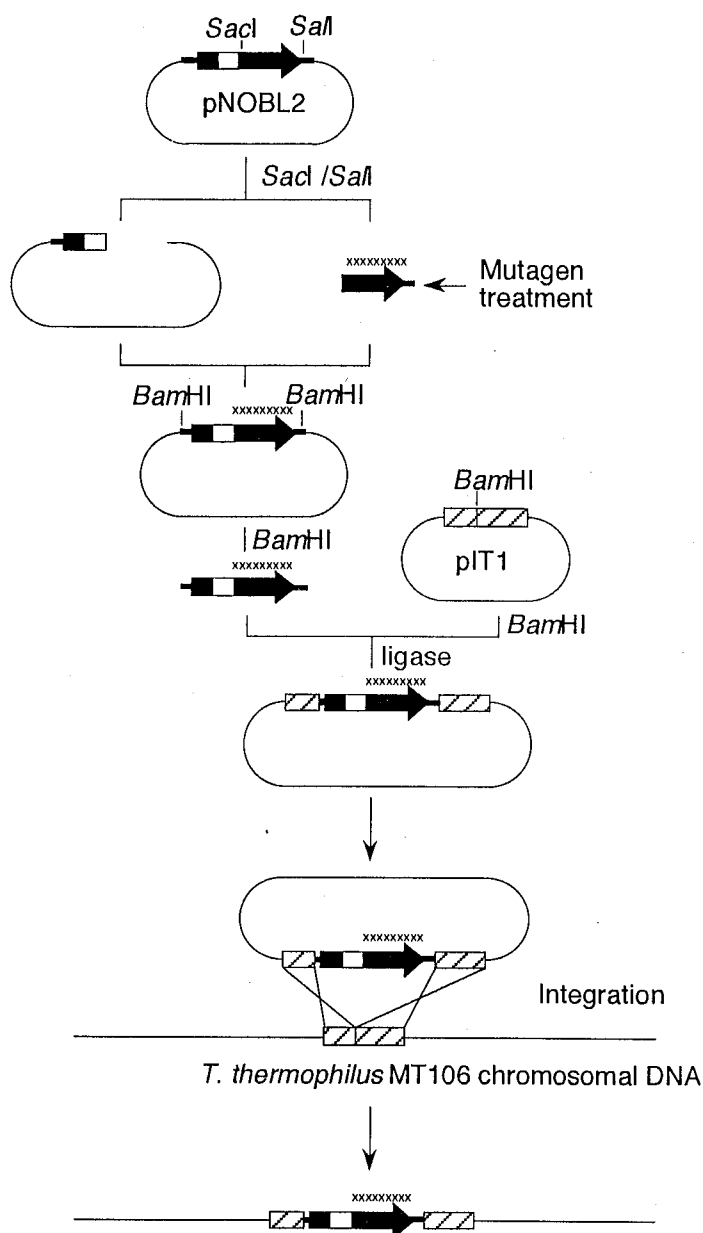


Figure 2-1. Construction of a randomly mutated DNA library and integration into *T. thermophilus*. Plasmid pNOBL2 was digested with *SacI* and *SalI*; the smaller fragment containing the 3' region of the *leuB* gene was treated with MNNG and ligated with the rest of the plasmid. The resulting plasmids 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* $\Delta leuB$ 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 $4,000\times g$ for 10 min, and the pellets were resuspended in 500 μ l 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 *Hind*III; 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 μ g/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 *Hind*III fragment was subcloned into the *Bam*HI site of pUC119 to prepare sequencing templates. Sequence was determined by the dideoxy-chain termination method (Sanger et al., 1977) 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 the genes encoding the mutant enzymes

For production of the mutant of the chimeric enzyme, the chimeric *leuB* gene containing the base substitution which results in an Ala172→Val replacement in the amino acid sequence was cloned into pUC119 as described by Numata et al. (1995). The base substitution was introduced into the *T. thermophilus leuB* gene by replacing the *Sad*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

Enzymatic activity was estimated from the increase in the rate of the absorbance at 340 nm in 100 mM potassium phosphate buffer (pH 7.6) containing 1 M KCl, 0.2 mM MnCl₂, 0.8 mM NAD, and 0.4 mM DL-IPM as described by Yamada et al. (1990).

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 μ g/ml). Cells were harvested and disrupted by sonication, and soluble fractions were obtained by centrifugation at $60,000\times g$ 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 $60,000\times g$ for 20 min. Each enzyme was purified from the supernatant by using columns of butyl-Toyopearl (Tosoh) and Mono-Q (Pharmacia) successively (Yamada et al., 1990). 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, pH7.6), heat-treated at various temperatures, and immediately chilled on ice for 10 min. The chilled samples were centrifuged at $15,000\times g$ for 10 min, and the remaining activity of the supernatants was measured at 50°C for the chimeric IPMDH and its mutant, or 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

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* JM109 and used as a randomly mutated DNA library (Fig. 2-1). 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 $\Delta leuB$ strain MT106 from *T. thermophilus* HB27 by gene disruption (Tamakoshi et al., 1995). We have also demonstrated the integration of the chimeric *leuB* gene into the chromosomal DNA of *T. thermophilus* MT106 by using the integration vector (Tamakoshi et al., 1995). 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 2-1). We transformed *T. thermophilus*

Table 2-1. Growth of *T. thermophilus* strains in minimum medium without leucine^a

<i>T. thermophilus</i> strain	Growth at:	
	70°C	76°C
HB27 (wild-type)	+	+
MT106 ($\Delta leuB$)	-	-
NB26 (chimeric <i>leuB</i>)	+	-
NK101 (heat resistant)	+	+
NK102 (heat resistant)	+	+
NK103 (heat resistant)	+	+

^aEach strain was plated on minimum medium without leucine and incubated for 36 h at the indicated temperatures.

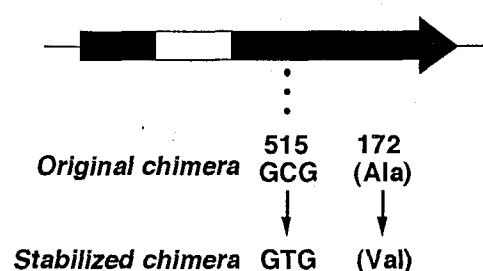


Figure 2-2. Sequence of the *leuB* gene encoding the mutant of 2T2M6T. DNA sequencing showed that the chimeric *leuB* genes from the thermostabilized mutants had thymine at 515 instead of cytosine. This mutation causes an Ala172→Val amino acid replacement.

MT106 with the randomly mutated DNA library (Fig. 2-1), 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 2-1).

The *leuB* gene was cloned from NK101 and sequenced. The sequencing revealed that the gene had only a single base replacement, C515→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 Ala172→Val amino acid replacement (Fig. 2-2).

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 (C515→T). The results suggest that the base point C515 is a hot spot of the MNNG treatment. Alternatively, the mutation Ala172→Val may be the only mutation that can improve the thermal stability of the chimeric enzyme. We have analyzed these possibilities and the results will appear in Chapter 3.

Thermal stability of 2T2M6T and its mutant

To examine the thermal stability of the chimeric enzyme with the Ala172→Val substitution, the mutant enzyme, 2T2M6T-A172V, was purified and the thermal properties were compared with those of the original enzyme, 2T2M6T. Figure 2-3A shows the activities of the enzymes after the 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. 2-4A. 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 (Imada et al., 1991). 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 (Iwasaki et al., 1996). 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 Ala172→Val substitution increased the thermal stability of the chimeric enzyme.

Improvement of the heat resistance of the thermophile enzyme

The amino acid substitution Ala172→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. 2-3B. 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. 2-4B). In contrast to the chimeric enzymes, which showed two peaks in the thermogram under the condition used (Fig. 2-4A), 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°C) was slightly higher than that of the

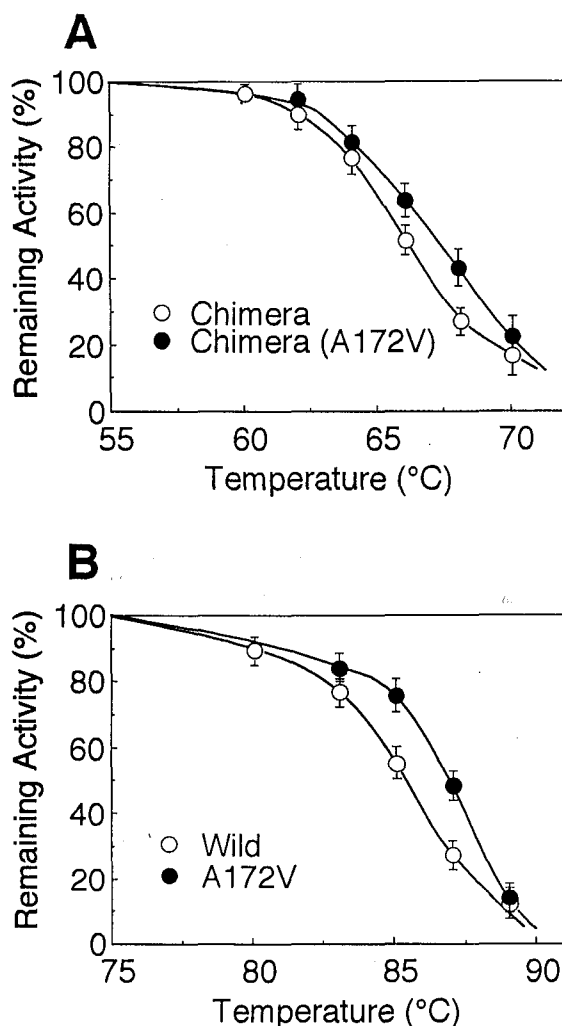


Figure 2-3. Activities of IPMDHs after heat treatment. (A) Thermal stabilities of the chimeric enzymes; (B) thermal stabilities of the wild-type and mutant enzymes. Each enzyme was purified, diluted to 0.1 mg/ml with 20 mM potassium phosphate (pH 7.6) containing 0.5 mM EDTA, and treated at the indicated temperatures for 10 min. The remaining activity was expressed as a percentage of the original activity.

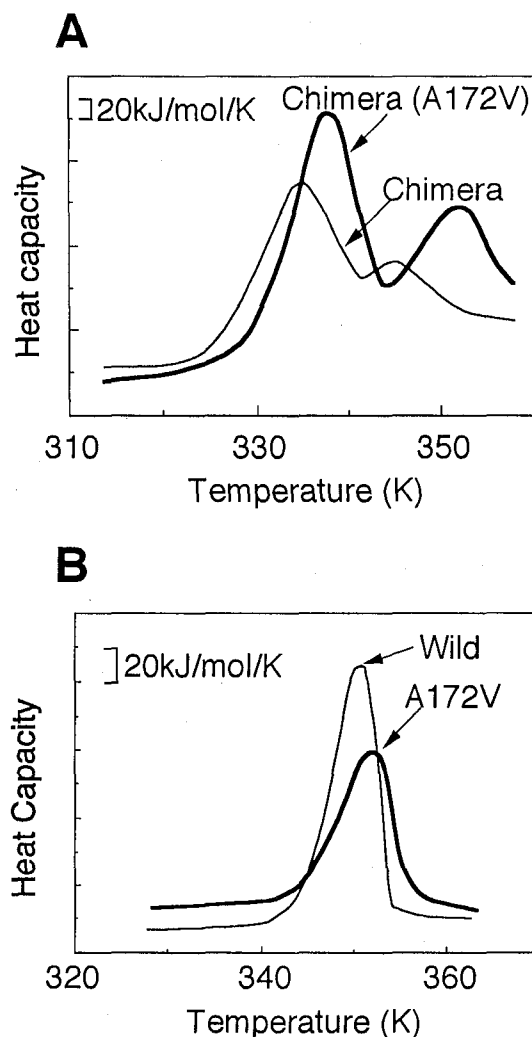


Figure 2-4. DSC curves for the chimeric IPMDHs (A) and for *T. thermophilus* wild-type and mutant enzymes (B). The protein concentration was 1.0 mg/ml in 20 mM NaHCO₃ (pH 10.2). The rate of temperature increase was 0.5°C/min.

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 2-2. While the Michaelis constant (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 Ala172→Val substitution. These results showed that the Ala172→Val substitution enhanced the thermal stability of the enzyme without significant changing its catalytic properties.

Table 2-2. Kinetic constants of *T. thermophilus* wild-type and mutant enzymes^a

Enzyme	K_m (μ M)		k_{cat} (s^{-1}) ^b
	D-3-IPM	NAD	
Wild-type	31 ($\pm$ 4)	130 ($\pm$ 23)	163 ($\pm$ 22)
A172V	22 ($\pm$ 6)	130 ($\pm$ 23)	220 ($\pm$ 25)

^aThe initial rates were measured at 60°C in the presence of various concentrations of D-3-IPM and NAD.

^b k_{cat} values are expressed as reaction per dimer.

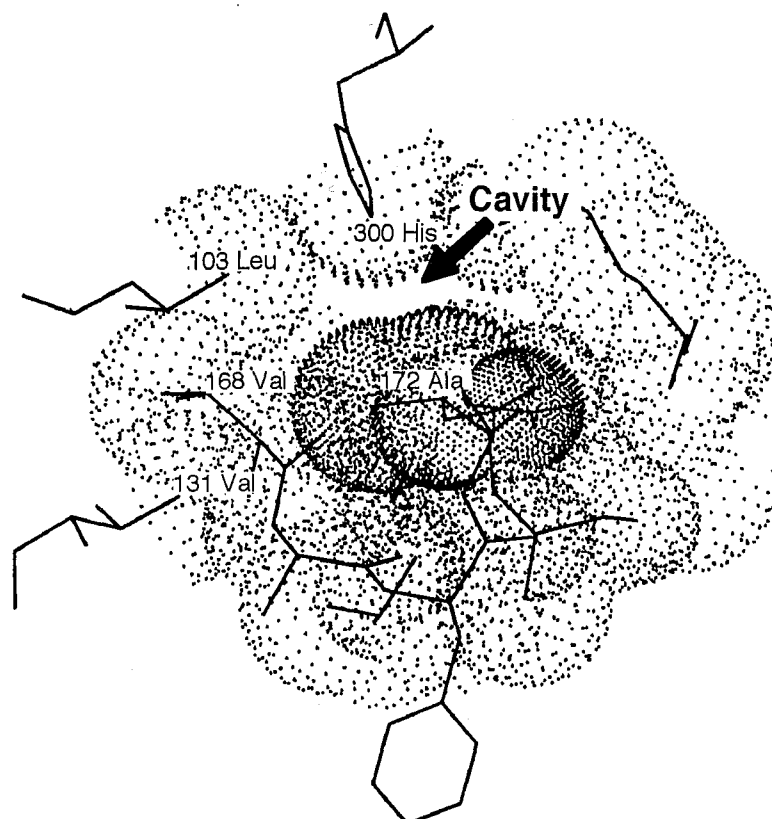


Figure 2-5. The cavity around residue 172 of *T. thermophilus* IPMDH. The shadows represent the van der Waals radii of side chains of the enzyme. The drawing was rendered for the Silicon Graphics Indigo with the program QUANTA (Molecular Simulations Inc.).

DISCUSSION

According to the three-dimensional structure determined by X-ray crystallography (Imada et al., 1991), *T. thermophilus* IPMDH is a homodimer and each subunit consists of two domains. Ala172 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 (Onodera et al., 1994). In both enzymes, the methyl side chain of Ala172 points to the hydrophobic core and is surrounded by hydrophobic residues such as Leu103, Val131 and Val168. A small cavity is, however, detected around this side chain (Fig. 2-5). Computer analysis revealed that the cavity volume illustrated in Fig. 2-5 was reduced by 25 Å³ upon the substitution of Ala172 to 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 (Yutani et al., 1987; Kellis et al., 1989; Matsumura et al., 1989). Combination of hydrophobic interaction and packing effect has also been discussed (Sandberg and Terwilliger, 1989).

The presence of a cavity inside the hydrophobic core has been searched for by computer-aided analyses (Tomuro et al., unpublished). However, the gap around Ala172 shown in Fig. 2-5 was not detected. In other words, the present random mutagenesis study found the small gap that could not be detected by the computer analysis.

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.

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Chapter 3: Multiple Mutations at a Single Position Increasing Thermal Stability of 3-Isopropylmalate Dehydrogenase

ABSTRACT

We succeeded in thermostabilization of a chimeric and a thermophilic 3-isopropylmalate dehydrogenases. The chimeric enzyme was previously constructed by combining parts of the enzymes from *Bacillus subtilis* and an extreme thermophile, *Thermus thermophilus*. Thermal stability of the chimeric enzyme is less than that of the *T. thermophilus* enzyme. A mutant strain of *T. thermophilus* which carries the chimeric *leuB* gene in its chromosome shows a temperature-sensitive growth phenotype under conditions lacking leucine. Eleven phenotypically thermostabilized variants evolved from the mutant strain contained either single-base replacement corresponding to Ile93→Leu or Ala172→Val substitution in its chimeric *leuB* gene. In order to induce other types of stabilization, the gene encoding a mutated chimeric enzyme, in which Ile93 and Ala172 were replaced by Ala and Ser, respectively, was constructed by site-directed mutagenesis. The directed molecular evolution using the mutated *leuB* gene produced a more thermostable variant that had Leu at position 172. Further stabilization of the inherent thermostable enzyme of *T. thermophilus* IPMDH was also achieved by changing Ala172 to more hydrophobic residues, such as Val, Ile, Leu and Phe.

INTRODUCTION

In the past decade, many workers have attempted to increase thermal stability of proteins and proposed several principles for protein stabilization (Ahern et al., 1987; Matthews et al., 1987; Yutani et al., 1987; Nicholson et al., 1988; Pantoliano et al., 1988; Kuroki et al., 1989; Matsumura et al., 1989a; Suzuki et al., 1989). There is, however, no general theory and we are still far from the comprehensive understanding of structure-stability relationship of proteins.

Evolutionary molecular engineering (Eighen and Gardiner, 1984) is a powerful tool for generating thermostable proteins. Based on this strategy, we can obtain mutant proteins with enhanced thermostability in the absence of any structural information. The strategy consists of two steps: introduction of forced random or spontaneous mutations into a target gene; and selection of mutated genes that express products with a desired property under strongly selective pressures. The first successful example of evolutionary improvement of protein properties was the thermostabilization of kanamycin nucleotidyltransferase by Matsumura and Aiba (Matsumura and Aiba, 1985). In that study, the gene encoding the enzyme was isolated from a mesophile and treated with a chemical mutagen. Then, the mutated gene encoding the stabilized enzyme was screened in a moderate thermophile, *Bacillus stearothermophilus*. Liao et al. (1986) also improved the thermal stability of the same enzyme by *in vivo* evolution using a transformed *B. stearothermophilus*.

3-Isopropylmalate dehydrogenase (IPMDH, EC 1, 1, 1, 85) is involved in leucine biosynthesis. The *leuB* gene encoding IPMDH of an extreme thermophile, *Thermus thermophilus* HB8, has been cloned and sequenced (Tanaka et al., 1981; Kagawa et al., 1984; Kirino et al., 1994). The enzyme from *T. thermophilus* (designated as 10T) has been purified and characterized

(Yamada et al., 1990). Its crystal structure has been determined at 2.2 Å resolution (Imada et al., 1991); the enzyme is a homodimer with its subunit consisting of 345 amino acid residues and each subunit can be divided into two domains. In addition, several chimeric enzymes have been constructed by combining the genes encoding the enzymes of a mesophile, *Bacillus subtilis*, and *T. thermophilus* (Numata et al., 1995). One of such chimeric enzymes, designated as 2T2M6T, was designed to have about 20% portions of the sequence (residues 74 to 133) from the enzyme of the mesophile, and the rest from the thermophilic enzyme. The chimeric enzyme has also been well characterized in terms of its function, stability and structure (Onodera et al., 1994; Numata et al., 1995).

Previously, the host-vector system for integration and expression of foreign genes in the *leu* operon of *T. thermophilus* was developed (Tamakoshi et al., 1995). Using this system, the chimeric *leuB* gene was integrated into *T. thermophilus* HB27 and two types of stabilized 2T2M6T were obtained by screening spontaneous mutations (Tamakoshi et al., 1995) or random point mutations induced with a chemical mutagen (Chapter 2). The first example had Leu instead of Ile at position 93 which was on the portion derived from the mesophile enzyme, while the second one had Val instead of Ala at position 172 which was from the thermophile enzyme. The thermal stability of the unusually thermostable IPMDH from *T. thermophilus* was also improved by changing Ala at position 172 to Val (Chapter 2). In this study, we further attempted to improve the thermal stability of the chimeric and thermophile IPMDHs by a similar evolutionary modification technique using the integration vector system for *T. thermophilus*.

EXPERIMENTAL PROCEDURES

Reagent

A mutagen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), was purchased from Sigma chemical Co., Ltd. Restriction and DNA modification enzymes were obtained from Takara shuzo Co., Ltd. or Toyobo Co., Ltd. DL-3-isopropylmalate (DL-3-IPM), a substrate of IPMDH, and coenzyme NAD were obtained from Wako Pure Chemical Industries, Ltd. and Oriental Yeast Co., Ltd., respectively.

Bacterial strains and media

A *leuB*-deficient strain *T. thermophilus* MT106 (Tamakoshi et al., 1995) was used as the host for integration of mutated *leuB* genes into its chromosomal DNA. *T. thermophilus* NB26 was derived from *T. thermophilus* MT106 by integration of the original chimeric *leuB* gene (Tamakoshi et al., 1995). These strains were grown in *Thermus* nutrient medium (Oshima and Imahori, 1974) or *Thermus* minimum medium (Sakaki and Oshima, 1975). Solid media contained 1.0% Gelrite (Wako Pure Chemical Industries, Ltd.). *Escherichia coli* strain JA221(*F*⁺, *hsdR*, Δ *trpE5*, *leuB6*, *lacY*, *recA1*) was used as the host for expression of the *leuB* genes and was cultured in 2YT medium (Sambrook et al., 1989) supplemented with ampicillin (150 µg/ml).

Isolation of thermostable variants by *in vivo* evolution

The mutant strain of *T. thermophilus* NB26, which carried the chimeric *leuB* gene within its chromosomal DNA, was pre-cultured in nutrient medium at 70°C for 12 hr. The culture (containing 10⁷ cells) was centrifuged at 5,000×*g*, the cell pellet was resuspended in 100 µl of sterilized water, and transferred to 10 test tubes of minimum medium containing MNNG (100 µg/ml). After a 3-day incubation at 76°C, growth was observed in four of the ten test tubes. The cultures were then spread on minimum medium plates and four independent colonies grown up at 76°C were isolated from the plates after another 3-day incubation.

Error-prone PCR and screening

Randomly mutated chimeric *leuB* genes were obtained by error-prone PCR according to the method of Leung et al. (1989). They have reported that the method produces a point mutation rate of 2.0%. Synthetic oligonucleotides, 5'AT CGG GGA GTC TTC TGG AGG CC3' and 5'TCA TAT CCC CAT CTT AGG CGA GGT3', were used as specific PCR primers for the *leuB* gene. The PCR products of the chimeric *leuB* gene, which should contain random base replacements, were used to transform *T. thermophilus* NB26 without ligating to the integration vector. The transformation was done as described by Koyama et al. (1986). The transformed cells were screened at 76°C on minimum medium plates without leucine and seven strains with phenotypically improved thermal stability were isolated after a 3-day incubation.

Other DNA manipulations

Recombinant DNA experiments were performed by standard methods (Sambrook et al., 1989). The nucleotide sequences of the mutant *leuB* genes were determined by dideoxy chain termination method (Sanger et al., 1977).

Some mutations of the *leuB* genes were generated by oligonucleotide-directed base substitutions according to the method of Kunkel et al. (1987). Oligonucleotides used for generating the mutations were 5'GG CTG CTC AGC GCT AGA AAA CAG CTT GA3' (Ile93→Ala), 5'CGG GTG GCC TTC GAA TCG GCG AGG AAG3' (Ala172→Ser), 5'CTG CGC TTC CTG GCG CCC TCA AAG GCC A3' (Ala172→Gly), 5'CG CTT CCT CGC GAT TTC GAA GGC CAC CCG3' (Ala172→Ile), and 5'CGC TTC CTC GCG AAT TCA AAG GCC ACC3' (Ala172→Phe). The mutations in the gene were screened by testing the digestion patterns with the restriction endonucleases, *Aor*51HI (Ile93→Ala), *Nsp*V (Ala172→Ser, Ile), *Nar*I (Ala172→Gly) and *Eco*RI (Ala172→Phe), and then confirmed by nucleotide sequencing. Single mutant enzymes designated as I93A, A172S and the like, respectively, represent those in which Ile93 is substituted to Ala, Ala172 is substituted to Ser, and such. For designation of double mutant enzymes, the two mutations within a single molecule are indicated with a slash, as like I93A/A172S.

Integration of mutated *leuB* gene into *T. thermophilus*

The 1.2-kb *Bam*HI fragment containing the chimeric *leuB* gene with mutations that corresponded to Ile93→Ala and Ala172→Ser was inserted into the integration vector pIT1 for *T. thermophilus*. The resulting plasmid was used to transform *T. thermophilus* MT106 by the method of Koyama et al. (1986). The transformed cells were screened at 57°C and two leucine autotrophic strains, RB101 and RB102, were isolated after a 6-day incubation. The strain RB101 was subjected to the evolutionary experiment to improve the thermal stability of the mutated chimeric enzyme.

Construction of the genes encoding the mutant enzymes

To produce the chimeric enzyme variants, the *leuB* genes encoding 2T2M6T-A172S, A172L, I93A/A172S, I93V/A172S, and I93V/A172L were cloned into pUC119 according to Numata et al. (1995). The base substitution corresponding to Ala172→Leu was introduced into the *T. thermophilus leuB* gene by replacing the *Aor*51HI-*Eco*RI fragment of the *T. thermophilus leuB* gene, which was inserted at the *Bam*HI site of pUC119, with the corresponding fragment of the chimeric genes encoding the mutant form, 2T2M6T-A172L. The *leuB* genes encoding the thermophilic enzyme variants, 10T-A172G, A172I and A172F were constructed by site-directed mutagenesis as described above. All of the resulting plasmids were used for overexpression of the genes encoding these enzymes.

Thermal stability measurement

T. thermophilus wild-type, chimeric and mutant IPMDHs were overproduced in *E. coli* JA221 harboring respective expression plasmids and purified as described by Yamada et al. (1990). Each enzyme was confirmed to be homogeneous as judged by SDS-gel electrophoresis. The thermal stabilities of the purified enzymes were determined by measuring the irreversible inactivation after heat treatment at various temperatures for 10 min as described in Chapter 2.

Thermal denaturation was measured with a JASCO J720C spectropolarimeter. To prevent aggregate formation after denaturation, the circular dichroism (CD) measurements were carried out in an alkaline buffer. The enzymes were dissolved in 20 mM NaHCO₃ (pH 10.9) and the enzyme concentration was 0.2 mg/ml. The temperature of the enzyme solution was controlled with a circulating bath and a programmable temperature controller. The precise temperature was monitored with a thermocouple and scan rate was 1.0°C/min. The thermal denaturation curves were normalized as described by Iwasaki et al. (1996).

Kinetic experiments

The kinetic parameters, Michaelis constant (K_m) and catalytic constant (k_{cat}), for D-3-IPM and NAD were determined in steady state experiments as described in Chapter 2.

RESULTS

Variants with improved thermal stability obtained by evolutionary experiments

Previously, the $\Delta leuB$ strain of *T. thermophilus* MT106 was constructed from the wild-type strain HB27 by gene disruption (Tamakoshi et al., 1995). The mutant strain *T. thermophilus* NB26 was obtained by introducing the chimeric *leuB* gene into the chromosomal DNA of *T. thermophilus* MT106 by using the integration vector system (Tamakoshi et al., 1995). Strain NB26 showed leucine-autotrophy at 70°C but not at 76°C (Tamakoshi et al., 1995) while the wild-type strain of *T. thermophilus* HB27 grew at 76°C under conditions lacking leucine (Table 3-1). Eleven phenotypically thermostabilized strains, which could grow at 76°C without leucine, were obtained either by *in vivo* evolution promoted with the mutagen, MNNG, or by error-prone PCR followed by phenotype selection (Table 3-1). The *leuB* genes were cloned from these

Table 3-1. Growth of *T. thermophilus* mutants in minimum medium

<i>T. thermophilus</i> strain	Growth ^a at:			
	57°C	60°C	70°C	76°C
HB27 (Wild-type)	+	+	+	+
MT106 ($\Delta leuB$)	-	-	-	-
NB26 (2T2M6T <i>leuB</i>)	+	+	+	-
SN7601 (Heat resistant)	+	+	+	+
SN7602 (Heat resistant)	+	+	+	+
SN7603 (Heat resistant)	+	+	+	+
SN7604 (Heat resistant)	+	+	+	+
PS112 (Heat resistant)	+	+	+	+
PS211 (Heat resistant)	+	+	+	+
PS212 (Heat resistant)	+	+	+	+
PS214 (Heat resistant)	+	+	+	+
PS322 (Heat resistant)	+	+	+	+
PS101 (Heat resistant)	+	+	+	+
PS201 (Heat resistant)	+	+	+	+
RB101 (2T2M6T-I93A/A172S <i>leuB</i>)	+	-	-	-
RB102 (2T2M6T-I93V/A172S <i>leuB</i>)	+	+	+	-
SN301 (Heat resistant)	+	+	+	+
SN302 (Heat resistant)	+	+	+	+

^aThe growth of each strain was scored after incubation for 72 h on a minimum medium-coated plate at the indicated temperatures.

Table 3-2. Mutations found from the evolutionary experiments ^a

<i>T. thermophilus</i> strain	Mutations in chimeric IPMDH
NB26	Original 2T2M6T
SN7601	Ala172→Val (GCG→GTG)
SN7602	Ala172→Val (GCG→GTG)
SN7603	Ala172→Val (GCG→GTG)
SN7604	Ala172→Val (GCG→GTG)
PS112	Ile93→Leu (ATC→CTC)
PS211	Ile93→Leu (ATC→CTC)
PS212	Ile93→Leu (ATC→CTC)
PS214	Ile93→Leu (ATC→CTC)
PS322	Ile93→Leu (ATC→CTC)
PS101	Ala172→Val (GCG→GTG)
PS201	Ala172→Val (GCG→GTG)
RB101	Ile93→Ala (ATC→GCT)
	Ala172→Ser (GCG→TCG)
RB102	Ile93→Ala→Val (ATC→GCT→GTT)
	Ala172→Ser (GCG→TCG)
SN301	Val93 (GTT)
	Ser172→Leu (TCG→TTG)
SN302	Val93 (GTT)
	Ser172→Leu (TCG→TTG)

^aCodons with mutation are shown in parentheses.

strains and sequenced. The DNA sequencing revealed two types of amino acid substitution, Ala172→Val (GCG→GTG) and Ile93→Leu (ATC→CTC) (Table 3-2). Both Ala172→Val and Ile93→Leu substitutions were generated through respective single-base replacements and were previously analyzed in detail (Chapter 2; Tamakoshi et al., 1995).

Experimental evolution of mutated 2T2M6T

Generally, single-base replacements occur more frequently than double- and triple-base replacements and, therefore, appearance of the mutants with a multiple-base replacement must be less frequent than that with a single-base replacement. To avoid the occurrence of amino acid substitutions Ile93→Leu and Ala172→Val through the single-base replacement, the chimeric *leuB* gene was mutated by site-directed mutagenesis so as to change Ile93 and Ala172 to Ala (GCT) and Ser (TCG), respectively, and the *leuB*-deficient strain of *T. thermophilus* MT106 was transformed with the mutated gene. Two leucine-autotrophic transformants, designated as RB101 and RB102, were isolated on the minimum medium plate after a 6-day incubation at 57°C. Unexpectedly, DNA sequencing revealed that the strain RB102 had Val (GTT) at position 93 instead of the introduced residue, Ala (GCT) or original one Ile (Table 3-2). This observation suggested that the introduction of Ala at position 93 of the chimeric enzyme so reduced its stability or/and activity at least *in vivo* that the strain harboring the *leuB* gene for the designed enzyme did not fully acquire the leucine autotrophy even at 57°C, thus indicating that this incubation screened the spontaneous mutant producing more functional enzyme at 57°C. Although the strain RB101 grew at 57°C in minimum medium without leucine (Table 3-1), the growth rate was significantly

slower than that of the strain RB102 (data not shown). The strain RB102 was confirmed to grow at the temperatures of up to 70°C on minimum medium plate without leucine (Table I) and we therefore used RB102 in the following experiment. Ten cultures of RB102 (containing 10^6 cells each) was transferred to 76°C, followed by a 3-day incubation in minimum medium containing MNNG. Growth was observed in two out of the ten test tubes and two phenotypically thermostable strains, SN301 and SN302, were isolated. The *leuB* genes were cloned from these strains and their sequences were determined, showing that Ser (TCG) at position 172, which had been introduced instead of Ala (GCG) by site-directed mutagenesis, was further substituted to Leu (TTG) in both mutant strains (Table 3-2).

Thermal stability of the mutant 2T2M6Ts

The chimeric *leuB* genes isolated from the mutant strains of *T. thermophilus* were over-expressed in *E. coli* and the products were purified to homogeneity. The thermal stabilities of the purified enzymes were estimated by measuring the activities of these enzymes after heat treatment for 10 min at various temperatures. Figure 3-1A shows that 2T2M6T-I93V/A172L, which is the product of the *leuB* gene from SN301, is more thermostable than the original chimeric enzyme, by 2.6°C in terms of the half inactivation temperature at which 50% inactivation is observed after heat treatment for 10 min. The stability of 2T2M6T-I93V/A172S is similar to that of the original enzyme. The variant 2T2M6T-I93A/A172S is less thermostable than 2T2M6T, but the decrease in half inactivation temperature is only 1.6°C. The mutant strain RB101, which harbors the gene encoding 2T2M6T-I93A/A172S, could not grow at 60°C although the mutant enzyme was sufficiently stable at 60°C *in vitro*, suggesting that factors other than the protein stability observed *in vitro* were involved in the phenotype of this strain; those factors probably included tolerance to

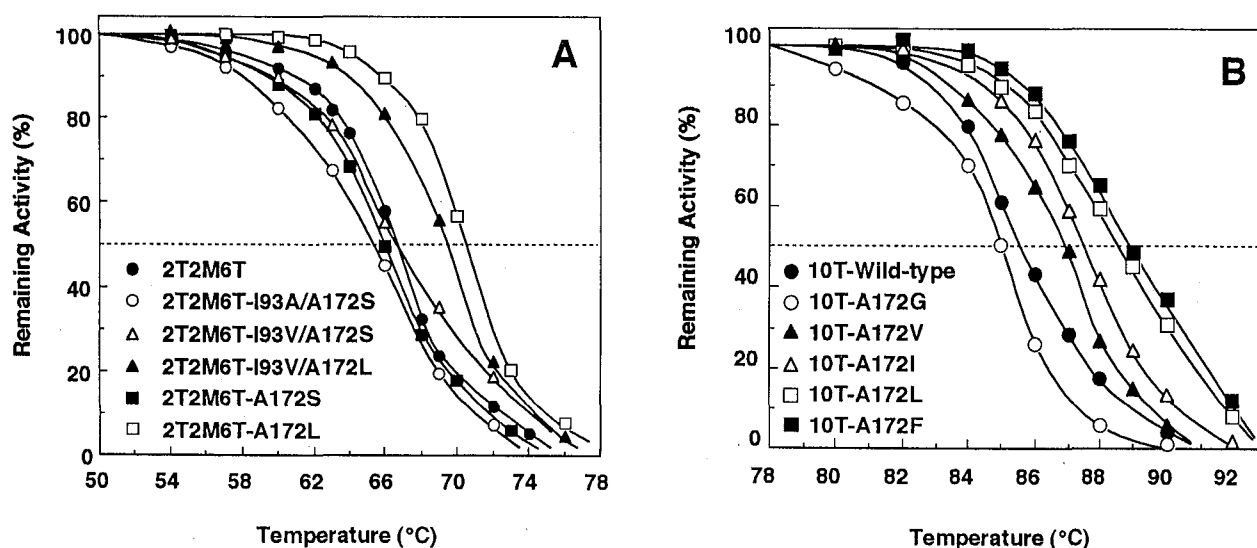


Figure 3-1. Activities of IPMDHs after heat treatment. (A) Thermal stabilities of the chimeric enzymes; (B) thermal stabilities of the thermophile wild-type enzyme and its variants. Each purified enzyme was diluted to 0.1 mg/ml with 20 mM potassium phosphate (pH 7.6) containing 0.5 mM EDTA and treated at the indicated temperatures for 10 min. The remaining activities are expressed as percentages of the original activities. Each value is a average of at least three determinations.

proteolysis and altered catalytic activity under the *in vivo* condition.

To examine the effect of the single mutations at position 172 on thermal stability of the chimeric enzyme, two variants with single mutation, 2T2M6T-A172S and 2T2M6T-A172L, were also purified and measured for their stabilities. Figure 3-1A shows that the single Ala172→Leu substitution increases the stability of the chimeric enzyme by 3.8°C. In contrast, the variant 2T2M6T-A172S shows the stability similar to the original 2T2M6T.

The kinetic parameters of the chimeric enzyme and its variants are listed in Table 3-3. The Ala172→Ser mutation slightly improves the K_m value for D-3-IPM. On the contrary, the K_m value of 2T2M6T-I93A/A172S for NAD is 1.5-fold higher than that of the original chimeric enzyme. The values of k_{cat} obtained for 2T2M6T-I93A/A172S and 2T2M6T-I93V/A172S are slightly decreased compared with the original 2T2M6T while a slight improvement of k_{cat} value is observed for two thermostable variants, 2T2M6T-I93V/A172L and 2T2M6T-A172L.

Table 3-3. Kinetic constants of chimeric IPMDHs

Enzyme	K_m (μ M) ^a		k_{cat} (s^{-1}) ^{a,b}
	D-3-IPM	NAD	
2T2M6T (Original chimera)	45.8 ($\pm$ 5.3)	176 ($\pm$ 25)	116 ($\pm$ 4)
2T2M6T-I93A/A172S	37.3 ($\pm$ 2.3)	273 ($\pm$ 9)	89 ($\pm$ 5)
2T2M6T-I93V/A172S	45.6 ($\pm$ 3.6)	177 ($\pm$ 10)	79 ($\pm$ 2)
2T2M6T-I93V/A172L	37.3 ($\pm$ 2.4)	217 ($\pm$ 19)	137 ($\pm$ 3)
2T2M6T-A172S	28.2 ($\pm$ 2.7)	193 ($\pm$ 22)	113 ($\pm$ 4)
2T2M6T-A172L	38.7 ($\pm$ 2.7)	227 ($\pm$ 28)	128 ($\pm$ 8)

^aThe kinetic constants were determined in steady-state experiments at 50°C in 100 mM potassium phosphate buffer containing 1 M KCl, 0.2 mM MnCl₂, and various concentrations of D-3-IPM and NAD. Each value is the average of three independent determinations. Standard deviations are shown in parentheses.

^b k_{cat} values are expressed as reaction per dimer.

Further stabilization of *T. thermophilus* IPMDH

Because the chimeric enzyme was indicated to be stabilized by the mutation at the position 172 which is in the segment derived from the thermophilic enzyme, a possibility arose that the thermophile enzyme was further stabilized by introducing a more hydrophobic residue at position 172. In Chapter 2, we demonstrated that the Ala172→Val substitution improved the thermal stability of *T. thermophilus* IPMDH. We therefore investigated whether Ala172→Ile, Leu, and Phe mutations also improved the stability of the thermophile enzyme. The mutant enzyme with Ala172→Gly substitution was also constructed for the comparison. The thermophile wild-type enzyme and its variants were purified and their thermal properties were compared in Fig. 3-1B, showing that 10T-A172I, 10T-A172L and 10T-A172F are more thermostable than the wild-type enzyme, by 2.0°C, 3.0°C and 3.3°C, respectively, as judged by the half inactivation temperature. It should be noted that these present variants are more stable against heat treatment than 10T-A172V which was obtained previously.

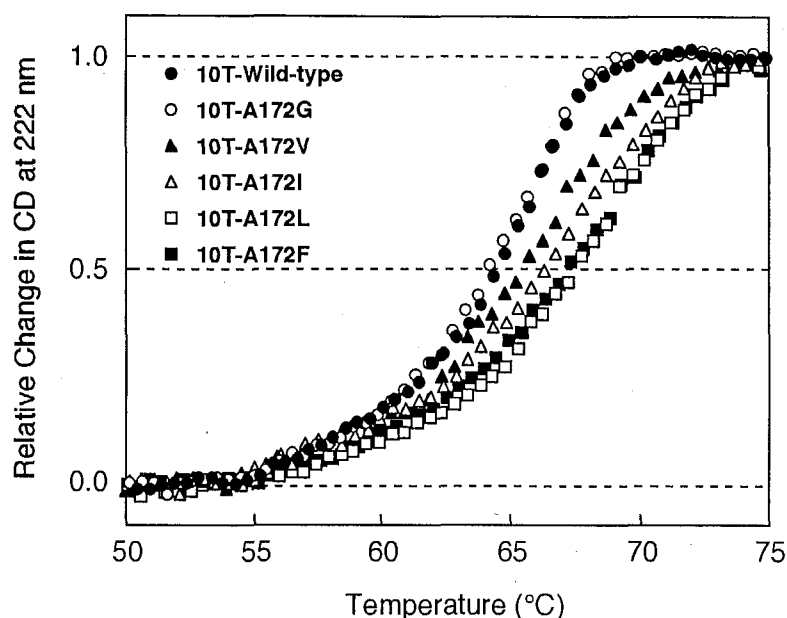


Figure 3-2. Thermal melting profiles of the thermophile IPMDHs. The denaturation was monitored by CD at 222 nm in 20 mM NaHCO₃ buffer (pH 10.9). The rate of temperature increase was 1.0°C/min. The melting curves were normalized with the baselines of the native and denatured states.

The thermal denaturation process was also analyzed by monitoring the change in CD at 222 nm. Figure 3-2 shows that all the mutations Ala-172→Val, Ile, Leu, and Phe increase the denaturation temperature of the enzyme. In contrast, the mutation Ala-172→Gly does not seriously affect the denaturation temperature of the enzyme. These results clearly show that the introduction of a residue with more hydrophobic side chain at position 172 further stabilizes the inherent thermostable enzyme as judged from the stability of the secondary structure.

The kinetic constants, K_m and k_{cat} , of each enzyme are summarized in Table 3-4. The largest change in K_m value is found with 10T-A172F, the value of the variant for NAD reduced to 0.6-fold that of the wild-type enzyme. On the other hand, k_{cat} value does not vary significantly among these enzymes. These results indicate that changing Ala-172 to Val, Ile, Leu and Phe improves the thermal stability of the thermophile enzyme without considerable loss of enzymatic activity.

Table 2-4. Kinetic constants of *T. thermophilus* IPMDH and its variants

Enzyme	K_m (μ M) ^a		k_{cat} (s ⁻¹) ^{a,b}
	D-3-IPM	NAD	
10T (Wild-type)	22.1 ($\pm$ 2.5)	116 ($\pm$ 26)	107 ($\pm$ 5)
10T-A172G	24.1 ($\pm$ 2.2)	93 ($\pm$ 20)	110 ($\pm$ 5)
10T-A172V	29.1 ($\pm$ 5.5)	122 ($\pm$ 8)	113 ($\pm$ 5)
10T-A172I	17.5 ($\pm$ 2.1)	120 ($\pm$ 27)	121 ($\pm$ 5)
10T-A172L	23.9 ($\pm$ 0.4)	100 ($\pm$ 24)	114 ($\pm$ 5)
10T-A172F	26.8 ($\pm$ 2.8)	66 ($\pm$ 15)	89 ($\pm$ 4)

^aThe kinetic constants were determined in steady-state experiments at 60°C in 100 mM potassium phosphate buffer containing 1 M KCl, 0.2 mM MnCl₂, and various concentrations of D-3-IPM and NAD. Each value is the average of three independent determinations. Standard deviations are shown in parentheses.

^b k_{cat} values are expressed as reaction per dimer.

DISCUSSION

Two basic methods have been reported to improve thermal stability of proteins. One is theoretical and only applicable to the proteins whose three dimensional structures have been determined at high resolution. The other way is to use evolutionary molecular engineering. The evolutionary methods do not require any structural information and give us the opportunity to obtain a stabilized mutant beyond our present knowledge. However, a problem is the fact that the evolutionary experiment produces mutant proteins with enhanced thermostability through only a single-nucleotide replacement. Although we analyzed eleven phenotypically thermostable strains which were evolved from *T. thermophilus* NB26, only two types of stabilizing mutation, Ile93→Leu and Ala172→Val, were found. It has already been observed that the thermal stability of the chimeric IPMDH was improved by several amino acid substitutions such as Ser82→Arg (Sakurai et al., 1995) and Asn125→Gly (Numata *et al.*, unpublished result) as well as Ile93→Leu and Ala172→Val. Nevertheless, we have failed to obtain these types of thermostable variant in this study. Whereas both Ile93→Leu and Ala172→Val substitutions are generated through respective single-base replacements, the other substitutions require respective two continuous nucleotide replacements within the codon. These results indicate that the evolutionary experiment produces variants with double-base replacements within a codon seriously less frequently than that with a single-base replacement.

We can expect that changing the codon to the one for some other amino acid which is not so much responsible for the stability of the enzyme will provide us with a chance to select stabilizing mutations other than that we have already known. In the present study, we changed the residues at 93 and 172 of the chimeric enzyme to Ala and Ser, respectively. Unexpectedly, two additional mutations were found from our evolutionary experiment. First mutation was found at position 93; we found Val at position 93 instead of the introduced residue of Ala, or the original one of Ile. The second mutation was found at position 172; the introduced residue Ser was further replaced by Leu. Thermal stability of the present variant, 2T2M6T-A172L, is higher than that of the previously obtained variant, 2T2M6T-A172V. The substitution Ser→Leu was generated through the single-base replacement. Thermal stability of 2T2M6T-A172S was similar to that of the original 2T2M6T and therefore mutation Ala172→Ser can be called a “neutral mutation”. We therefore suggest that introduction of neutral mutations prior to induce random nucleotide substitutions facilitates to select thermostable variants with an amino acid substitution that results from two contiguous base replacements within the same codon.

According to the three dimensional structure of *T. thermophilus* IPMDH determined by X-ray crystallography (Imada et al., 1991), Ala172 is in the interface between two domains and the methyl side chain of the residue is surrounded by hydrophobic residues such as Leu103, Leu129, Val131, Val169, and Val232. However, a small gap is recognized around the methyl group. The bulky side chains of hydrophobic amino acid residues generally contribute to protein stability through hydrophobic interactions (Dill, 1990; Sharp, 1991). So far, several proteins have been thermostabilized by increasing the intramolecular hydrophobicity (Kellis et al., 1988; Matsumura et al., 1988; Kellis et al., 1989; Matsumura et al., 1989b; Sandberg and Terwilliger, 1989;

Shortle et al., 1990; Lim and Sauer, 1991; Sandberg and Terwilliger, 1991; Ishikawa et al., 1993). Pace (1992) has reported that a single methylene group buried in the intramolecule contributes about 1.3 kcal/mol to the protein stability. We previously proposed that changing Ala172 to Val filled up the gap, which were observed in the wild-type enzyme, without significant change of the backbone structure and therefore reduced free energy attributable to the intramolecular hydrophobicity of the enzyme (Chapter 2).

The side chain of Leu is a little too large for those gaps mentioned above and, therefore, the replacement with Leu is likely to enlarge the hydrophobic core of the relevant domain, hence such a replacement may rather bring about a substantial conformation alteration. Very recently, the crystal structure of 10T-A172L was determined, showing that both the hydrophobic cavity and the cleft between the two domains were extended compared to those of the wild-type enzyme.

The small-angle X-ray scattering analysis of a liganded structure of the thermophile wild-type enzyme with its substrate, D-3-IPM, in solution (Kadono et al., 1995) suggested that the substrate bound to the enzyme induced a small conformational change that closed the active site by reorientation of the two domains. The crystal structure of the thermostable variant 10T-A172L shows a similar domain movement. The conformation change induces the reduction of the solvent accessible surface area of hydrophobic side chains and formation of new hydrogen bonds between the domains, thereby increasing the thermal stability of the enzyme.

Because aromatic ring of Phe is also too large to fill up the gaps without significant steric rearrangement, it can readily be inferred that the Ala172→Phe substitution, analogous to Ala172→Leu, changes the conformation to the closed form and stabilizes the thermophile enzyme. On the other hand, Ile has β -branched side chain as Val does, but the number of the methylene group is equal to Leu. In addition, 10T-A172I comes in-between 10T-A172V and 10T-A172L with respect to thermostability. It is, therefore, of interest to understand how the alanine-to-isoleucine substitution improves the stability of the thermophile enzyme. The two thermostable variants, 10T-A172I and 10T-A172F, have been under the X-ray crystallographic analyses and the results will be published elsewhere.

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Chapter 4: Effect of Polar Side Chains at Position 172 on Thermal Stability of 3-Isopropylmalate Dehydrogenase from *Thermus thermophilus*

ABSTRACT

To understand the role of the amino acid residue at position 172 in the conformational stability, four mutant enzymes of *Thermus thermophilus* 3-isopropylmalate dehydrogenase in which Ala172 was replaced with Asp, Glu, Asn, and Gln were prepared by site-directed mutagenesis. Three mutants were more stable than the wild-type enzyme. No significant change in catalytic properties was found in the mutant enzymes. The molecular modeling studies suggested that the enhanced thermostability of the mutant enzymes resulted from formation of extra electrostatic interactions and/or improvement of hydrophobic packing of the interior core.

INTRODUCTION

Improvement of protein thermostability is one of the important topics of protein engineering. Several strategies have been proposed to increase the thermostability of proteins: for examples, by introducing disulfide bonds or metal binding site to reduce the difference in entropy between native and denatured states (Matthews et al., 1987; Pantoliano et al., 1988; Kuroki et al., 1989; Matsu-mura et al., 1989); changing specific residues to stabilize the dipoles of α -helices (Nicholson et al., 1988); improving intramolecular hydrophobic packing (Yutani et al., 1987); or reducing the solvent accessible hydrophobic surface area (Wigley et al., 1987). However, these strategies are only applicable to the proteins of which three-dimensional structures are known at high resolution. Even when such structural information is available, application of these strategies does not always improve the protein stability. To establish the general principles of molecular design for protein stabilization, more information is required with respect to structure-stability relationship of proteins.

3-Isopropylmalate dehydrogenase (IPMDH, EC 1, 1, 1, 85), a product of the *leuB* gene, is an enzyme involved in leucine biosynthesis. The *leuB* genes have been cloned from a variety of microorganisms including an extreme thermophile, *Thermus thermophilus* HB8 (Tanaka et al., 1981). The unusually thermostable enzyme of the thermophile IPMDH has been purified and characterized (Yamada et al., 1990). The three-dimensional structure of the enzyme has been determined at 2.2 Å resolution by X-ray diffraction (Imada et al., 1991), showing that the enzyme is a dimeric protein composed of two identical subunits, each consisting of 345 amino acid residues. The subunit can be divided into two structural domains; domain 1 consists of residues 1-99 and 255-345, including N- and C- termini, and domain 2 is involved in the subunit interaction.

Previously, we have further stabilized the thermostable enzyme of *T. thermophilus* IPMDH by the combination of a suppressor mutation method and an evolutionary modification technique using *T. thermophilus* as a host cell (Chapters 2, 3). In these previous studies, we found that single amino acid replacements of Ala172 with more hydrophobic residues, such as Val, Ile, Leu and Phe, contribute to increase in thermal stability of the thermophilic enzyme. X-ray crystallographic analyses of the stabilized variants have suggested that the substitutions of Ala172 to Val and to Leu

improve the stability of the enzyme through different molecular mechanisms: the Ala-to-Val substitution fills up the cavity around the mutated site without apparent change of the backbone structure (Qu, 1997); the Ala-to-Leu substitution contributes to the enhanced stability through inducing a domain movement similar to a ligand-induced conformation change that closes the active site (Qu et al., 1997).

In this chapter, we constructed additional mutant enzymes of *T. thermophilus* IPMDH by site-directed mutagenesis, in which Ala172 was replaced with polar residues. In contrast to our expectation, three mutant enzymes, in which Ala172 was changed to Glu, Gln, and Asn, were more thermostable than the wild-type enzyme. Based on the modeled structures of these mutant enzymes, we proposed the mechanisms for increase in thermostability, which were involving electrostatic and/or hydrophobic interactions.

EXPERIMENTAL PROCEDURES

Construction of mutant enzymes

Restriction- and modification-enzymes used in this study were purchased from Takara Shuzo, Toyobo or New England Biolabs. *Escherichia coli* JA221 (*F*⁺ *hsdR*, Δ *trpE5*, *leuB6*, *lacY*, *recA1*) was used for DNA amplification and expression of mutant *leuB* genes of *T. thermophilus* HB8. Site-directed mutagenesis was carried out according to the method of Kunkel et al. (1987). Oligonucleotides used for generating mutations were 5'GCT TCC TCG CCT CTT CGA AGG CCA CCC G3' for substituting Ala172 to Glu, 5'CTT CCT GCG CTT GCG CGC ATC CTC AAA GGC CA3' for substituting Ala172 to Asp, 5'GCT TCC TCG CCT GTT CGA AGG CCA CCC G3' for substituting Ala172 to Gln and 5'CTT CCT GCG CTT GCG CGC GTT CTC AAA GGC CAC3' for substituting Ala172 to Asn. Mutations in the gene were confirmed by nucleotide sequencing (Sanger et al., 1977). Expression and purification of the wild-type and mutant IPMDHs were done as described previously (Yamada et al., 1990). All the enzymes used in this study were purified to homogeneity as judged by SDS-polyacrylamide gel electrophoresis. Mutant enzymes designated as A172E, A172D, A172Q and A172N represent those in which Ala172 is substituted to Glu, Asp, Gln and Asn, respectively.

Thermal stability measurement

The stability against thermal inactivation of the purified enzymes was determined as described in Chapter 2.

Thermal denaturation was measured with a JASCO J720C spectropolarimeter. To prevent aggregate formation after denaturation, the circular dichroism (CD) measurements were carried out in an alkaline buffer as described in Chapter 3.

Kinetic experiments

The kinetic parameters, K_m and k_{cat} for D-3-isopropylmalate (D-3-IPM), a substrate of IPMDH, and for coenzyme NAD were determined in steady state experiments at 60°C as described in Chapter 2.

Modeling studies

Molecular modeling was carried out on Silicon Graphics Indigo 2 with program QUANTA (Molecular Simulation Inc.) by replacing Ala172 of *T. thermophilus* IPMDH with Gln and Asn. Then, the mutant structures were energy-minimized using the CHARMM procedure (Brooks et al., 1983), under the conditions in which all the atoms except those within 6 Å from the mutation site were fixed. The modeled structures of A172E and A172D were recently built by Qu (Qu, 1997).

RESULTS

Thermal stabilities of mutant IPMDHs

Previously, we successfully stabilized the thermophilic IPMDH by replacing Ala172 with more hydrophobic residues (Chapter 2, 3). According to the three dimensional structure of the wild-type enzyme determined by X-ray crystallography (Imada et al., 1991), the Ala172 residue is on an α -helix and its side chain is buried in a hydrophobic core. In addition to several hydrophobic residues, two basic residues of Lys175 and His300 are surrounding the methyl side chain of Ala172. Therefore, we examined the effect of introducing acidic residues at the position 172 on the thermal stability of the *T. thermophilus* enzyme. Two mutant enzymes, A172E and A172D, were constructed by site-directed mutagenesis and their thermal properties were compared with that of the wild-type enzyme. The remaining activities of these enzymes after the heat treatment at various temperatures are shown in Figure 4-1A, showing that A172E is more stable than the wild-type enzyme by 1.0°C in terms of the half inactivation temperature ($T_{1/2}$) at which the 50 % inactivation is observed after the heat treatment for 10 min. In contrast, it is also indicated that A172D is more labile than the wild-type enzyme.

For further comparison, additional two mutant enzymes A172N and A172Q were prepared and their thermal stabilities were also determined. The results are shown in Figure 4-1A. Both A172N and A172Q are more resistant against heat treatment than the wild-type enzyme: $T_{1/2}$ s of A172N and A172Q are higher than that of the wild-type enzyme by 1.5°C and 2.3°C, respectively.

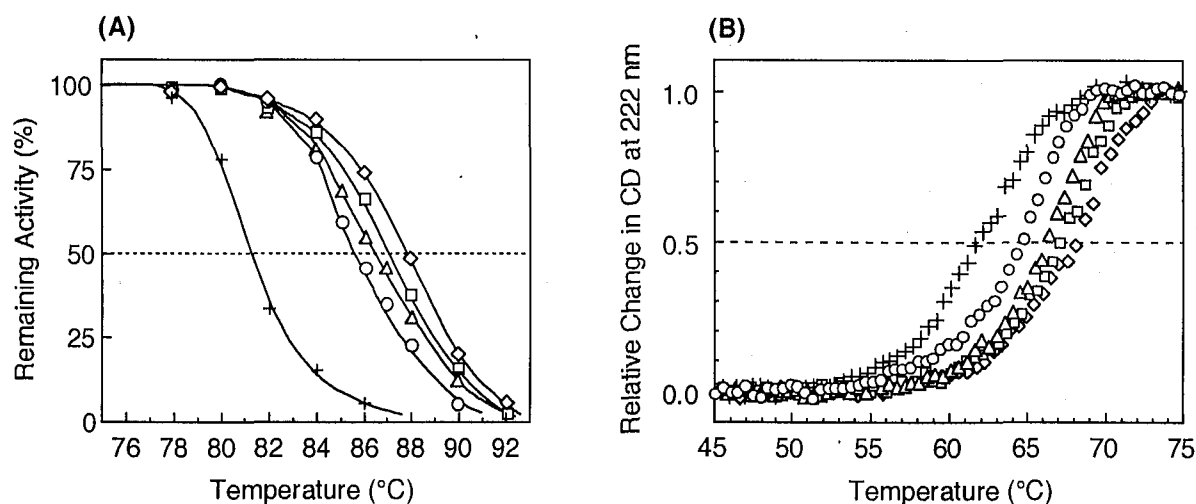


Figure 4-1. Thermal stability of *T. thermophilus* IPMDHs. (A) Remaining activities of IPMDHs after heat treatment. Each enzyme was diluted to 0.1 mg/ml with 20 mM potassium phosphate, pH 7.6, containing 0.5 mM EDTA and incubated at the indicated temperature for 10 min. The remaining activities were expressed as percentages of the original activities. The values are the averages of at least three measurements. (B) Thermal melting profiles of IPMDHs. The denaturation was monitored by CD at 222 nm as described in "Experimental procedures". The melting curves were normalized with respect to the baselines of the native and denatured states. Open circles, wild-type; "+", A172D; open triangles, A172E; open squares, A172N; open diamonds, A172Q.

The thermal denaturation processes of the wild-type and the mutant enzymes were also analyzed by measuring the change in ellipticity at 222 nm in the alkaline buffer. The temperature for 50% denaturation (T_m) was used to compare the thermal stability of the enzymes. Figure 1B indicates that the mutations of Ala172→Glu, Asn and Gln increase T_m by 1.5°C, 2.5°C and 3.3°C, respectively, while Ala172→Asp decreases the thermostability by 3.0°C in T_m . Essentially the same results were obtained in the two separate experiments. The results also show that the mutant enzymes, A172E, A172N and A172Q, are more thermostable than the wild-type enzyme as judged from the stability of the secondary structure.

Steady-state kinetic parameters of the wild-type and mutant enzymes are listed in Table 4-1. Slight changes in the Michaelis constant (K_m) are observed only with A172D; the K_m values of A172D for D-3-IPM and for NAD are reduced to 0.75- and 0.65-fold those of the wild-type enzyme, respectively. The catalytic constant (k_{cat}) does not vary significantly among these enzymes. These results indicate that the mutations Ala172→Glu, Asn and Gln enhanced the stability of the thermophilic enzyme without significant change in the enzymatic activity and that the mutation Ala172→Asp slightly improved the affinity both for D-3-IPM and for NAD at the cost of decrease in thermostability.

Table 4-1. Kinetic parameters of IPMDHs^a

Enzyme	K_m (μ M)		k_{cat} (s^{-1}) ^b
	D-3-IPM	NAD	
Wild-type	22.1 ($\pm$ 2.5)	116 ($\pm$ 26)	107 ($\pm$ 5)
A172D	16.9 ($\pm$ 1.8)	76 ($\pm$ 17)	100 ($\pm$ 13)
A172E	25.7 ($\pm$ 0.5)	100 ($\pm$ 22)	117 ($\pm$ 2)
A172N	23.4 ($\pm$ 0.5)	105 ($\pm$ 30)	117 ($\pm$ 2)
A172Q	26.7 ($\pm$ 2.2)	94 ($\pm$ 10)	111 ($\pm$ 2)

^aEach value is the mean of three independent determinations.

^b k_{cat} values are expressed as reaction per dimer.

Structural analyses

In order to analyze the structural basis of altering the thermal stability through the mutations, crystallization of the mutant enzymes has been attempted in our laboratory. Unfortunately, the crystallization experiments have been unsuccessful so far, precluding crystallographic analyses. Alternatively, molecular modeling of the mutant structures of A172E and A172D has recently been carried out by Qu (1997). In this study, we also carried out the molecular modeling of the structures of A172Q and A172N. The proposed structure of A172E suggests that the Glu172 side chain fills up the hydrophobic core with its two methylene groups and its terminal carboxylate group points to His300, possibly forming a salt bridge or a hydrogen bond with the side chain of His300 (Fig. 4-2A). Thereby, A172E might acquire the increased thermostability.

In the proposed structures of A172Q and A172N, the side chains of Gln172 and Asn172 similarly interact with the surrounding residues (Fig. 4-2C, D). Their methylene groups might be involved in the hydrophobic interaction, and their side chain carbonyl groups form hydrogen bonds

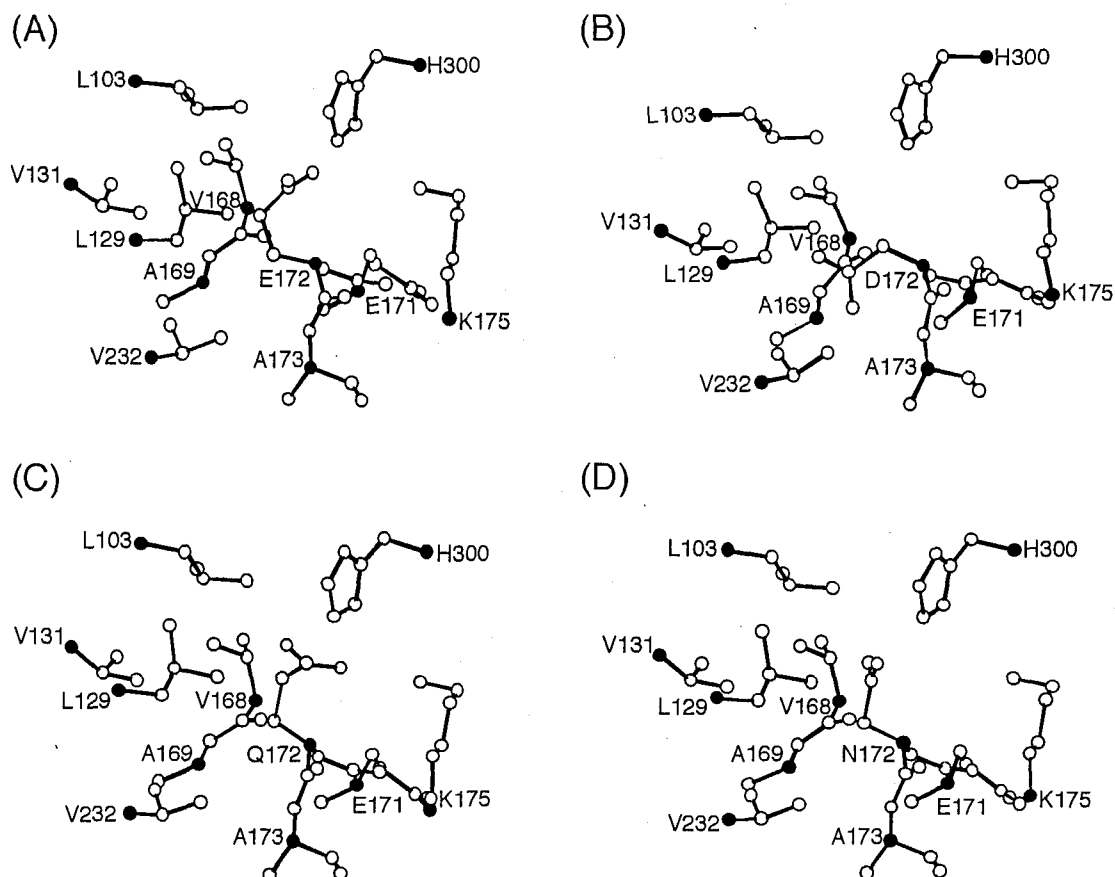


Figure 4-2. Modeled structures around mutation site of A172E (A), A172D (B), A172Q (C) and A172N (D). The α -carbons were represented with solid circles. Amino acid residues are designated by the one-letter code and numbers represent the positions of the amino acid residues.

with His300 and/or Lys175. In addition, their side chain amides are close to the main chain carbonyl of Val168 within 2.8 Å (A172N) and 3.0 Å (A172Q), presumably forming a new hydrogen bond. Thereby, the stabilities of the mutant enzymes are also enhanced. We note that A172Q is the most stable variant among the mutant enzymes prepared in this study. The side chain of Gln172 fills up the hydrophobic core with two methylene group whereas that of Asn172 does with one methylene group. In addition, the longer side chain of glutamine makes its amide group possible to locate more suitable position to interact with Lys175. Thereby, the A172Q mutant is more thermostable than the A172N mutant. In the modeled structures of A172Q and A172E, the amide group of Gln172 is hydrogen-bonding with the main chain carbonyl of Val168 whereas there is no hydrogen bond between the side chain of Glu172 and Val168. That can be why the A172Q mutant is more thermostable than the A172E mutant.

In contrast to those mutant enzymes, A172D is more thermolabile than the wild-type IPMDH. From the modeled structure, it seems that the decreased thermostability is caused by unfavorable side chain interactions; the charged side chain of Asp172 is located in the hydrophobic core and thereby aspartic acid is not preferred in this position (Fig. 4-2).

DISCUSSION

A correlation between the sensitivity of a residue position to amino acid substitutions and the solvent exposed surface area of the residue has been reported base on the mutagenesis studies of λ repressor, lac repressor, T4 lysozyme and gene V protein of bacteriophage f1 (Bowie et al., 1990; Rennell et al., 1991; Markiewicz et al., 1994; Terwilliger et al., 1994). These studies indicated that residues buried in hydrophobic core tend to be more sensitive to amino acid substitutions than exposed residues. Recently, Huang et al. (1996) showed from the saturation mutagenesis studies of β -lactamase that interior hydrophobic residues can be substituted for other hydrophobic amino acids to some extent. In our previously studies, it was shown that the thermostability of *T. thermophilus* IPMDH could be improved by the amino acid replacements of Ala172, which was completely buried in the hydrophobic core, with other hydrophobic residues such as Val, Ile, Leu and Phe (Chapters 2, 3). We speculated that the substitution of Ala172 with polar amino acid residues might reduce the stability of the enzyme. To our surprise, the substitutions of Ala172 with several polar residues, such as Glu, Gln and Asn, also improved the thermostability of the thermophilic enzyme without significantly changing in the catalytic properties.

Based on these observations, the structural mechanisms for stabilization of the thermophilic enzyme could be categorized into three types. First as shown in the case of Val172 mutant, a larger hydrophobic side chain filled up the intramolecular hydrophobic cavity without significant change in the overall structure, thereby increasing the enzyme stability (Qu, 1997). Second mechanism was found in the mutant with Leu172; the Ala-to-Leu substitution induced the domain movement to close the active site, thereby reducing the solvent accessible surface area of hydrophobic side chains which were involved in the substrate binding (Qu et al., 1997). In addition, the conformation change might improve the domain-domain interaction through formation of additional hydrogen bonds between the domains (Qu et al., 1997). The third type of the stabilization mechanism was suggested from the present mutant enzymes. Even though the amino acid residue is buried in a hydrophobic pocket, if it is possible to form a new electrostatic interaction, the substitution with a polar side chain may improve the stability of the enzyme; in the present study, the polar groups of the glutamate, glutamine and asparagine residues form electrostatic interactions with the surrounding residues and their methylene groups contribute to packing the hydrophobic core.

Generally, proteins are well optimized in their structures for their physiological conditions and, therefore, it is reasonable that the *T. thermophilus* enzyme is inherent thermostable. Nevertheless, changing the residue at position 172 to at least seven other residues further improves the stability of the enzyme, indicating that this position has not fully optimized with respect to thermal stability in the course of evolution.

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Chapter 5: Spontaneous tandem sequence duplications reverse the thermal stability of carboxyl-terminal modified 3-isopropylmalate dehydrogenase

ABSTRACT

A mutant strain of *Thermus thermophilus* which contained deletions in the 3' terminal region of its *leuB* gene showed a temperature-sensitive growth phenotype in the absence of leucine. Three phenotypically thermostable mutants were isolated from the temperature-sensitive strain by spontaneous evolution. Each pseudorevertant carried a tandem sequence duplication in the 3' region of its *leuB* gene. The mutated 3-isopropylmalate dehydrogenases encoded by the *leuB* genes from the pseudorevertants were more thermostable than the enzyme from the temperature-sensitive strain. Structural analyses suggested that the decreased thermostability of the enzyme from the temperature-sensitive strain was caused by reducing hydrophobic and electrostatic interactions in the carboxyl-terminal region and that the recovered stability of the enzymes from the pseudorevertants was due to the restoration of the hydrophobic interaction. Our present results indicate that tandem sequence duplications are the general genetic way to alter protein characteristics in evolution.

INTRODUCTION

3-Isopropylmalate dehydrogenase (IPMDH, EC 1, 1, 1, 85) is an enzyme in the leucine biosynthetic pathway and catalyzes the oxidative decarboxylation of (2*R*,3*S*)-3-isopropylmalate to 2-oxoisocaproate. Its gene (*leuB*) from an extreme thermophile, *Thermus thermophilus* HB8, has been cloned (Tanaka et al., 1981) and sequenced (Kagawa et al., 1984; Kirino et al., 1994). The well-characterized enzymatic properties show that the enzyme has unusually high level of thermal stability (Yamada et al., 1990).

Previously, we developed an integration vector system that enables the expression of foreign genes in *T. thermophilus* (Tamakoshi et al., 1995). This thermophilic species can grow at high temperatures up to 85°C (Oshima and Imahori, 1974) and shows high efficiency of genetic recombination (Koyama et al., 1986). These characteristics provide us with a screening system for modified enzymes with enhanced thermal stability. The gene encoding a chimeric IPMDH (Numata et al., 1995) which was constructed by combining the parts of the enzymes of *T. thermophilus* and a mesophile, *Bacillus subtilis*, was introduced in *T. thermophilus* (Tamakoshi et al., 1995). Several thermostabilized mutants derived from the chimeric enzyme were isolated by an *in vivo* selection technique based on the culture of the mutant strain harboring the chimeric gene under strongly selective pressure (Chapters 2, 3; Tamakoshi et al., 1995). These stabilizing mutations were assigned to each of the single amino acid replacements: Ile93→Leu, Ala172→Val, or Ala172→Leu.

In this chapter, we reversed the stability of another thermolabile IPMDH by a similar *in vivo* evolutionary technique in order to investigate how enzymes are adapted to the culture conditions. The gene of the temperature-sensitive *T. thermophilus* strain used in our study had deletions in

its 3' region, and the enzyme showed a decreased thermal stability. We attempted to restore the thermal stability of the modified enzyme by a spontaneous second-site mutation which complemented temperature-sensitive growth, and we found that the insertion of tandem sequence duplications near the 3' terminus in the *leuB* gene restored the thermal stability of the encoded enzyme.

EXPERIMENTAL PROCEDURES

Bacterial strains and media

The *leuB*-deficient strain *T. thermophilus* MT106 (Tamakoshi et al., 1995) was cultured in *Thermus* nutrient medium (Oshima and Imahori, 1974) or *Thermus* minimum medium (Sakaki and Oshima, 1975). Solidification was performed by mixing the double-strength media and the Gelrite solution after autoclave sterilization (Takada et al., 1993). *Escherichia coli* HB101 (*F'*, *hsdS*20 (*rB*⁻, *mB*⁻), *recA*13, *ara*-13, *proA*2, *lacY*1, *galK*2, *rpsL*20 (*Sm*^r), *xyl*-5, *mtl*-1, *supE*44, λ) and JA221 (*F'*, *hsdR*, *trpE*5, *leuB*6, *lacY*, *recA*1, λ) were used as hosts for plasmid amplification and for expression of the *leuB* genes, respectively. They were cultured in 2YT medium (Sambrook et al., 1989) supplemented with ampicillin (150 mg/liter).

Deletion of the 3' terminus of the leuB gene

A plasmid, pUTL-AL118, harboring the *leuB* gene with the point mutation which results in a thermostabilizing mutation of *T. thermophilus* IPMDH (Ala172→Leu) was constructed in Chapter 3. Two restriction sites, *Bln*I (CCTAGG) and *Eco*T22I (ATGCAT), were introduced immediately upstream of the termination codon of the *leuB* gene on the plasmid by the method of Kunkel et al. (1987). An oligonucleotide 5'TAT CCC CAT CTT ATG CAT GCC TAG GTG GCG GAG3' was used for generating the restriction sites. The resulting plasmid was digested with *Bln*I and *Eco*T22I and purified by successive extractions with phenol/chloroform and chloroform. The DNA was precipitated with ethanol, resuspended in 50 μ l of exonuclease III buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl₂, 10 mM β -mercaptoethanol), and treated with 150 U of exonuclease III at 16°C for various times ranging from 10 to 100 sec. The DNA solution was added to 50 μ l of mung bean buffer (40 mM sodium acetate, pH 4.5, 100 mM NaCl, 2 mM ZnCl₂, 10 % Glycerol), and the mixture was heat treated at 65°C for 5 min. Twenty-five units of mung bean nuclease was then added, and the solution was incubated at 37°C for 5 min. After extractions with phenol/chloroform and chloroform, the DNA was recollected by ethanol precipitation and resuspended in 25 μ l of Klenow buffer (7 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 20 mM NaCl, 7 mM MgCl₂, 0.1 mM each dNTP). Two units of Klenow Fragment of DNA polymerase I was added into the DNA solution, and then the mixture was incubated at 37°C for 15 min. The DNA was self-ligated using a ligation kit (Takara syuzo Co. LTD.) and amplified in *E. coli* HB101.

Isolation of a temperature-sensitive strain

The 1.2-kb *Bam*HI fragments, containing the *leuB* genes with deletions in their 3' region, were recovered from the library and inserted into the integration vector for *T. thermophilus*, pIT1 (Tamakoshi et al., 1995). The resulting plasmids were used to transform *T. thermophilus* MT106 by the method of Koyama et al. (1986) with slight modifications as described previously (Takada et al., 1993). The transformation mixture was incubated at 65°C for 3 h, and cells were harvested by centrifugation at 5,000 $\times g$ for 5 min and resuspended in 1 ml of *Thermus* minimum medium. To select quiescent cells at 72°C, the suspension was treated with ampicillin (300 mg/liter) at 72°C, spread on the minimum medium plate, and incubated for 3 days at 65°C. A temperature-sensitive mutant, CD071, which could grow at 65°C but not at 72°C without leucine, was obtained from 50 colonies.

Spontaneous pseudorevertant from the temperature-sensitive mutant

The temperature-sensitive strain CD071 was pre-cultured in the nutrient medium at 70°C for 12 h. The culture was centrifuged at 5,000 $\times g$ and the cell pellet was resuspended in 100 μ l of sterilized water, and the suspension was transferred to 2 ml of the minimum medium and then incu-

bated at 65°C. When cell growth was saturated, the culture was centrifuged at 5,000×g, the cell pellet was resuspended in 100 µl of sterilized water and then plated onto the minimum medium. The plate was incubated at 75°C, and three independent pseudorevertants, HD177, HD708 and HD711, were isolated after a 3-day incubation.

PCR and DNA sequencing

About 200 ng of the genomic DNA prepared from *T. thermophilus* mutant strains as described in Chapter 2 was dissolved in 100 µl of PCR amplification buffer (20 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 25 mM KCl, 0.05% Tween 20) supplemented with bovine serum albumin (0.1 mg/ml), 50 µM each dNTP, and 20 pmol each of the specific PCR primers for the *leuB* gene. After heat treatment at 100°C for 10 min, 2.5 U of *Taq* DNA polymerase was added and amplification was carried out as follows: a cycle consisting of denaturation at 95°C for 1 min, annealing at 55°C for 0.5 min and polymerization at 72°C for 1 min was repeated 30 times, and the amplification mixture was finally incubated at 72°C for 5 min. The amplified *leuB* gene was then treated with T4 DNA polymerase to generate blunt ends and digested with *Sac*I, which cleaves inside the gene. The resulting two fragments were subcloned into the phage vector M13mp18 or M13mp19 to prepare sequencing templates. Sequencing was performed by the dideoxy chain termination method (Sanger et al., 1977) with AutoRead Sequencing kit (Pharmacia) and a Pharmacia ALF DNA Sequencer II.

Thermal stability measurement

For preparation of IPMDHs, respective *leuB* genes were inserted at the *Bam*HI site of pUC119, the genes were expressed in *E. coli* JA221, and the products were purified to homogeneity as described by Yamada et al. (1990). The thermal stability of the purified enzymes was determined as described in Chapter 2.

RESULTS

Isolation and characterization of temperature-sensitive mutant and pseudorevertants

Since *T. thermophilus* IPMDH is known to be stabilized by the replacement of Ala172 with a more hydrophobic residue (Chapters 2, 3), we used the gene encoding the mutant IPMDH with an increased thermostability by the replacement of Ala172 with Leu in this study in order to analyze the stabilizations other than the point mutation at 172.

The experimental procedures are shown in Fig. 5-1. The *leuB* gene (Ala172→Leu) was first treated with an exonuclease to remove its 3' terminal nucleotides and then integrated into the $\Delta leuB$ strain of *T. thermophilus*. The resulting transformants were screened at 65 and 75°C, and a temperature-sensitive mutant was isolated. This strain, named CD071, could grow at 65 but not at 75°C under conditions lacking leucine, but it could grow at 75°C with leucine (Table 5-1). The DNA sequence of the *leuB* gene amplified from the genomic DNA of CD071 was determined. The gene carried a 22-base deletion around the original termination codon of the *leuB* gene (Fig. 5-2). This deletion results in the replacement of 5 carboxyl-terminal residues (Leu-Arg-His-Leu-Ala) with 2 new ones (Gly-Ile) in the encoded enzyme.

The resulting temperature-sensitive mutant, CD071, was cultured at the restrictive temperature, 75°C, and three independent pseudorevertants named HD177, HD708, and HD711 were isolated as leucine autotrophic mutants at 75°C. The DNA sequencing of the *leuB* genes of these revertants revealed different lengths of tandem sequence duplications, which are shown in Fig. 5-2: the sequences of 6, 12 and 21 bases were duplicated in HD177, HD708 and HD711, respectively.

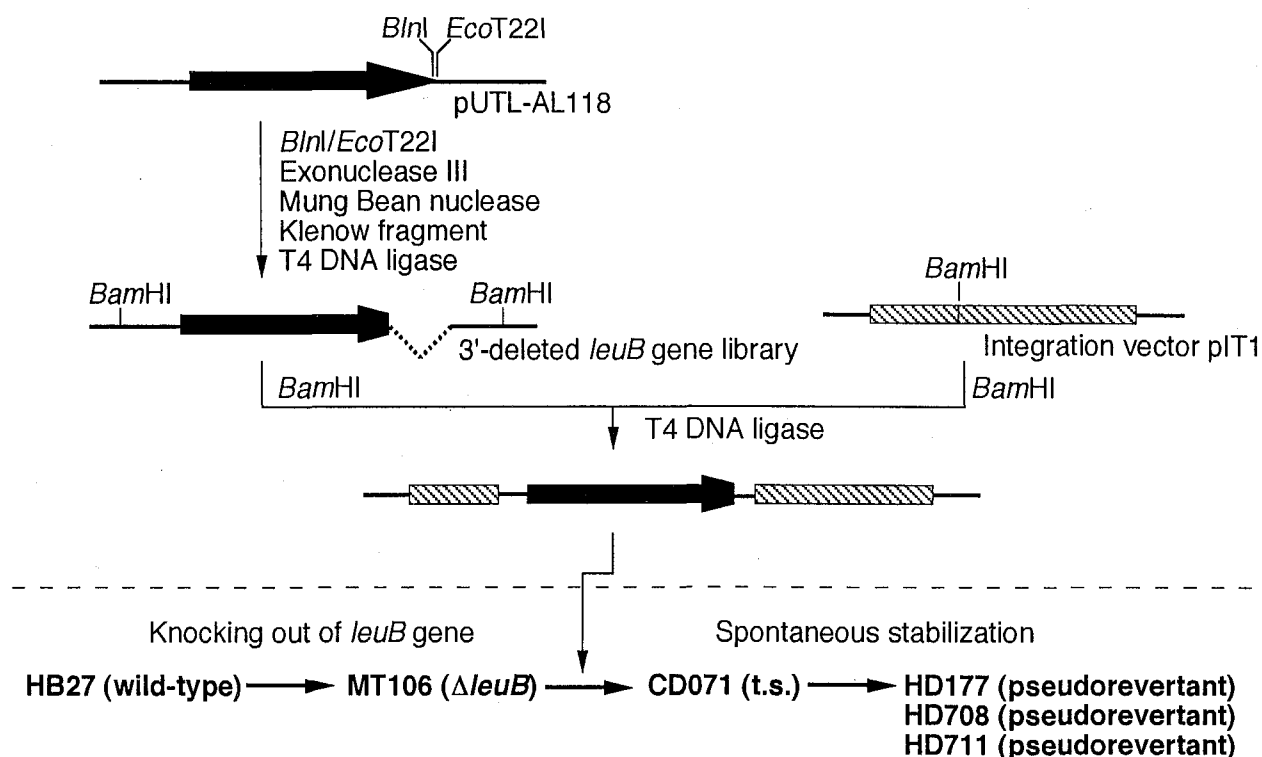


Figure 5-1. Construction of a library for *T. thermophilus* with *leuB* genes with 3' deletions and selection in *T. thermophilus*. Plasmid pUTL-AL118 was digested with *BlnI* and *EcoT22I*; the larger fragment was successively treated with exonuclease III, mung bean nuclease, and Klenow fragment of DNA polymerase I and then self-ligated. The resulting plasmids were digested with *BamHI*; the 1.2-kb fragments containing the *leuB* gene were excised and inserted into the integration vector, pIT1, for *T. thermophilus*. The *leuB* genes with 3' deletions were integrated into the chromosomal DNA of the *T. thermophilus* $\Delta leuB$ strain MT106 by homologous recombination. The temperature-sensitive (ts) strain CD071 was isolated by phenotype selection. Three pseudorevertants, HD177, HD708, and HD711, were obtained after a 3 day-incubation of CD071 at 75°C. The details of the procedures are described in "Experimental Procedures". Filled arrows, *T. thermophilus leuB* gene; hatched lines, flanking sequence of the *leuB* gene.

Table 5-1. Growth of *T. thermophilus* mutants in minimum medium

<i>T. thermophilus</i> strain	Growth ^a at the indicated temp		
	With leucine	Without leucine	
	75°C	65°C	75°C
HB27 (wild-type)	+	+	+
CD071 (temperature sensitive)	+	+	-
HD177 (pseudorevertant)	+	+	+
HD708 (pseudorevertant)	+	+	+
HD711 (pseudorevertant)	+	+	+

^aThe growth of each strain was scored after incubation for 24 h on a minimum medium-coated plate at the indicated temperatures.

Strain	333	345
HB27 (Wild-type)	ACGGAGGCCTTCACGGCCACGGTCCTCCGCCACCTCGCCTAAGATGGGGATATGA T E A F T A T V L R H L A *	
CD071 (ts mutant)	ACGGAGGCCTTCACGGCCACGGT-----GGGGATATGA T E A F T A T V	G I *
HD177 (pseudorevertant)	ACGGAGGCCTTCACGGCCACGGT-----GGGGATGGGGATATGA T E A F T A T V	G M G I *
HD708 (pseudorevertant)	ACGGAGGCCTTCACGGCCACGGTCACGGCCACGGT-----GGGGATATGA T E A F T A T V T A T V	G I *
HD711 (pseudorevertant)	ACGGAGGCCTTCACGGCCACGGTGGAGGCCTTCACGGCCACGGT-GGGGATATGA T E A F T A T V E A F T A T V	G I *

Figure 5-2. Nucleotide and putative amino acid sequences between residues 333 and the carboxyl termini of IPMDHs in wild-type HB27, temperature-sensitive (ts) mutant CD071, and three pseudorevertants (HD177, HD708, and HD711) of *T. thermophilus*. The temperature-sensitive mutant CD071 carried a 22-base deletion, indicated by the broken line. All of the revertants contained the respective inserted sequences generated by tandem sequence duplication, which are underlined by arrows. Possible nicking sites are indicated by downward-pointing arrows; open downward-pointing arrows indicate sites of deletion.

In vitro analyses of the mutant IPMDHs

The *leuB* genes obtained from the mutants of *T. thermophilus* were overexpressed in *E. coli*, and the products were purified to homogeneity. The A172L enzyme with intact carboxyl terminus (A172L-LRHLA) was also prepared for comparison. The levels of thermal stability of the enzymes were estimated by measuring the remaining activities after heat treatment for 10 min at various temperatures ranging from 65 to 95°C. Figure 5-3 shows that the product of the gene from the temperature-sensitive mutant CD071 (A172L-GI) is less thermostable than the original enzyme, A172L, by 14°C in terms of the half-inactivation temperature. The enzymes of the pseudorevertants HD177, HD708, and HD711 (A172L-GMGI, A172L-TATVGI, and A172L-EAFTATVGI, respectively), which contained the respective inserted sequences in the carboxyl terminal region, restored thermal stability, by 3, 6, and 8°C, respectively, from the thermal stability of the enzyme of CD071. It is noteworthy that the enzyme carrying the longest insertion shows the highest level of stability among the three mutants.

The kinetic parameters of the mutant IPMDHs are listed in Table 5-2. The Michaelis constants (K_m s) for D-3-isopropylmalate (D-3-IPM), a substrate of IPMDH, of the enzymes from CD071 and HD711 are similar to that of the original enzyme A172L, while the K_m s obtained for the enzymes from HD177 and HD708 are 1.5-fold higher than that for A172L. The K_m for coenzyme, NAD, is slightly improved over that of the enzyme from CD071, while the K_m values of the enzymes from the pseudorevertants are increased by 1.2- to 1.6-fold compared with that of

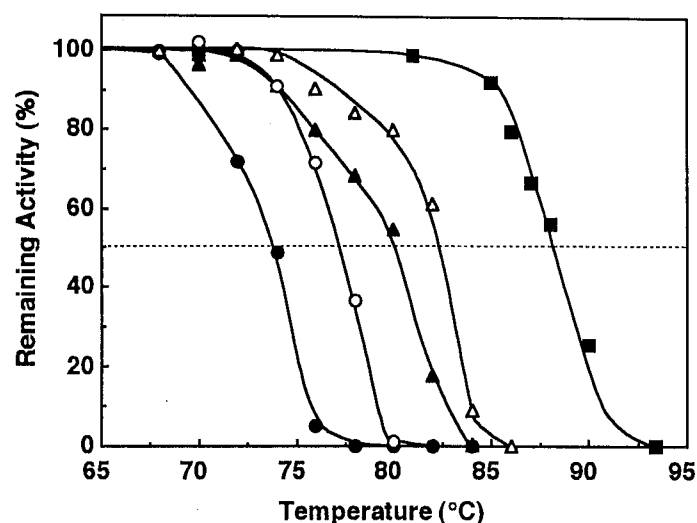


Figure 5-3. Activities of IPMDHs after heat treatment. Each purified enzyme was diluted to 0.1 mg/ml with 20 mM potassium phosphate buffer (pH 7.6) containing 0.5 mM EDTA and treated at the indicated temperatures for 10 min. The remaining activities were expressed as percentages of the original activities. The values are the averages of three independent experiments. closed squares, A172L-LRHLA (original A172L); closed circles, A172L-GI (CD071); open circles, A172L-GMGI (HD177); closed triangles, A172L-TATVGI (HD708); open triangles, A172L-EAFTATVGI (HD711).

Table 5-2. Kinetic constants of mutant IPMDHs^a

Enzyme	Source	K_m (μ M)		k_{cat} (s^{-1}) ^b
		D-3-IPM	NAD	
A172L-LRHLA ^c		23.9 ($\pm$ 0.4)	100 ($\pm$ 24)	114 ($\pm$ 5)
A172L-GI	CD071	22.8 ($\pm$ 2.0)	73 ($\pm$ 4)	104 ($\pm$ 12)
A172L-GMGI	DH177	33.0 ($\pm$ 4.1)	116 ($\pm$ 6)	115 ($\pm$ 8)
A172L-TATVGI	HD708	33.0 ($\pm$ 4.1)	158 ($\pm$ 34)	121 ($\pm$ 8)
A172L-EAFTATVGI	HD711	19.5 ($\pm$ 3.4)	150 ($\pm$ 32)	129 ($\pm$ 9)

^aThe kinetic constants were determined in steady-state experiments at 60°C in 100 mM potassium phosphate buffer containing 1 M KCl, 0.2 mM MnCl₂, and various concentrations of D-3-IPM and NAD. Each value is the mean of three independent experiments.

^b k_{cat} , catalytic constant. Values are expressed as reaction per dimer.

^cOriginal A172L IPMDH with intact carboxyl-terminal sequence.

the original enzyme, A172L. The catalytic constant did not vary significantly among these enzymes. These results indicate that the tandem sequence duplications restore thermal stability without any drastic change of the catalytic properties.

Structural analyses of the carboxyl-terminal modification

The three-dimensional structure of *T. thermophilus* IPMDH has been determined as 2.2 Å (Imada et al., 1991), showing that the enzyme is a homodimer, with its subunit consisting of 345 amino acid residues. The subunit of the enzyme has two structural domains; domain 1 consists of residues 1 to 99 and 255 to 345, thus including N and C termini, and domain 2 contains the subunit interface. The refined structure around the carboxyl-terminal region of wild-type

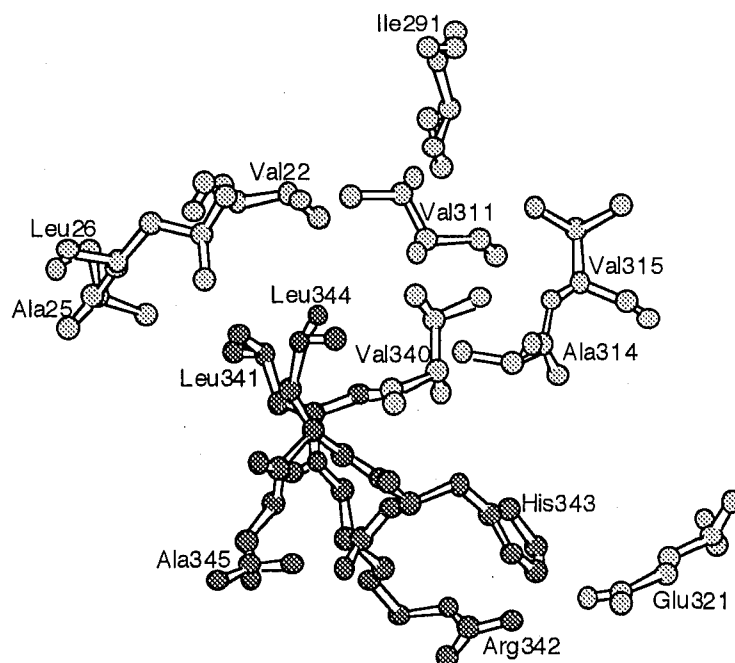


Figure 5-4. Structural arrangement of residues in the carboxyl-terminal region of wild-type *T. thermophilus* IPMDH. The crystal structure of the wild-type enzyme was determined by Imada et al. (3). The residues from 341 to 345 (Leu-Arg-His-Leu-Ala) in the wild-type enzyme were replaced with Gly-Ile, Gly-Met-Gly-Ile, Thr-Ala-Thr-Val-Gly-Ile, and Glu-Ala-Phe-Thr-Ala-Thr-Val-Gly-Ile in the enzymes of CD071, HD177, HD708, and HD711, respectively.

IPMDH is shown in Fig. 5-4. The side chains of the two bulky residues (Leu341 and Leu344), which were truncated in the enzyme of CD071, point to the hydrophobic core and are surrounded by Val22, Ala25, Leu26, Val311, and Val340 within 4.5 Å, suggesting that the hydrophobic residues in the carboxyl-terminal region contribute to stability by intramolecular hydrophobic interaction in the original enzyme. In addition, the distance between Arg342 and Glu321 is 2.4 Å and that between His343 and Glu321 is 3.0 Å, suggesting that they form salt bridges or hydrogen bonds. The enzyme from CD071 lacks the 5 residues in the carboxyl terminus; thus, it lacks the region encoding a part of the hydrophobic interaction and the electrostatic interactions, dramatically reducing its stability. Although we have not fully investigated the mechanism of the reversion of stability in the enzymes of the pseudorevertants, it is conjectured that the reversed stabilities of the enzymes are due to the restoration of the hydrophobic interaction involving the carboxyl-terminal residues inserted by the sequence duplications.

DISCUSSION

Previously, Hall (1981) reported an evolutionary experiment; a strain of *E. coli* from which *lacZ* was deleted was cultured with lactose as the sole carbon source. The wild-type second β -galactosidase does not hydrolyze the β -galactoside bond and therefore does not allow *E. coli* with *lacZ* deleted to grow in the lactose medium. However, several spontaneous mutant strains which could grow in the lactose medium were isolated, and the second β -galactosidase from the

spontaneous mutant strain hydrolyzed lactose effectively. On the other hand, Tamakoshi et al. (1995) cultured a mutant strain of *T. thermophilus* which carries the chimeric *leuB* gene instead of the wild-type *leuB* gene in *Thermus* minimum medium without leucine and selected a spontaneous mutation which contributed to an increased thermal stability. We also improved the thermal stability of the carboxyl-terminus-modified IPMDH by the evolutionary technique using *T. thermophilus* in the present study. These results suggest that the artificial evolution technique can be used to produce mutant enzymes with improved catalytic activities and/or thermal stabilities.

Stabilization of proteins by evolutionary modification technique with a thermophile as a host cell was reported by Liao et al. (1986) and Tamakoshi et al. (1995) with kanamycin nucleotidyltransferase and the chimeric IPMDH, respectively. In both cases, the stabilizations were attributed to the respective single point mutations. However, we observed here that the tandem sequence duplications are the cause of stabilization of the enzyme; no mutant with nucleotide replacements has been isolated so far.

Recently, Kless and Vermaas (1995) reported that the spontaneous tandem sequence duplications restored photoautotrophic growth of cyanobacterium *Synechocystis* sp. mutants carrying deletions of conserved residues in the D1 protein of Photosystem II. They suggested a mechanism for the sequence duplication, namely, that a nick generated in a DNA strand provides DNA polymerase with a priming site for extension and that the following polymerization synthesizes a new strand instead of the old one: the displaced strand folds back and religates to the end of the newly extended strand [see reference of Kless and Vermaas (1995) for more details] We consider that the tandem duplications observed in this study may be caused by a similar genetic mechanism in *T. thermophilus*. On the basis of this mechanism, the sites of initial nick formation, which are indicated by the downward-pointing arrows in Fig. 5-2, can be either 3' or 5' of the duplicated sequences. One of the two sites, indicated by an open arrow, was the exact site of deletion in all of the three revertants which we isolated. The same was true in the revertants from a deletion strain reported by Kless and Vermaas (1995).

The occurrence of sequence duplication leading to restoration of activity (Kless and Vermaas) and thermal stability (the present case) suggests that the sequence duplication is one of the general ways new proteins and enzymes evolve. Furthermore, we believe that the generation of tandem sequence duplications, as well as point mutations, is a useful strategy to improve protein characteristics by protein engineering or evolutionary molecular engineering.

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Chapter 6: Serial Increase in the Thermal Stability of 3-Isopropylmalate Dehydrogenase from *Bacillus subtilis* by Experimental Evolution

ABSTRACT

We improved the thermal stability of 3-isopropylmalate dehydrogenase from *Bacillus subtilis* by an *in vivo* evolutionary technique using an extreme thermophile, *Thermus thermophilus* as a host cell. The *leuB* gene encoding *B. subtilis* 3-isopropylmalate dehydrogenase was integrated into the chromosome of a *leuB*-deficient strain of *T. thermophilus*. The resulting transformant showed a leucine-autotrophy at 56°C but not at 61°C and above. Phenotypically thermostabilized strains which can grow at 61°C without leucine were isolated from spontaneous mutants. Screening temperature was stepwise increased from 61 to 66 and then to 70°C and mutants which showed a leucine-autotrophic growth at 70°C were obtained. DNA sequence analyses of the *leuB* genes from the mutant strains revealed three stepwise amino acid replacements, threonine-308 to isoleucine, isoleucine-95 to leucine and methionine-292 to isoleucine. The mutant enzymes with these amino acid replacements were more stable against heat treatment than the wild-type enzyme. Furthermore, the triple-mutant enzyme showed significantly higher specific activity than that of the wild-type enzyme.

INTRODUCTION

Enhancement of protein thermostability is one of the major subjects in protein engineering. Recently, many successful examples of protein stabilization have been reported and several principles to increase the stability of proteins have been proposed, for example: (i) introducing disulfide bonds to reduce the difference in entropy between native and denatured states (Matthews et al., 1987; Matsumura et al., 1989), (ii) increasing intramolecular hydrophobic interaction (Yutani et al., 1987), (iii) changing specific residues to stabilize the dipoles of α -helices (Nicholson et al., 1988) and (iv) introducing metal binding sites (Pantoliano et al., 1988; Kuroki et al., 1989). However, the application of these principles is limited to the proteins whose three-dimensional structures have been determined.

Evolutionary molecular engineering technique is an alternative approach to obtain mutant proteins with enhanced thermostability. The concept of the evolutionary technique was first proposed by Eighen and Gardiner (1984). Using the evolutionary engineering strategy, we can feasibly obtain stabilized proteins even in the absence of information on their three-dimensional structures. This strategy consists of two steps; (i) introduction of random or spontaneous mutations to a target gene, and (ii) selection and reproduction of the mutant genes with the desired properties under selective pressure. We previously constructed an integration vector system that enables the expression of foreign genes in an extreme thermophile, *Thermus thermophilus* (Tamakoshi et al., 1995). This thermophilic species is able to grow at temperatures of up to 80°C (Oshima and Imahori, 1974) and shows high efficiency of homologous recombination with foreign DNAs (Koyama et al., 1986). These characteristics provide us with an opportunity to screen for mutant proteins with en-

hanced thermostability.

3-Isopropylmalate dehydrogenase (IPMDH), a product of the *leuB* gene, catalyzes the oxidative decarboxylation of (2R,3S)-3-isopropylmalate to 2-oxoisocaproate, which is involved in leucine biosynthesis. The gene have been cloned from *Bacillus subtilis* (Nagahari and Sakaguchi, 1978) and *T. thermophilus* (Tanaka et al., 1981), and their sequences have been determined (Kagawa et al., 1984; Imai et al., 1987; Kirino et al., 1994). The enzyme from *T. thermophilus* has been well characterized in terms of its catalytic properties, thermal stability (Yamada et al., 1990) and tertiary structure (Imada et al., 1991). We have recently constructed several chimeric enzymes by combining parts of the enzymes of *T. thermophilus* and *B. subtilis* (Numata et al., 1995). The gene encoding one of such chimeric enzymes, designated as 2T2M6T, was integrated into the chromosome of a *leuB*-deficient strain of *T. thermophilus* by using the integration vector system for the thermophile (Tamakoshi et al., 1995). Because the chimeric enzyme is less thermostable than the thermophilic enzyme, the resulting transformant shows a temperature-sensitive autotrophy; the strain can grow at 70°C but not at 79°C without leucine. However, a phenotypically thermostabilized strain was obtained by selecting a leucine-autotrophic mutant at 79°C. It was confirmed that the variant of the chimeric enzyme isolated from the spontaneous mutant was more thermostable than the original chimeric enzyme (Tamakoshi et al., 1995).

In the present study, we attempted to stabilize a thermolabile IPMDH from *B. subtilis* by a similar evolutionary technique. The amino acid sequence of the enzyme shows 56% identity with that of *T. thermophilus* IPMDH, whereas thermal stability of the enzyme is less than that of *T. thermophilus* IPMDH by 40°C. The enzyme has been purified and characterized (Numata et al., 1995) but crystallographic data have not been available. We applied the evolutionary technique to *B. subtilis* IPMDH in order to obtain mutant enzymes with enhanced thermostability.

EXPERIMENTAL PROCEDURES

Plasmids

The *EcoRI*-*Bam*HI fragment encoding the *leuB* gene of *B. subtilis* was previously cloned into pUC118 and the resulting plasmid was designated as pEB118 (Numata et al., 1995). Plasmid pIT*leuB*BNV (Tamakoshi et al., 1997) was also previously constructed by inserting the 1.2-kb *Bam*HI fragment containing *T. thermophilus leuB* gene into pIT1, which is an integration vector for *T. thermophilus* (Tamakoshi et al., 1995). The plasmid pIT*leuB*BNV has *Nde*I and *EcoRV* sites at the initiation codon and at immediately downstream of the termination codon of the *T. thermophilus leuB* gene, respectively.

Bacterial strains and media

The *leuB*-deficient strain of *T. thermophilus* MT106 was isolated by Tamakoshi et al. (1995). *Thermus* strains were cultured in *Thermus* nutrient medium (Oshima and Imahori, 1974) or *Thermus* minimum medium (Sakaki and Oshima, 1975) supplemented with 25 mg/liter each of isoleucine and valine because the integration process may affect the isoleucine and valine biosyntheses (Takada et al., 1993). Solidification of the media was performed by mixing the double-strength media and the Gelrite solution after autoclave sterilization. *Escherichia coli* JA221 (*F*⁺, *hsdR*, *trpE5*, *leuB6*, *lacY*, *recA1*, λ) was used as a host for plasmid amplification and for expression of the *leuB* gene, and was cultured in 2YT medium (Sambrook et al., 1989) supplemented with 150 mg/liter ampicillin.

DNA manipulation procedures

Recombinant DNA experiments were done by standard methods (Sambrook et al., 1989).

Restriction endonucleases and modification enzymes were purchased from Takara shuzo, Toyobo, or New England Biolab. To generate a *NdeI* site at the initiation codon of the *B. subtilis leuB* gene harbored by plasmid pEB118, site-directed mutagenesis was carried out using an oligonucleotide, 5'AAT ACG TTT CTT CAT ATG AAC CGT TCT CCT3', by the method of Kunkel et al. (1987). The resulting plasmid was designated as pEB118-*NdeI*.

Transformation of T. thermophilus

Integration of *B. subtilis leuB* gene into the *leuB*-deficient strain of *T. thermophilus* MT106 was carried out according to the transformation procedure described by Koyama et al. (1986) with slight modifications reported by Takada et al. (1993). The transformation mixture was incubated at 65°C for 3 h and then plated on *Thermus* minimum medium without leucine. Five colonies appeared after a 7-day incubation at 51°C. One of the colonies were designated as BTH5601 and used for further study.

Isolation of spontaneous mutants

The strain BTH5601 was precultured in 2 ml of *Thermus* minimum medium at 56°C for 3 days. One hundred microliter of the culture was spread on each of 3 plates coated with *Thermus* minimum medium and incubated at 61°C. One out of about 1,000 colonies grown on each plate after a 3-day incubation was randomly selected. The selected colonies were arbitrarily named BTH6121, BTH6141 and BTH6151. The strains BTH6121, BTH6141 and BTH6151 were precultured at 61°C in 2 ml of *Thermus* minimum medium for 3 days. One hundred microliter each of the cultures was plated on *Thermus* minimum medium and mutants strains BTH6622, BTH6623, BTH6642, BTH6643 and BTH6652, which showed leucine-autotrophy at 66°C, were isolated after a 3-day incubation at 66°C. Similarly, BTH6622 and BTH6642 were cultured at 66°C in 2 ml of *Thermus* minimum medium and one hundred microliter of each culture was spread on a plate of *Thermus* minimum medium followed by a 3-day incubation at 70°C. Strains BTH7024 and BTH7044 which showed leucine-autotrophy at 70°C evolved from BTH6622 and BTH6642, respectively.

PCR and sequencing

The *leuB* genes of the spontaneous mutants were amplified by PCR from the genomic DNAs prepared from the mutant strains as described in Chapter 2. Oligonucleotides 5'ACC GCA CCG CTC CCC TCT TCA TCC3' and 5'AGG CAA GAT CTT TTA TTT CCT CCTCT AC3' were used as specific PCR primers for the *leuB* gene. The details of the PCR conditions were described in Chapter 3. The PCR products were cloned into M13mp18 or M13mp19 to prepare templates for DNA sequence analysis. The DNA sequences of the mutant *leuB* genes were determined by the dideoxy chain termination method (Sanger et al., 1977) with AutoRead sequencing kits (Pharmacia) and a Pharmacia ALF DNA Sequencer II.

Thermal stability measurement

For production of the wild-type and mutant *B. subtilis* IPMDHs, the respective *leuB* genes were recloned into *Bam*HI site of pUC119 according to Numata et al (1995). Expression of the genes in *E. coli* and purification of the enzymes were also carried out as described by Numata et al (1995). All the enzymes used in this study were purified to homogeneity as judged by SDS-gel electrophoresis. The remaining activities of the purified enzymes after heat treatment over temperature range 40-63°C were measured at 40°C as described in Chapter 2.

Single-mutant enzymes designated as I95L, M292I, and T308I represent those in which Ile95 is replaced by Leu, Met292 is replaced by Ile and Thr308 is replaced by Ile, respectively. For designation of the mutant enzymes with double- or triple-mutations, the two or three mutations within a single molecule are indicated with a slash, i.e. I95L/T308I and I95L/M292I/T308I.

Enzyme assay

The enzymatic activities were measured in 100 mM potassium phosphate buffer (pH 7.6) containing 1 M KCl, 0.2 mM MnCl₂, 0.4 mM D-3-IPM and 8 mM NAD over temperature range 40-75°C by monitoring the production of NADH at 340 nm as described by Yamada et al (1990). The enzyme unit was defined as 1 μmol NADH formed per min (Yamada et al., 1990).

The kinetic parameters, Michaelis constant (K_m) for D-3-isopropylmalate (D-3-IPM), a substrate of IPMDH, and catalytic constant (k_{cat}), were determined in steady state experiments at 40°C in 100 mM

potassium phosphate buffer (pH 7.6) containing 1 M KCl, 0.2 mM MnCl₂ and 4 mM NAD with varying concentrations of D-3-IPM, ranging 10-100 μ M. In order to determine K_m for NAD, the concentrations of the coenzyme were varied in the range of 50-400 μ M employing a fixed D-3-IPM concentration (400 μ M). Initial rates were measured according to Yamada et al. (1990).

RESULTS

Integration of *B. subtilis* *leuB* gene into *T. thermophilus*

Figure 6-1 shows a procedure for the integration of *B. subtilis* *leuB* gene into the chromosome of *T. thermophilus*. Plasmid pEB118-NdeI was constructed from pEB118 by generating *Nde*I site at the initiation codon of the *leuB* gene by means of the site-directed mutagenesis procedure. The 2.2-kb *Nde*I-*Hinc*II fragment containing the *B. subtilis* *leuB* gene was cut out from plasmid pEB118-NdeI and then ligated with the larger *Nde*I-*Eco*RV fragment of pITleuBNV. The *leuB*-deficient strain of *T. thermophilus* MT106 was transformed with the resulting plasmid pITBSleuB at 65°C, followed by selection at 53°C on a plate coated with *Thermus* minimum medium without leucine. A leucine-autotrophic transformant BTH5601 was isolated on the plate after 7 days of in-

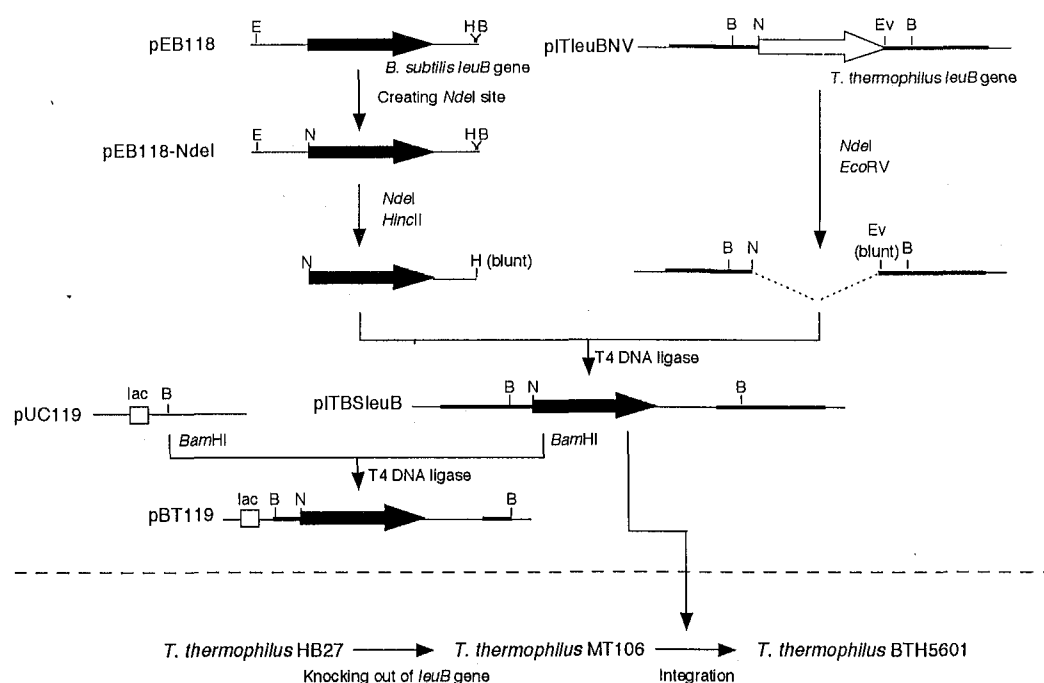


Figure 6-1. Construction of plasmids. The recognition site for *Nde*I was generated at the initiation codon of the *leuB* gene carried by plasmid pEB118, and the resulting plasmid was designated as pEB118-NdeI. Plasmid pEB118-NdeI was digested with *Nde*I and *Hinc*II, and the 2.2-kb fragment containing *B. subtilis* *leuB* gene was isolated. Plasmid pITleuBNV was digested with *Nde*I and *Eco*RV to remove the 1.2-kb fragment containing *T. thermophilus* *leuB* gene. The resulting 5.7-kb fragment was ligated with the 2.2-kb fragment containing *B. subtilis* *leuB* gene. The resulting plasmid designated as pITBSleuB was used as an integration vector. *B. subtilis* *leuB* gene was integrated into the chromosomal DNA of *T. thermophilus* Δ *leuB* strain MT106 by homologous recombination. The 2.1-kb *Bam*HI fragment containing *B. subtilis* *leuB* gene was excised from pITBSleuB and inserted into the *Bam*HI site of pUC119. The resulting plasmid pBT119 and its derivatives were used for expression of the *leuB* genes in *E. coli*. Thick lines, flanking sequences of the *leuB* gene from the wild-type strain of *T. thermophilus*; filled arrows, *B. subtilis* *leuB* gene; open arrow, *T. thermophilus* *leuB* gene; open box, lac promoter of pUC119. Restriction sites: E, *Eco*RI; H, *Hinc*II; B, *Bam*HI; N, *Nde*I; Ev, *Eco*RV.

cubation. Strain BTH5601 could grow at 56°C but not at 61°C under the conditions lacking leucine, but it could grow at 75°C with leucine, indicating that the *B. subtilis* IPMDH retains enzymatic activity up to 56°C under the *in vivo* condition.

Isolation of phenotypically thermostabilized strains

T. thermophilus mutant strains obtained in this study were summarized in Table 6-1. The strain BTH5601 was precultured in 2 ml of *Thermus* minimum medium at 56°C for 3 days. One hundred microliter of the culture containing about 10^7 cells was spread on each of 3 plates coated with *Thermus* minimum medium and then incubated at a restrictive temperature of 61°C. After a 3-day incubation, about 1,000 colonies appeared on each plate. One colony was randomly selected from each plate and the selected colonies were named BTH6121, BTH6141 and BTH6151, respectively. These strains could grow at 61°C but not at 66°C on the minimum medium plate without leucine.

The strains BTH6121, BTH6141 and BTH6151 were subjected to screening at 66°C. Each of BTH6121, BTH6141 and BTH6151 was precultured at 61°C in 2 ml of *Thermus* minimum medium for 3 days and each preculture containing about 10^7 cells was plated on *Thermus* minimum medium. Five colonies appeared after a 3-day incubation at 66°C and were designated as BTH6622, BTH6623, BTH6642, BTH6643 and BTH6652, respectively. Similarly, the strains BTH7024 and BTH7044, leucine-autotrophic mutants at 70°C, were obtained by screening 5×10^7 cells each of BTH6622 and BTH6642, respectively, on *Thermus* minimum medium at 70°C for 3 days.

The strains BTH7024 and BTH7044, leucine-autotrophic mutants at 70°C, were obtained by plating 5×10^7 cells each of BTH6622 and BTH6642, respectively, on *Thermus* minimum medium. Therefore, the apparent frequency to obtain the leucine-autotrophic mutants at 70°C was estimated to be 2×10^{-8} .

Table 6-1. Growth of *T. thermophilus* mutants in minimum medium

Strain	Parent	Selection Temp.	Growth ^a at:					
			+Leu	-Leu				
			76°C	56°C	61°C	66°C	70°C	76°C
HB27 (wild-type)			+	+	+	+	+	+
MT106 ($\Delta leuB$)	HB27		+	-	-	-	-	-
BTH5601	MT106	53°C	+	+	-	-	-	-
BTH6121	BTH5601	61°C	+	+	+	-	-	-
BTH6141	BTH5601	61°C	+	+	+	-	-	-
BTH6151	BTH5601	61°C	+	+	+	-	-	-
BTH6622	BTH6121	66°C	+	+	+	+	-	-
BTH6623	BTH6121	66°C	+	+	+	+	-	-
BTH6642	BTH6141	66°C	+	+	+	+	-	-
BTH6643	BTH6141	66°C	+	+	+	+	-	-
BTH6652	BTH6151	66°C	+	+	+	+	-	-
BTH7024	BTH6622	70°C	+	+	+	+	+	-
BTH7044	BTH6642	70°C	+	+	+	+	+	-

^aThe growth of each strain was scored after incubation for 72 h on a minimum medium-coated plate at the indicated temperatures.

Table 6-2. Mutations found from the evolutionary experiments ^a

<i>T. thermophilus</i> strain	Mutations found in <i>B. subtilis</i> IPMDH
BTH6121	Thr308→Ile (ACA→ATA)
BTH6141	Thr308→Ile (ACA→ATA)
BTH6151	— ^b
BTH6622	Thr308→Ile (ACA→ATA), Ile95→Leu (ATC→CTC)
BTH6623	Thr308→Ile (ACA→ATA), Ile95→Leu (ATC→CTC)
BTH6642	Thr308→Ile (ACA→ATA), Ile95→Leu (ATC→CTC)
BTH6643	Thr308→Ile (ACA→ATA), Ile95→Leu (ATC→CTC)
BTH6652	Thr308→Ile (ACA→ATA)
BTH7024	Thr308→Ile (ACA→ATA), Ile95→Leu (ATC→CTC), Met292→Ile (ATG→ATA)
BTH7044	Thr308→Ile (ACA→ATA), Ile95→Leu (ATC→CTC), Met292→Ile (ATG→ATA)

^aCodons with mutation are shown in parentheses.

^bNo mutation was found in *B. subtilis* IPMDH gene.

Sequence of mutant *leuB* genes

The chromosomal DNA of each mutant strain was isolated. The *leuB* gene was amplified by PCR, cloned and sequenced as described in "Materials and Methods". Table 6-2 shows the amino acid replacements revealed by the DNA sequencing of the *leuB* genes from the thermostabilized strains. The DNA sequence of the *leuB* gene from strain BTH6151 showed no nucleotide replacement, suggesting that mutations at positions outside of the *leuB* gene led the phenotype of leucine autotrophic growth at 61°C. One possible explanation is that a mutation improving the promoter activity of the *leu* operon was generated and thereby the expression efficiency of the *leuB* gene was increased.

The sequences of the gene from BTH6121, BTH6141 and BTH6652 contained a same base substitution which resulted in Thr308→Ile replacement. The *leuB* genes from BTH6622, BTH6623, BTH6642 and BTH6643 contained an additional base substitution which resulted in Ile95→Leu replacement. Namely, these *leuB* genes encode the mutant enzyme with double-replacements, Ile95→Leu and Thr308→Ile substitutions. The DNA sequence analyses of BTH7024 and BTH7044 revealed that the *leuB* genes of these strains contained three point mutations corresponding to Ile95→Leu, Met292→Ile and Thr308→Ile.

Thermal stabilities of mutant enzymes

The wild-type and mutant *leuB* genes were recloned into pUC119 and expressed in *E. coli* JA221. The cells were lysed by sonication and the soluble fractions were used for enzyme purification. The levels of thermal stability of the purified enzymes were estimated by measuring the remaining activities at 40°C after heat treatment for 10 min at various temperatures ranging 40-63°C. The thermal inactivation processes of the wild-type and mutant enzymes were irreversible under the conditions employed in the present study. Figure 6-2 shows that the mutant enzymes T308I, I95L/T308I and I95L/M292I/T308I are more stable against heat treatment than the wild-type enzyme by 4.5, 8.0 and 10.0°C, respectively, in terms of the half-inactivation temperature at which 50%-inactivation is observed after heat treatment for 10 min.

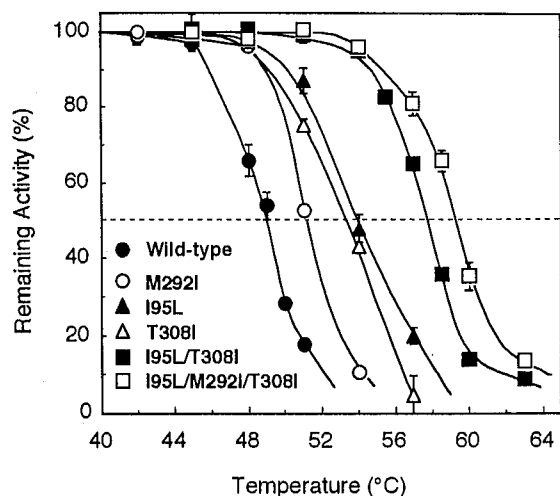


Figure 6-2. Activities of *B. subtilis* IPMDHs after heat treatment. Each purified enzyme was diluted to 0.4 mg/ml with 20 mM potassium phosphate buffer (pH 7.6) containing 0.5 mM EDTA and treated at the indicated temperatures for 10 min. The remaining activities were expressed as percentages of the original activities. Each value is the average of three independent determinations.

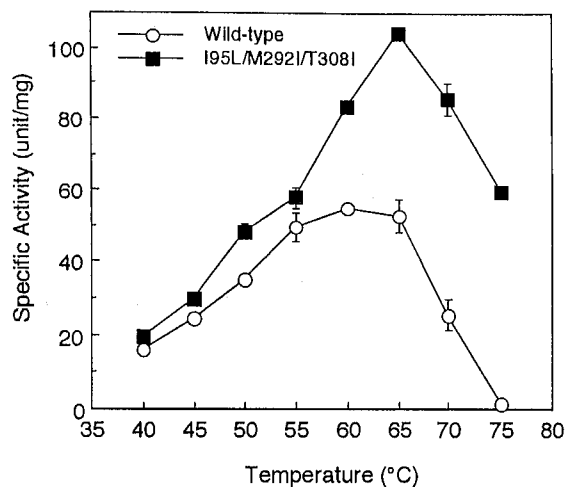


Figure 6-3. Effect of temperature on activities of the wild-type and triple-mutant *B. subtilis* IPMDHs. The specific activities were measured in 100 mM potassium phosphate buffer (pH 7.6) containing 1 M KCl, 0.2 mM MnCl₂, 0.4 mM D-3-IPM and 8 mM NAD. Each value is the average of three independent determinations.

To examine the effect of single mutations Ile95→Leu and Met292→Ile on thermal stability of the enzyme, the mutant enzymes I95L and M292I were also purified and their thermal inactivations were analyzed. Figure 6-2 shows that the I95L and M292I mutants are more thermostable than the wild-type enzyme by 5.0°C and 2.0°C, respectively, in half-inactivation temperature. These results indicate that the three amino acid replacements independently contributed to the enhanced thermostability of the enzyme and additive effect of these amino acid replacements was observed in the variants with the double or triple mutations.

Enzymatic activities of the mutant enzymes

The specific activity of the wild-type enzyme and its mutant with the triple-amino acid replacement were measured over temperature range 40-75°C in the presence of fixed concentrations of D-3-IPM (0.4 mM) and NAD (8 mM). Figure 6-3 indicates that the mutant enzyme shows slightly higher temperature optimum for its activity than that of the wild-type enzyme. Moreover, although the mutant enzyme shows level of activity only 1.3-fold that of the wild-type enzyme at 40°C, the mutant enzyme is 3.4-fold more active than the wild-type enzyme at 70°C. These results clearly show that our evolutionary experiment generated the mutant IPMDH that is more stable and active than the wild-type enzyme at high temperatures.

Table 6-3. Kinetic constants of *B. subtilis* IPMDH and its variants^a

Enzyme	K_m (μ M)		k_{cat} (s^{-1}) ^b	k_{cat}/K_m ($M^{-1}s^{-1}$)	
	D-3-IPM	NAD		D-3-IPM	NAD
Wild-type	30.5 ($\pm$ 2.9)	333 ($\pm$ 3)	21.2 ($\pm$ 0.3)	6.95×10^5	6.36×10^4
M292I	22.6 ($\pm$ 0.2)	248 ($\pm$ 6)	22.0 ($\pm$ 0.3)	9.73×10^5	8.87×10^4
I95L	56.8 ($\pm$ 2.4)	546 ($\pm$ 34)	23.9 ($\pm$ 1.2)	4.21×10^5	4.38×10^4
T308I	32.8 ($\pm$ 2.2)	332 ($\pm$ 20)	23.2 ($\pm$ 0.8)	7.07×10^5	6.98×10^4
I95L/T308I	60.1 ($\pm$ 2.5)	433 ($\pm$ 35)	22.6 ($\pm$ 0.9)	3.76×10^5	5.21×10^4
I95I/M292I/T308I	37.4 ($\pm$ 1.8)	241 ($\pm$ 6)	26.0 ($\pm$ 0.6)	6.95×10^5	1.07×10^5

^aThe kinetic constants were determined in steady-state experiments at 40°C in 100 mM potassium phosphate buffer containing 1 M KCl, 0.2 mM MnCl₂, and various concentrations of D-3-IPM and NAD. Each value is the average of three independent determinations. Standard deviations are shown in parentheses.

^b k_{cat} values are expressed as reaction per dimer.

The steady-state kinetic parameters of the wild-type and mutant enzymes are listed in Table 6-3. The K_m values for D-3-IPM and for coenzyme NAD of the I95L mutant increased to 1.9- and 1.6-fold those of the wild-type enzyme, respectively. In contrast, the single Met292→Ile replacement improves K_m values for both D-3-IPM and NAD; the both values decreased to 0.75-fold those of the wild-type enzyme. For the enzyme with the triple-mutations, in contrast to the K_m value for D-3-IPM which is almost similar to that of the wild-type enzyme, the K_m values for NAD is slightly improved compared with that of the wild-type enzyme. Slight increase in k_{cat} value is also observed in the mutant enzyme with the triple-amino acid replacements.

DISCUSSION

Evolutionary modification technique using a thermophile as a host cell is one of the useful approaches to improve thermal stability of proteins. The first *in vivo* selection of an enzyme with enhanced thermostability was carried out with kanamycin nucleotidyltransferase by Liao et al. (1986). We previously reported another evolutionary engineering experiment (Tamakoshi et al., 1995). We developed an integration vector system for *T. thermophilus* and introduced the gene encoding the chimeric IPMDH 2T2M6T into the extreme thermophile. This thermophilic species shows the highest growth temperature among microorganisms whose genetic manipulation system has been developed. The thermal stability of the chimeric enzyme was improved by selecting a spontaneous mutant at 79°C. In this chapter, we introduced *B. subtilis leuB* gene into *T. thermophilus* using the integration vector system and successfully thermostabilized the mesophilic IPMDH.

The contribution of a single amino acid replacement to protein stabilization is not so large in general and therefore the combination of multiple stabilizing mutations is necessary to obtain hyperthermostable mutant proteins (Stearman et al., 1988; Pantoliano et al., 1989; Akasako et al., 1995). In this study, the selection of mutations was repeated three times as selection temperature was increased from 61 to 66 and then to 70°C and the mutant enzyme with the triple amino acid replacements was obtained. The thermal stability of the mutant enzyme was higher than that of the wild-type enzyme by 10°C in half-inactivation temperature, indicating that the successive selection of

thermostabilized variants is a powerful strategy to obtain mutant proteins with significant increase in thermal stability.

In order to further stabilize the mutant enzyme with the triple amino acid replacements, the strain BTH7024 has been cultured at 73°C on the minimum medium plate without leucine. Unfortunately, no colony with improved thermal stability was obtained by this method. A limitation to the evolutionary modification technique used in this study is that the method rarely generates contiguous double-base replacements in one codon. Because of this rare incidence, several types of amino acid substitution cannot be practically produced. Recently, a saturation mutagenesis approach was used to identify amino acid residues critical for the function of β -lactamase (Huang et al., 1996). The strategy consisted of randomizing three contiguous codons to create a random library containing all possible amino acid substitutions for the region randomized. An application of this saturated randomization technique may be effective to identify additional stabilizing substitutions which are generated through two simultaneous base replacements within a codon.

Although stabilization of an enzyme is sometimes achieved with a cost of reduced activity (Shoichet et al., 1995), the evolutionary technique produces variants with increased stability without significant loss of catalytic activity because the variants are selected for their activities *in vivo* at a higher growth temperature. The variant with the triple mutations obtained in this study showed specific activity slightly higher than that of the wild-type enzyme at 40°C (Fig. 6-3). Moreover, the maximum value of the specific activity of the variant at 65°C was 2-fold higher than that of the wild-type enzyme at 60°C (Fig. 6-3). These findings clearly show that our evolutionary experiment generated mutant enzymes with increased activity as well as increased stability compared to those of the original enzyme.

In the present study, we selected three mutations that stepwise increased thermal stability of *B. subtilis* IPMDH. The mutations were generated in the order of Thr308→Ile (ACA→ATA, at 61°C), Ile95→Leu (ATC→CTC, at 66°C) and Met292→Ile (ATG→ATA, at 70°C) in most cases of the independent experiments (Fig. 6-4). The increase in thermal stability by the Thr308→Ile mutation is almost similar to that by the Ile95→Leu mutation (Fig. 6-2). Nevertheless, the mutant enzyme with Thr308→Ile substitution was selected as the first mutation, i.e. BTH6121, BTH6141 and BTH6652. The difference in the mutation rate of C→T (Thr308→Ile) and A→C (Ile95→Leu) base replacements may be responsible for that the Thr308→Ile mutation was selected prior to the Ile95→Leu mutation. Li et al. (1984) previously reported that the frequency of C→T base replacement is significantly higher than that of A→C base replacement.

The increase in thermal stability by the Met292→Ile mutation is less than that by the other two mutations. However, only the Met292→Ile mutation significantly improves K_m values for D-3-IPM and NAD of the enzyme. Generally, K_m value of an enzyme for its substrate increases with increase in reaction temperature, indicating that production of a mutant enzyme functional at an unusually high temperature requires an improved K_m value as well as an enhanced thermal stability. Therefore, it is reasonably speculated that the *in vivo* substrate concentration is as such that the Met292→Ile mutation effectively contributes to make the enzyme functional at 70°C rather than the mutation does at 61 and 66°C. This can be why the Met292→Ile mutation was selected at 70°C.

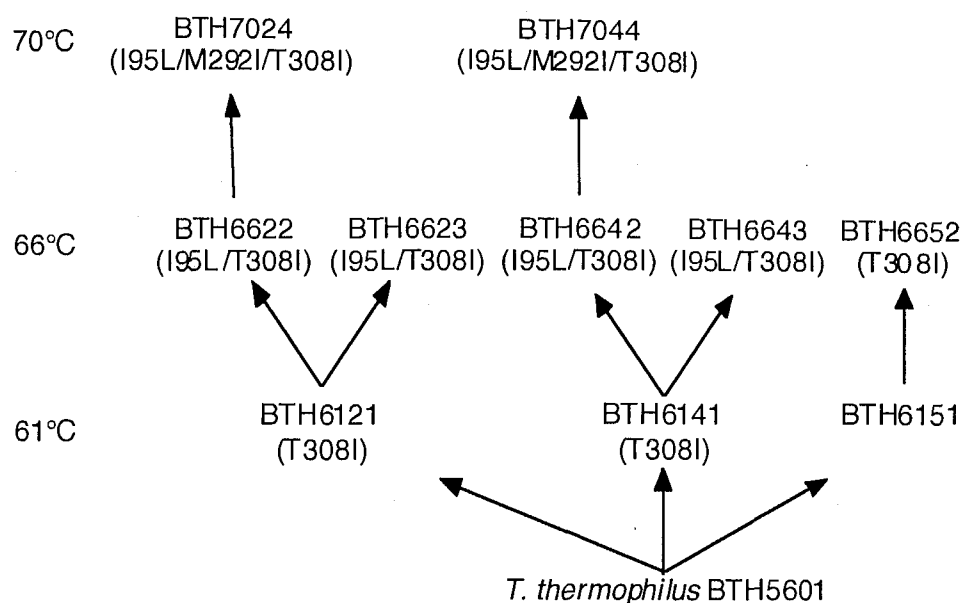


Figure 6-4. Evolution pathway of *T. thermophilus* mutant strain which carried *B. subtilis leuB* gene. Temperatures at which mutant strains were selected are shown.

Figure 6-5 shows the amino acid sequence of *B. subtilis* IPMDH, which is composed of 365 amino acid residues, aligned with that of the thermophilic counterpart *T. thermophilus* IPMDH. The amino acid sequence of *T. thermophilus* IPMDH shows 56% identity to that of *B. subtilis* IPMDH. In addition to the types of amino acid replacement, the localization in the primary sequence of the amino acid residues with mutations that increase the protein stability are also shown in Fig. 6-5. The replacements of the residues with the corresponding ones of *T. thermophilus* enzyme are found at positions 95 and 292. However, the residue at position 308 found in the thermostabilized variant of *B. subtilis* IPMDHs is different from that in the sequence of *T. thermophilus* enzyme: *T. thermophilus* IPMDH sequence contains His at this position, whereas it is Ile in the stabilized *B. subtilis* IPMDH. The result indicates that stabilization of thermolabile proteins does not always require to change a residue to corresponding one found in the sequence of the thermophilic counterpart.

The high-resolution three-dimensional structure of *T. thermophilus* IPMDH has been determined by X-ray crystallographic studies (Imada et al., 1991), showing that the enzyme is a dimeric protein composed of two identical subunit each consisting of two structural domains; one (domain 1) contains the N- and C-termini, and the other (domain 2) contains the subunit interface (Fig. 6-6). In contrast, the efforts to crystallize *B. subtilis* IPMDH have been unsuccessful so far, precluding any crystallographic analyses. However, the significant sequence homology between *B. subtilis* and *T. thermophilus* IPMDHs on their primary structure suggests that both enzymes have similar secondary and tertiary structures. The crystal structure of *T. thermophilus* IPMDH suggests that all of the mutated residues are located in domain 1 which is not involved in the subunit interaction. Iwasaki et al. (1996) previously studied the thermal unfolding process of the chimeric IPMDH

2T2M6T by differential scanning calorimetry and CD spectrophotometry. They reported the biphasic thermal unfolding transition of the chimeric enzyme; the first phase corresponds to the unfolding of domain 1 and the second to that of domain 2. Similar biphasic thermal denaturations were observed in the CD melting profiles of *B. subtilis* IPMDHs (data not shown), suggesting that the thermal denaturation of domain 1 is occurred prior to that of domain 2. Iwasaki et al. also suggested that the improvement of the thermal stability of domain 1 affects to the stability of the whole molecule more effectively than that of domain 2 does. Our present results support the previous finding that the mutations in the thermolabile domain (domain 1) can efficiently improve the thermostability of the whole enzyme.

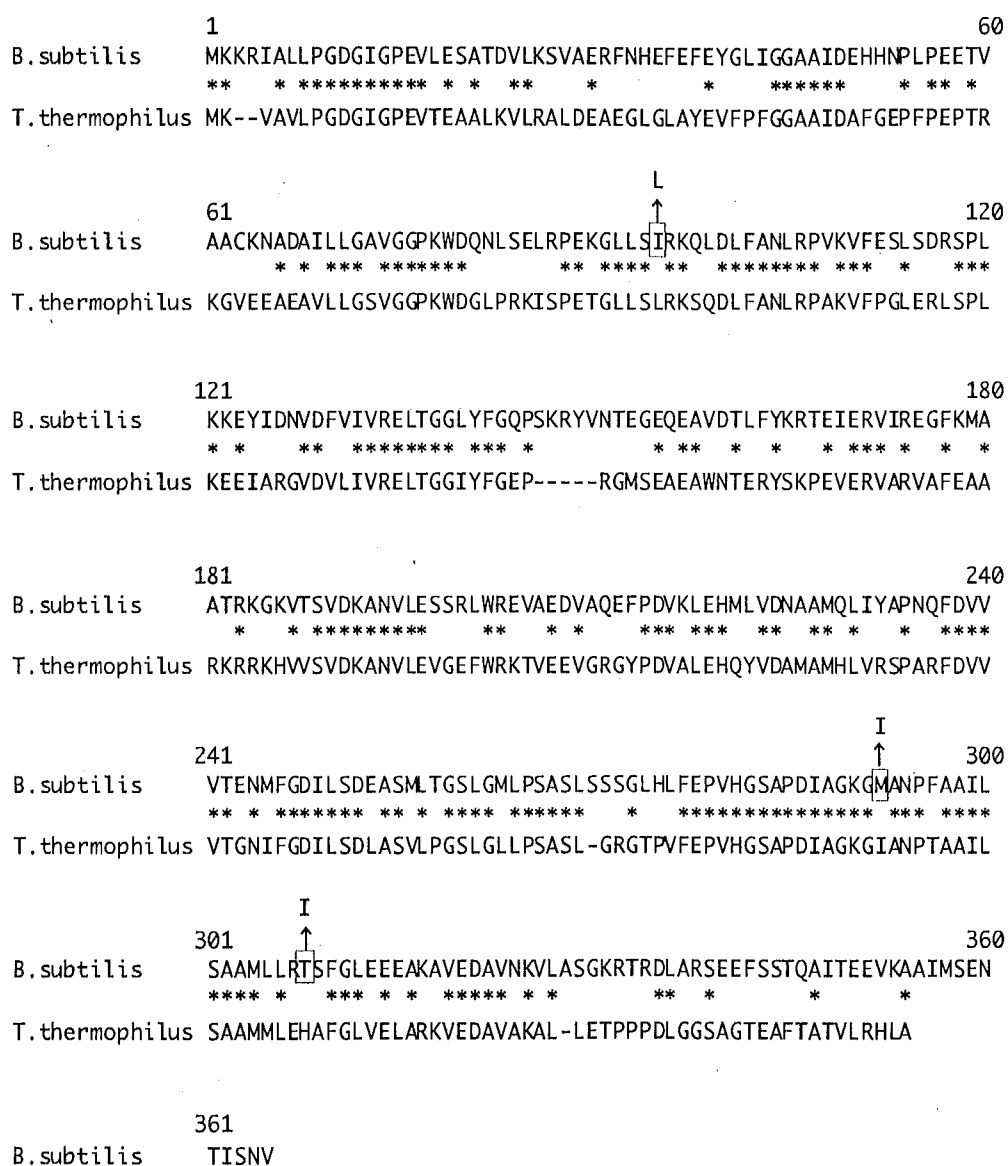


Figure 6-5. Alignment of the amino acid sequences of *B. subtilis* and *T. thermophilus* IPMDHs. Numbers above the sequences are those of *B. subtilis* IPMDH. Identical amino acid residues between *B. subtilis* and *T. thermophilus* IPMDHs are indicated by an asterisk. The residues found in the mutants with enhanced thermostability are indicated by arrows, and the original residues are boxed.

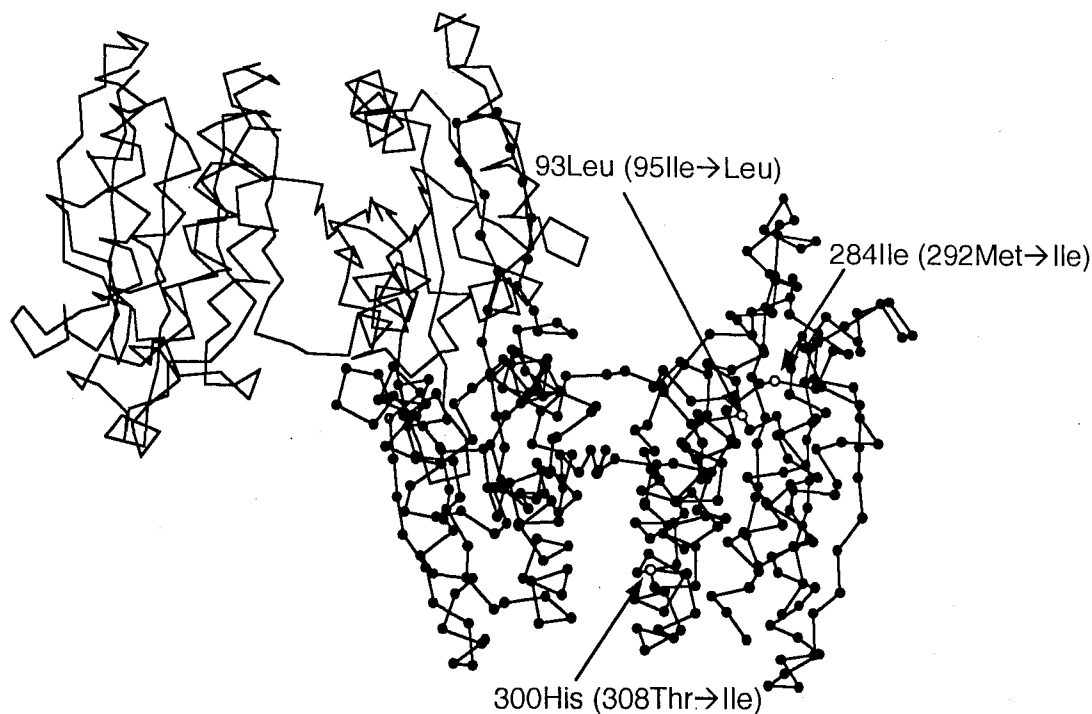


Figure 6-6. Backbone structure of *T. thermophilus* IPMDH. α -Carbons in one subunit of the dimer are represented with filled circles. Amino acid substitutions found in the mutant IPMDHs are shown in parentheses with residue number in *B. subtilis* IPMDH. Corresponding residues in *T. thermophilus* IPMDH are represented by open circles and are indicated by an arrow with the position of the residue.

Several authors have reported improvement of protein thermostability by increasing intramolecular hydrophobicity of the proteins (Yutani et al., 1987; Matsumura et al., 1988; Kellis et al., 1989). We have previously found a mutation Ala172→Val in a thermostabilized *T. thermophilus* IPMDH variant which was obtained by means of a suppressor mutation method (Chapetr 2). A crystal structure of the thermostabilized variant revealed that the Ala172→Val mutation filled up a gap between domains 1 and 2, which was surrounded by His300 and several hydrophobic residues (Qu et al., unpublished result). Thereby, we believe that the enhanced thermal stability of the *T. thermophilus* IPMDH variant was caused by the improved hydrophobic interaction between the domains. Since the residue at position 308 in the sequence of *B. subtilis* IPMDH corresponds to His300 in that of the *T. thermophilus* enzyme (Fig. 6-5), the Thr308→Ile substitution is also expected to improve the domain-domain interaction through the increased hydrophobicity. To test this possibility, it must be a useful approach to randomize the codon for the residue 308 to generate all possible amino acid substitutions. The study is under investigation.

On the other hand, the effect of the mutation at amino acid position 95 can not be explained by hydrophobicity of the residues; the introduced residue, leucine, is slightly less hydrophobic than the original one, isoleucine. Previously, the same type of amino acid replacement was found in the thermostabilized variant of chimeric IPMDH (Tamakoshi et al., 1995), that was also obtained by the evolutionary experiment. The chimeric enzyme variant with enhanced thermostability had also leucine at 93, the site corresponds to Ile95 in the sequence of *B. subtilis* IPMDH, instead of the original residue isoleucine. The crystal structures of the original and mutant chimeric enzymes have been determined (Onodera et al., 1994; Sakurai et al., 1995), and the results suggest that the re-

removal of the steric repulsion by the mutation plays a crucial role in the increased thermostability. A similar mechanism may be involved in the increased thermostabilities of *B. subtilis* IPMDHs with the Ile95→Leu mutation.

The amino acid sequence of *T. thermophilus* IPMDH has Ile at position 284 which corresponds to Met292 in the sequence of *B. subtilis* IPMDH (Fig. 6-5). The residue is in a loop linking a β -strand and an α -helix in the crystal structure of the thermophile enzyme (Fig. 6-6). Although the detailed mechanism of the stabilization by the Met292→Ile mutation has not been apparent yet, it is most likely that the improved hydrophobic interaction is responsible for the increased thermostability of the *B. subtilis* IPMDH variants with the Met292→Ile mutation.

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Chapter 7: Stepwise increasing the thermal stability of 3-isopropylmalate dehydrogenase from *Bacillus subtilis* by random and site-directed mutagenesis

ABSTRACT

A thermostabilized mutant of *Bacillus subtilis* 3-isopropylmalate dehydrogenase obtained in the previous study contained a set of triple amino acid substitutions. To further improve the stability of the mutant, we used a random mutagenesis technique and identified two additional thermostabilizing substitutions, Thr22→Lys and Met256→Val, that separately endowed the protein with further stability. Then, we introduced the two mutations into the single enzyme molecule, thus constructing a mutant with the overall quintuple mutations. Other studies have suggested that an improved hydrophobic subunit interaction and a rigid type II β -turn play important roles in enhancing the protein stability. Based on those observations, we successively introduced amino acid substitutions into the mutant with the quintuple mutations by site-directed mutagenesis: Glu253 at the subunit interface was replaced by Leu to increase the hydrophobic interaction between the subunits; Glu112, Ser113 and Ser115 that were involved in the formation of the turn were replaced by Pro, Gly and Glu, respectively, to make the turn more rigid. The thermal stability of the mutant at each replacement step was determined based on remaining activity after heat treatment and first-order rate constant of thermal unfolding, which showed a gradual increases in thermal stability as more mutations were included.

INTRODUCTION

It is usually assumed that protein thermostability can be improved through a single amino acid replacement without apparent change of backbone structure. However, the contribution of a single amino acid replacement to protein stabilization is not so large in general. Therefore, the combination of multiple stabilizing mutations is required to create hyperthermostable proteins.

3-Isopropylmalate dehydrogenase (IPMDH, EC 1.1.1.85), encoded by a *leuB* gene, catalyzes the oxidative decarboxylation of (2*R*,3*S*)-3-isopropylmalate to 2-oxoisocaproate, which is involved in leucine biosynthesis. The *leuB* genes have been cloned from a variety of microorganisms, including *Bacillus subtilis*, *Escherichia coli* and an extreme thermophile, *Thermus thermophilus*, and their sequences have been determined (Nagahari and Sakaguchi, 1978; Tanaka et al., 1981; Kagawa et al., 1984; Imai et al., 1987; Kirino et al., 1994). The *T. thermophilus* enzyme has been well-studied in terms of its catalytic properties and thermal stability (Yamada et al., 1990). The enzyme from *B. subtilis* has also been purified and characterized (Numata et al., 1995). While the high resolution X-ray crystallographic structure has been determined for *T. thermophilus* IPMDH (Imada et al., 1991), crystallization experiments of *B. subtilis* IPMDH have been unsuccessful so far, precluding any crystallographic analysis of the mesophilic enzyme. However, sequence alignment analysis of *T. thermophilus* and *B. subtilis* IPMDHs shows significant amino acid sequence similarity, indicating that both enzymes must have similar secondary and tertiary structures. Several chimeric IPMDHs have also been constructed by combining parts of the enzymes from *T.*

thermophilus and *B. subtilis* (Numata et al., 1995).

We previously developed an integration and expression host-vector system which makes us possible to introduce foreign DNAs into the *leuB* locus within the chromosomal DNA of *T. thermophilus* strains by homologous recombination (Tamakoshi et al., 1995). We demonstrated the integration of *B. subtilis leuB* gene into a *leuB*-deficient strain of *T. thermophilus* (Chapter 6). The resulting transformant is able to grow at 56 but not at 61°C without leucine because *B. subtilis* IPMDH is not sufficiently stable at 61°C and above. Phenotypically thermostabilized strains which can grow at 70°C without leucine were isolated by stepwise-selection of spontaneous mutant at 61, 66 and then 70°C under the condition lacking leucine. The thermostabilized strains carried the gene for the mutant enzyme with three of single amino acid substitutions, Ile95→Leu, Met292→Ile and Thr308→Ile. The thermal stability of the mutant enzyme was significantly higher than that of the wild-type enzyme (Chapter 6).

In this chapter, we further improved the thermal stability of the mutant enzyme with the three amino acid substitutions by using error-prone PCR mutagenesis technique. In addition, site-directed mutagenesis was carried out to successively introduce a single and a set of triple mutations that were expected to improve a subunit-subunit interaction and to make a turn structure more rigid, respectively. The resulting mutant contained nine point mutations and exhibited a large increase in thermal stability compared to the wild-type enzyme.

The mutant enzymes of *B. subtilis* IPMDH present in this chapter are designated as shown in Table 7-1.

Table 7-1. Designation of *B. subtilis* IPMDH variants

Variant	Mutations
Trip	T308I, I95L, M292I
Quad-TK	T308I, I95L, M292I, T22K
Quad-MV	T308I, I95L, M292I, M256V
Pent	T308I, I95L, M292I, T22K, M256V
Hex	T308I, I95L, M292I, T22K, M256V, E253L
Nona	T308I, I95L, M292I, T22K, M256V, E253L, E112P, S113G, S115E

EXPERIMENTAL PROCEDURES

Strains, media and plasmids

T. thermophilus strain BTH7024 carries the mutant *leuB* gene encoding *B. subtilis* IPMDH with the triple amino acid substitutions, isoleucine to leucine at position 95, methionine to isoleucine at 292 and threonine to isoleucine at 308 (Chapter 6). The strain was cultured in *Thermus* nutrient medium (Oshima and Imahori, 1974) or *Thermus* minimum medium (Sakaki and Oshima, 1975) supplemented with isoleucine and valine (25 mg/liter each). Solid media contained 1.0 % (w/v) Gelrite (Wako Pure Chemical Industries, Ltd.). *E. coli* strain JA221 (*F'*, *hsdR*, *DtrpE5*, *leuB6*, *lacY*, *recA1*) was used as the host for expression of the *leuB* genes and was cultured in 2YT medium (Sambrook et al., 1989). Plasmid pBT119 (Chapter 6) and its derivatives were used to express *B. subtilis leuB* genes in *E. coli* JA221.

DNA procedures

Recombinant DNA experiments were performed by standard methods (Sambrook et al., 1989). Restriction endonucleases and modification enzymes were purchased from Takara shuzo, Toyobo, and New England Biolab. To generate randomly mutated *leuB* genes, error-prone PCR mutagenesis was performed by the method of Leung et al. (1989) using oligonucleotides, 5'ACC GCA CCG CTC CCCTCT

TCA TCC3' and 5'AGG CAA GAT CTT TTATTT CCT CCC TCT AC3', as specific PCR primers for the *leuB* gene. Site-directed mutagenesis was carried out by the method of Kunkel et al. (1987) using oligonucleotides, 5'CGA GCC TGT AAG TAC TGA CGC TAG ATC GCT TAA AAT ATC3' and 5'GGC GAA CGG TCT TCT AGA CCT GGA AAT ACC3', to generate amino acid substitutions, Glu253→Leu and Glu112→Pro+Ser113→Gly+Ser115→Glu, respectively. Nucleotide sequencing was carried out by the dideoxy chain termination method (Sanger et al., 1977). The template DNAs for the nucleotide sequencing were prepared as described in Chapter 6.

Transformation of T. thermophilus

The randomly mutated *leuB* genes were directly used to transform *T. thermophilus* BTH7024. The transformation was carried out as described by Koyama et al. (1986), with slight modifications reported by Takada et al. (1993). The transformation mixture was incubated at 65°C for 3 h and then plated on *Thermus* minimum medium without leucine. Five colonies appeared after a 3-day incubation at 73°C.

Enzyme assay

Production and purification of *B. subtilis* IPMDHs were carried out as described by Numata et al. (1995). All the enzyme used in this study were purified to homogeneity as judged by SDS-gel electrophoresis. The enzymatic activity was measured as described by Yamada et al. (1990). To determine the stability against thermal inactivation, the remaining activities of the purified enzymes were measured at 40°C after heat treatment for 10 min at various temperatures ranging 40-70°C as described in Chapter 2. The kinetic parameters, Michaelis constants (K_m s) for D-3-isopropylmalate (D-3-IPM), a substrate of IPMDH, and coenzyme NAD, and catalytic constant (k_{cat}) were determined in steady state experiments at 40°C as described in Chapter 2.

Temperature-jump experiments

Temperature-jump relaxation experiments were performed by manual mixing technique and thermal denaturation reaction was monitored by change in circular dichroism (CD) values at 222 nm with a Jasco J-720C spectropolarimeter equipped with a circulating bath and a temperature controller. The buffer used for the unfolding experiments was 20 mM potassium phosphate, pH 7.6. In contrast to the remaining activity measurements, where the protein concentration used was 0.4 mg/ml, the purified enzymes were diluted to 0.05 mg/ml for the temperature-jump experiments in order to prevent aggregation, which can occur at higher protein concentrations. The temperature-jump relaxation profiles were fitted to a single-exponential decay and then the rate constants for the thermal unfolding reaction of the wild-type and mutant enzymes were determined. The activation enthalpy ($\Delta H^\ddagger$) and activation free energy ($\Delta G^\ddagger$) at 70°C were calculated using the transition state theory.

RESULTS

Stabilization by random mutagenesis

The mutant strain of *T. thermophilus* BTH7024 carries the *leuB* gene encoding *B. subtilis* IPMDH with the three amino acid substitutions, Trip, and shows the phenotype of leucine-autotrophic growth at 70°C but not at 73°C, while the wild-type strain of *T. thermophilus* grows at 73°C without leucine (Chapter 6). The *leuB* gene encoding the Trip mutant was amplified by PCR under the condition of reduced *Taq* DNA polymerase fidelity and the pool of amplified DNA fragments was used to transform *T. thermophilus* BTH7024. The transformed cells were plated onto the minimum medium without leucine and then 5 leucine-autotrophic colonies were grown after a 3-day incubation at 73°C. The DNA sequencing of the *leuB* genes isolated from the phenotypically thermostabilized mutants, designated as BTH7325, BTH7326, BTH7327, BTH7328 and BTH7329, revealed additional two types of thermostabilizing substitutions, Thr22→Lys and Met256→Val, which are shown in Table 7-2. These mutant enzymes are named Quad-TK and

Quad-MV, respectively (Table 7-1).

The *leuB* genes encoding the mutant enzymes, Quad-TK and Quad-MV, were overexpressed in *E. coli* and the products were purified to homogeneity. The thermal stabilities of the enzymes were estimated by measuring the remaining activities after heat treatment for 10 min at various temperatures. The thermal inactivation processes of the enzymes were irreversible under the conditions employed here. Half-inactivation temperatures at which 50% inactivation is observed after heat treatment for 10 min are used to compare the thermal stabilities of the enzymes. Figure 7-1 shows that Quad-TK and Quad-MV are more stable against heat treatment than the Trip mutant by 1.0 and 2.2°C, respectively.

The two amino acid substitutions, Thr22→Lys and Met256→Val, were simultaneously introduced into the Trip mutant, thus constructing a mutant enzyme with the overall quintuple mutations. The thermal inactivation of the mutant enzyme, Pent, was also analyzed. Figure 7-1 shows that the two stabilizing effect are additive; the Pent mutant is more resistant against heat treatment than both Quad-MV and Quad-TK mutants: the half-inactivation temperature of Pent is higher than that of Trip by 3.0°C.

Table 7-2. Additional mutations found in the *leuB* gene from the thermostabilized mutant strains

<i>T. thermophilus</i> strain	Mutations ^a
BTH7325	Thr22→Lys (ACA→AAA)
BTH7326	Thr22→Lys (ACA→AAA)
BTH7327	Thr22→Lys (ACA→AAA)
BTH7328	Met256→Val (ATG→GTG)
BTH7329	Met256→Val (ATG→GTG)

^aAll the *leuB* genes have original mutations corresponding to Ile95→Leu, Met292→Ile and Thr308→Ile in addition to that listed in the table. Codons with mutation are shown in parentheses.

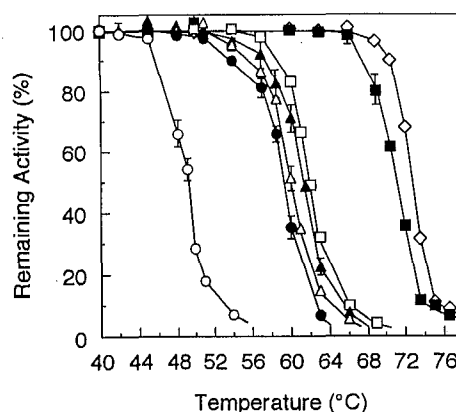


Figure 7-1. Activities of *B. subtilis* IPMDHs after heat treatment. Each purified enzyme was diluted to 0.4 mg/ml with 20 mM potassium phosphate buffer (pH 7.6) containing 0.5 mM EDTA and treated at the indicated temperatures for 10 min. The remaining activities were measured at 40°C in 100 mM potassium phosphate buffer (pH 7.6) containing 1 M KCl, 0.2 mM MnCl₂, 0.4 mM D-3-IPM and 0.8 mM NAD, and were expressed as percentages of the original activities. The values are the averages of three independent determinations. Open circles, wild type; closed circles, Trip; open triangles, Quad-TK; closed triangles, Quad-MV; open squares, Pent; closed squares, Hex; open diamonds, Nona.

Improvement of subunit-subunit interaction

Previously (Kirino et al., 1994), two residues at the subunit interface of *E. coli* IPMDH, Glu256 and Met259, were substituted with corresponding residues found in *T. thermophilus* IPMDH, Leu and Val, respectively. The mutated *E. coli* IPMDH was more thermostable than the wild-type *E. coli* enzyme. Amino acid sequence of *B. subtilis* IPMDH also contains residues Glu253 and Met256 at the positions corresponding to Glu256 and Met259 of *E. coli* IPMDH, respectively. Our random mutagenesis study revealed that the Met256→Val substitution improved the thermal stability of the Trip mutant of *B. subtilis* IPMDH. Therefore, we examined the effect of mutation Glu253→Leu on thermal stability of *B. subtilis* IPMDH by site-directed mutagenesis. The substitution was made on the Pent enzyme and the resulting mutant was named Hex. Figure 7-1 shows that the Glu253→Leu substitution improves the protein thermostability by 9.0°C in terms of the half-inactivation temperature. The result indicates that the thermal stability of the *B. subtilis* enzyme can also be improved by the increased hydrophobic interaction between the subunits.

Making turn structure more rigid

A chimeric IPMDH 2T2M6T, whose 74th-133rd residues of peptide sequence is coded by the *B. subtilis* gene and the rest coded by the *T. thermophilus* gene, has been constructed and mutated to improve its stability (Kawaguchi et al., 1994; Numata et al., 1995). The region around residue 110 forms a turn and three amino acids differ between the chimera and *T. thermophilus* IPMDH in this region: Glu110, Ser111 and Ser113 for the chimera and Pro110, Gly111 and Glu113 for the *T. thermophilus* enzyme. The mutated chimeric enzyme, in which Glu110, Ser111 and Ser113 were replaced by Pro, Gly and Glu, respectively, exhibited improved thermostability compared to the original chimera (Kawaguchi et al., 1994). We introduced the triple amino acid substitutions at the turn into the Hex mutant of *B. subtilis* IPMDH: Glu112, Ser113 and Ser115 of the Hex mutant were substituted with Pro, Gly and Ser, respectively. Figure 7-1 shows that the resulting mutant, Nona, is significantly more resistant to heat treatment than the Hex mutant. The half-inactivation temperature of the Nona mutant was higher than that of the wild-type enzyme by 23°C.

Kinetic stability of the mutant enzymes

The thermal unfolding of the wild-type and mutant enzymes were analyzed by temperature-jump experiments. The kinetic parameters of the thermal denaturation reaction were determined by change in CD at 222 nm. Figure 2 shows the Arrhenius plot of the unfolding rate constants of the enzymes. The first-order rate constants, activation enthalpies and activation Free energies between the native and transition state of the unfolding reaction at 70°C are summarized in Table 7-3. The activation free energy is successively increased as more mutations are included in the mutants. However, no correlation was observed between activation enthalpies and activation free energies. The best improvement in the unfolding rate constant was observed by the introduction of the Glu253→Leu substitution; the rate constant of Hex is 19-fold less than that of Pent. The rate constant for the thermal unfolding at 70°C of the Nona mutant is about 900-fold less than that of the wild-type enzyme. The kinetic stabilities of the enzymes are in agreement with those determined

from the remaining activity measurement shown in Fig. 7-1.

Catalytic activities

The steady-state kinetic parameters of the enzymes at 40°C are listed in Table 7-4. All the mutant enzymes showed improved K_m values for coenzyme NAD compared with that of the wild-type enzyme. On the contrary, improved K_m values for D-3-IPM were shown only with the Quad-MV and Pent mutants. Slight increase in k_{cat} values were observed with Trip and Quad-TK whereas Quad-MV, Pent, Hex and Nona exhibited significantly reduced in k_{cat} values at 40°C. The

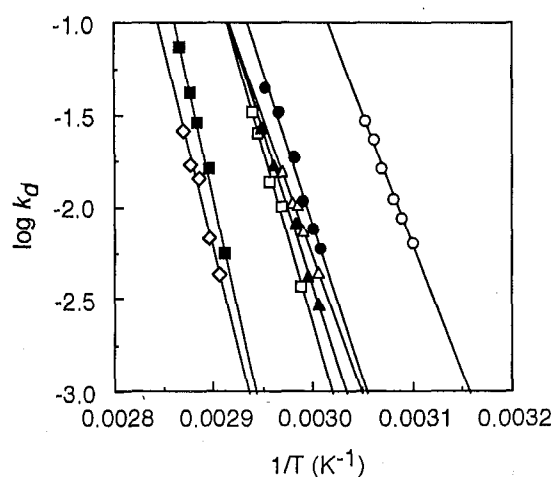


Figure 7-2. Arrhenius plots of rate constants of thermal unfolding for wild-type and mutant enzymes. The thermal denaturation rate constants were determined from the temperature-jump experiments by measuring the CD values at 222 nm. Open circles, wild type; closed circles, Trip; open triangles, Quad-TK; closed triangles, Quad-MV; open squares, Pent; closed squares, Hex; open diamonds, Nona.

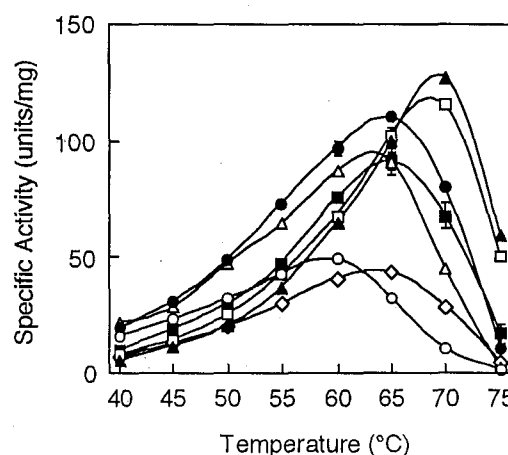


Figure 7-3. Effect of temperature on activities of *B. subtilis* IPMDHs. The specific activities were determined at the indicated temperatures in 100 mM potassium phosphate buffer (pH 7.6) containing 1 M KCl, 0.2 mM $MnCl_2$, 0.8 mM D-3-IPM and 4 mM NAD. The values are the averages of three independent determinations. Open circles, wild type; closed circles, Trip; open triangles, Quad-TK; closed triangles, Quad-MV; open squares, Pent; closed squares, Hex; open diamonds, Nona.

Table 7-3. First-order rate constants, and activation free energies and enthalpies of thermal unfolding of the wild-type and mutant enzymes

Enzyme	k_d^a	k_{wt}/k_{mut}^b	$\Delta G^\ddagger$ (kJ/mol) ^a	$\Delta H^\ddagger$ (kJ/mol)
Wild-type	2.74	1.00	81.6	271
Trip	0.227	12.1	88.7	323
Quad-TK	0.105	26.1	90.9	284
Quad-MV	0.109	25.1	90.8	325
Pent	9.81×10^{-2}	27.9	91.1	362
Hex	5.22×10^{-3}	525	99.4	469
Nona	3.05×10^{-3}	898	101.0	406

^aFirst-order rate constants and activation free energies of the unfolding reaction at 70°C are estimated from Figure 7-2.

^b k_{wt}/k_{mut} represents the ratio of the unfolding rate constant of the wild-type enzyme to that of the mutants.

Thr22→Lys and Met256→Val substitutions, that were found from the random mutagenesis, did not seriously reduce the k_{cat}/K_m values for both D-3-IPM and NAD whereas the Hex and Nona enzyme, that were constructed by site-directed mutagenesis, showed significantly decreased k_{cat}/K_m values for D-3-IPM.

The specific activity of the wild-type and mutant enzymes were measured over temperature range 40-75°C in the presence of fixed concentrations of D-3-IPM (0.8 mM) and NAD (4 mM). Figure 7-3 shows that the Quad-MV and Pent mutants exhibit significantly higher temperature optimum for their activity than that exhibited by the wild-type enzyme. In addition, the maximum values of the specific activity of Quad-MV and Pent at 70°C are higher than that of the wild-type enzyme at 60°C by 2.5-fold and 2.3-fold, respectively. On the other hand, the maximum values of the specific activity of the final construct, Nona, at 65°C is slightly less than that of the wild-type enzyme at 60°C.

Table 7-4. Kinetic constants of the wild-type and mutant enzymes

Enzyme	K_m (μ M) ^a		k_{cat} (s^{-1}) ^{a,b}	k_{cat}/K_m ($M^{-1}s^{-1}$)	
	D-3-IPM	NAD		D-3-IPM	NAD
Wild-type	30.5 ($\pm$ 2.9)	333 ($\pm$ 3)	21.2 ($\pm$ 0.3)	6.95x10 ⁵	6.36x10 ⁴
Trip	37.4 ($\pm$ 1.8)	241 ($\pm$ 6)	26.0 ($\pm$ 0.6)	6.95x10 ⁵	1.07x10 ⁵
Quad-TK	51.5 ($\pm$ 4.8)	319 ($\pm$ 6)	28.4 ($\pm$ 0.4)	5.51x10 ⁵	8.90x10 ⁴
Quad-MV	16.1 ($\pm$ 0.8)	172 ($\pm$ 19)	7.3 ($\pm$ 0.2)	4.53x10 ⁵	4.24x10 ⁴
Pent	22.4 ($\pm$ 3.0)	155 ($\pm$ 20)	11.3 ($\pm$ 0.6)	5.04x10 ⁵	7.29x10 ⁴
Hex	81.4 ($\pm$ 5.9)	256 ($\pm$ 14)	13.1 ($\pm$ 0.5)	1.61x10 ⁵	5.12x10 ⁴
Nona	57.1 ($\pm$ 3.6)	245 ($\pm$ 10)	9.2 ($\pm$ 0.0)	1.61x10 ⁵	3.76x10 ⁴

^a K_m and k_{cat} were determined in steady-state experiments at 40°C in 100 mM potassium phosphate buffer containing 1 M KCl, 0.2 mM MnCl₂, and various concentrations of D-3-IPM and NAD. Each value is the average of three independent determinations.

^b k_{cat} values are expressed as reaction per dimer.

DISCUSSION

In Chapter 6, we have improved the thermal stability of *B. subtilis* IPMDH by stepwise-selection of spontaneous mutant enzymes in *T. thermophilus* at 61, 66 and then 70°C. However, we have failed to obtain spontaneous mutants which is stable and active at 73°C. To increase the frequency of mutation, we carried out *in vitro* random mutagenesis using error-prone PCR technique in the present study. The genes with the mutation corresponding to either Thr22→Lys or Met256→Val were isolated by screening the randomly mutated *leuB* genes at 73°C. These substitutions were confirmed to increase the thermostability of the enzymes *in vitro*, indicating that the random mutagenesis is an effective strategy for the stabilization of the enzyme.

Although tertiary structure of *B. subtilis* IPMDH has not been apparent yet, the crystal structure of *T. thermophilus* IPMDH has been determined (Imada et al., 1991). The high sequence similarity between *B. subtilis* and *T. thermophilus* IPMDHs suggests that both enzymes have highly similar secondary and tertiary structures. The sequence alignment suggests that Thr22 is located in

	1	10	20	30	40	50
<i>B. subtilis</i>	M-K-KRIALLPGDGIGPEVLESA		DVLKSVAERFNHEFEFE	-YGLIGGAAIDEHNNPLPE		
<i>E. coli</i>	MSKNYHIAVLPGDGIGPEVMTQALKVLDAVRNRFAMRITTS		-HYDVGGAAIDNHGQPLPP			
<i>T. thermophilus</i>	M----KVAVLPGDGIGPEVTEAALKVLRALDEAEGGLAYEVFPF		-GGAADAFGEFPPE			
	SSSSS	HHHHHHHHHHHHHHHHHHHH	SSSSSS	HHHHHHH		
	60	70	80	90	100	110
<i>B. subtilis</i>	ETVAACKNADAILLGAVGGPKWDQNLSELRPEKG		-LLS	IRKQLDLFANLRPVKVF	ESL	SD
<i>E. coli</i>	ATVEGCEQADAVLFGSVGGPKWEHLPPDQQPERGALLPLRKHFKLFSNLRPAKLYQGLEA					
<i>T. thermophilus</i>	PTRKGVEEAEAVLLGSVGGPKWDGLPRKISPETG		-LLSLRKSQDLFANLRPAKVFPGLER			
	HHHHHHHH	SSSS	HHHHHHHHHHHH	SSSSSSSSSS		
	120	130	140	150	160	170
<i>B. subtilis</i>	RSPLKKE-YIDNVDFVIVRELTTGGLYFGQPSKRYVNTGEQEAVDTLFYKRTEIERVIRE					
<i>E. coli</i>	FCPLRADIAANGFDILCVRELTTGGIYFGQPKGREGSGQYE-KAFDTEVYHRFEIERIARI					
<i>T. thermophilus</i>	LSPLKEE-IARGVDVLIVRELTTGGIYFGEP--R--GM-SEAEAWNTERYSKPEVERVARV					
	SSSSSSSS	S--SS-S	SSSSSSSSSSHHHHHHHHHH			
	180	190	200	210	220	230
<i>B. subtilis</i>	GFKMAATRKGKVTSDKANVLESSRLWREVAEDVAQEPDVKLEHMLVDNAAMQLIYAPN					
<i>E. coli</i>	AFESARKRRHKVTSIDKANVLQSSILWREIVNEIATEYDPVELAHMYIDNATMQLIKDPS					
<i>T. thermophilus</i>	AFEAARKRRKHVVSVDKANVLEVGEFWRKTVEEVGRGYPDALEHQYVDAMAMHLVRSPA					
	HHHHHHH	SSSSSSS	HHHHHHHHHHHHHHHH	SSSSSSSSHHHHHHHHHH		
	240	250	260	270	280	
<i>B. subtilis</i>	QFDVVVTENMFGDILSD	EA	SLTGSLGMLPSASLSSSGLHLFEPVHGSAPDIAGKGMANP			
<i>E. coli</i>	-FDVLLCSNLF	GDILSD	CAMITGSMGMLPSASLNEQG	-GLYEPAGGSAPDIAGKNIANP		
<i>T. thermophilus</i>	RFDVVVTGNIF	GDILSD	LASVLP	GLSLGMLPSASL-GRGTPVFEPVHGSAPDIAGKGIANP		
	SSSSSHHHHHHHHHHHHHHH		SSSS	SSSSS		
	300	320	330	340	350	
<i>B. subtilis</i>	FAAILSAAMLLRT	SFGL	EEEEAKAVEDAVNKVLASGKRT	LDLARSEEFSSSTAITEEVKAA		
<i>E. coli</i>	IAQILSLALLRLRYSLDADDAACAIERAINRALEE	GRTGDLARGAAVSTDEMGDII-AR				
<i>T. thermophilus</i>	TAAILSAAMMLEHAFGLVELARKVEDAVAKALL-ETPPPDLG-G-S-AGTEAFTATV-LR					
	HHHHHHHHHHHH	HHHHHHHHHHHHHHHHHH-H		HHHHHHHHH-HH		
	360					
<i>B. subtilis</i>	IMSENTISNV					
<i>E. coli</i>	YVAE-GV---					
<i>T. thermophilus</i>	HLA-----					
	H					

Figure 7-4. Alignment of the amino acid sequences of *B. subtilis*, *E. coli* and *T. thermophilus* IPMDHs. Numbers above the sequences are those of *B. subtilis* IPMDH. The amino acid substitutions present in the Nona mutant are indicated. The secondary structures of *T. thermophilus* IPMDH are shown below their corresponding sequences. H, α -helix; S, β -strand.

an α -helix comprising residues 14-32 (Fig. 7-4). The new residue Lys at 22 has a positive charge that probably interacts with the negatively charged residue of Glu18 at the next position in the helix, thus leading to the protein stabilization. Alternatively, the Thr22→Lys substitution may have stabilized the α -helix because of the relative helical propensities; Lys is clearly a better helix former than Thr (Chou and Fasman, 1978).

The importance of strong hydrophobic interaction at the subunit-subunit interface for the unusual stability of *T. thermophilus* IPMDH has been speculated by Imada et al. (1991) from the crystal structure of the thermophile enzyme. Kirino et al. (1994) confirmed this speculation experimentally; they substituted Glu256 and Met259, which are located at the subunit interface of *E. coli*

IPMDH, with Leu and Val, respectively, to improve the hydrophobic interaction between the subunits and successfully increased the protein stability. The present mutagenesis study revealed that both Met256→Val and Glu253→Leu substitutions, which corresponds to the Met259→Val and Glu256→Leu substitution in *E. coli* IPMDH, respectively, were also responsible for the increased stability of the *B. subtilis* IPMDH variant. The results indicate that hydrophobic monomer-monomer interaction is an important stability determinant of IPMDHs.

Although the Glu253→Leu increased the protein thermostability more largely than the Met256→Val substitution, the Glu253→Leu substitution was not found by the random mutagenesis and *in vivo* screening procedures. This may be explained by the following reasons: i) Glu→Leu substitution is rarely generated by the error-prone PCR method because such a substitution requires two simultaneous base replacements in one codon; ii) the catalytic activity of the enzyme is reduced by the Glu253→Leu substitution and, therefore, the mutant enzyme with the Glu253→Leu substitution may not be active sufficiently to maintain the leucine biosynthesis *in vivo*.

The X-ray crystallographic study of the chimeric IPMDH 2T2M6T (Onodera et al., 1994) indicates that the backbone structure of the chimera is highly similar to that of *T. thermophilus* IPMDH, and the high root mean square deviation and the increment of the *B*-factor are concentrated into two regions originating from *B. subtilis* IPMDH, around residues 82 and 110. One of the regions, residues 110-113, forms a turn structure. The amino acid sequence of *T. thermophilus* IPMDH contains Pro110, Gly111, Leu112 and Glu113 while that of the chimeric enzyme does Glu110, Ser111, Leu112 and Ser113 in this region. The crystal structure of the thermophile enzyme (Imada et al., 1991) indicates that Pro110 restricts flexibility of the backbone in this region, Gly111 takes a typical conformation to form a type II β -turn and Glu113 forms a hydrogen bond with a main chain NH group of Gly111. Kawaguchi et al. (1994) reported that the triple mutations, Glu110→Pro+Ser111→Gly+Ser113→Glu, resulted in a remarkable increase in thermal stability of the chimera although each single mutation did not improve the stability. They concluded that the rigid type II β -turn in this region was crucial for the unusual stability of the *T. thermophilus* enzyme. Based on this finding, we changed Glu112, Ser113 and Ser115 to Pro, Gly and Glu, respectively, in combination with the sextuple mutations and successfully increased the stability of the *B. subtilis* IPMDH variant. This result strongly supports the idea that making the rigid β -turn in this region leads to the protein stabilization.

It is generally assumed that small change in the sequence of a protein should not have large effects on its stability. Therefore, an accumulation of multiple stabilizing substitutions is expected to produce mutant proteins with dramatically increased stability when the substitutions are independent on each other. Dramatic increases in protein stability were reported based on the mutagenesis studies of subtilisin (Pantoliano et al., 1989), T4 lysozyme (Matsumura et al., 1989), λ repressor (Stearman et al., 1988), barnase (Serrano et al., 1993) and ribonuclease HI (Akasako et al., 1995). These studies have indicated that the combination of multiple thermostabilizing mutations is a useful strategy for creating the highly stabilized proteins. The overall results of the present study also shows that the successive introduction of multiple mutations, each of which leads to protein stabilization, can create mutant enzymes with a greatly improved thermostability.

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Chapter 8: Concluding Remarks

Improvement of protein stability by evolutionary molecular engineering

It is generally believed that the best strategy for improving the stability of a specific protein by site-directed mutagenesis would require an information of its detailed three-dimensional structure. However, there is a limited number of proteins that satisfy this requirements. On the other hand, evolutionary molecular engineering techniques can be used to modify any protein without any knowledge of its three-dimensional structure. Moreover, the evolutionary techniques often produce modified enzymes that cannot be designed from our present knowledge. For example, the stability of *Thermus thermophilus* 3-isopropylmalate dehydrogenase (IPMDH) was improved by a Ala172→Val substitution (Chapter 2). The change was likely to fill up a small cavity present around the residue at 172. In an earlier work, the presence of a cavity inside the hydrophobic core had been searched for by computer-aided analysis (Tomuro et al., unpublished). However, the cavity around Ala172 had not been detected by that way. In other words, the evolutionary study found the small cavity that could not be detected by the computer analysis.

Another advantage of the evolutionary technique is that the method produces thermostabilized mutants without significant loss of catalytic activity. It has often been observed that the increase in thermal stability of engineered enzymes is accompanied by a decrease in their catalytic activity (Imoto et al., 1994; Shoichet et al., 1995). However, this was not the case for the mutant enzymes obtained in the present evolutionary experiments because the mutants enzymes with the increased stability were selected based on their enzymatic activity.

A limitation to the evolutionary modification technique used in this study is that the method rarely generates contiguous double-base replacements in one codon, as discussed in Chapter 3. Because of this problem, several types of amino acid substitution, such as Ala→Leu, cannot be produced. Recently, a saturation mutagenesis approach was used to identify amino acid residues critical for the function of β -lactamase (Huang et al., 1996). The strategy consisted of randomizing three contiguous codons to create a random library containing all possible amino acid residue substitutions for the region randomized. An application of this saturated randomization technique may be effective to identify additional stabilizing substitutions which are generated through two simultaneous base replacements within the codon.

Further stabilization of *T. thermophilus* IPMDH by changing residue at 172

In Chapters 2 and 3, the thermal stability of *T. thermophilus* IPMDH was successfully stabilized by a suppressor mutation method. In addition, several mutant enzymes at residue 172 were constructed and investigated in terms of their stability (Chapters 3, 4). From these studies, it was found that changing Ala172 to any of seven other residues, such as Val, Leu, Ile, Phe, Glu, Gln and Asn, led to the stabilization of the enzyme.

To analyze the molecular basis of increasing the thermal stability through these replacements, structural analyses were carried out by means of X-ray crystallography and molecular modeling. Crystal structure of the thermophile wild-type enzyme (Imada et al., 1991) shows that Ala172 is

located at a domain-domain interface and the vicinity around Ala172 is involved in the hinge part which connects the two domains. The methyl side chain of the residue is surrounded by several hydrophobic side chains in addition to two basic residues. The structural analyses of the mutant enzymes indicate that the molecular mechanism for stabilization of the thermophile enzyme can be classified into three types. First mechanism was found in the Val172 mutant. As shown in Chapter 2, the larger bulky side chain filled up a small cavity, that was detected around Ala172 in the wild-type structure, without significant change of the overall conformation, thus improving the enzyme stability. Second, in the case of the mutants with Leu172 or Phe172, the side chains of Leu and Phe are too large for the cavity mentioned above and therefore the replacements are likely to bring about a substantial conformation alteration. Crystal structures have recently been determined for the A172L and A172F mutants, showing that both replacements induced a domain movement that close the active site cleft (Qu, 1997; Qu et al., 1997). The conformation change might reduce the solvent accessible surface area of hydrophobic side chains and improve the domain-domain interaction through formation of additional hydrogen bonds between the domains (Chapter 3). The third type of the stabilization mechanism was suggested from the molecular modeling studies for the A172E, A172Q and A172N mutants. In the case of these mutant enzymes, the polar groups of the glutamate, glutamine and asparagine residues form electrostatic interactions with the surrounding residues and their methylene groups improve the hydrophobic packing of the interior core (Chapter 4).

It is well assumed that proteins are well optimized in their structures and functions for their physiological environment and, therefore, it is reasonable that the *T. thermophilus* enzyme is significantly stable before introduction of mutation. Nevertheless, it was demonstrated that the inherent thermostable enzyme could be further stabilized by changing the residue at position 172 to at least seven other residues. The result means that even the stability of the enzyme from the extreme thermophile is not fully optimized and can be improved to some extent.

Increasing thermal stability of *B. subtilis* IPMDH

The thermal stability of a naturally occurring protein is clearly a result of natural selection. On the other hand, there is no selective pressure to make proteins stable at temperatures above those which they encounter *in vivo*. Accordingly, it is reasonable that a mesophile protein has a potential to improve its thermostability. Several amino acid replacements, each of which increased the stability of *B. subtilis* IPMDH, are described in Chapters 6 and 7.

The comparison between homologous proteins from mesophiles and thermophiles has provided some insight into increased stability of thermostable proteins. Previously, Argos et al. (1979) reported that Gly, Ser, Ser, Lys and Asp in mesophile enzymes tend to be replaced by Ala, Ala, Thr, Arg and Glu, respectively, in thermophile enzymes. However, no replacement recommended by Argos et al. was found in the thermostabilized variants of *B. subtilis* IPMDH, indicating that the Argos' replacements is not always the way to improve the stability of mesophile proteins.

One of the factors that have been suggested as contributors to enhanced thermostability is the

decrease of main chain flexibility from additional proline side chains. *T. thermophilus* IPMDH has the largest number and percentage of proline residues among IPMDH sequences that have been determined, suggesting the importance of proline residues for the increased stability. However, changing to proline was not found throughout the present experiments. This leads us to conclude that either introducing proline residues is not responsible for increase in the protein stability or reduced flexibility caused by introduction of additional proline residue decreases the catalytic efficiency.

Hydrophobic interaction is thought to be the strongest driving force in protein tertiary structure. Several authors have reported the importance of the hydrophobicity of side chains to enhanced protein thermostability (Yutani et al., 1987; Matsumura et al., 1988; Kellis et al., 1989). The analyses of the amino acid sequences show that *T. thermophilus* IPMDH contains 61% of hydrophobic, 11% of neutral and 28% of hydrophilic residues in its sequence while the *B. subtilis* enzyme does 52 % of hydrophobic, 18% of neutral and 30% of hydrophilic residues. This difference in hydrophobic amino acid content may be one of the important determinants of thermal stability, which make the difference between the thermophile and mesophile enzymes. Five of the thermostabilizing replacements found in the *B. subtilis* enzymes involved hydrophobic amino acid residues and four of them increased hydrophobicity of the side chains (Chapters 6, 7). Accordingly, as long as the *B. subtilis* enzyme is concerned, introduction of a hydrophobic residue into an adequate position may be a rational way to improve its thermostability.

It is known that improvement of monomer-monomer interactions makes a contribution of the stability of proteins. The Glu253→Leu and Met256→Val replacements increased protein stability probably through improvement of the subunit-subunit interaction (Chapter 7). The result, as well as those obtained for lactate dehydrogenase (Kallwass et al., 1992), for xylose isomerase (Mrabet et al., 1992) and for triosephosphate isomerase (Ahern et al., 1987; Casal et al., 1987; Sun et al., 1992), points to the interaction between subunits as an important stability determinant. Similarly, improvement of domain-domain interaction is also expected to enhance protein stability. The enhancement of the stability upon the Thr308→Ile replacement may be because of more favorable domain-domain contact (Chapter 6). These observations suggest that improvement of interaction between structural units is one of the general strategy to increase protein stability.

Conclusion

This thesis describes directed molecular evolutions which improved thermostabilities of the thermophile and mesophile enzymes. The results described here indicate that the evolutionary strategy is a useful tool for increasing protein stability. Structural analyses for thermostabilized enzymes provided us with several principles for stabilization of the two enzymes. Particularly, it is suggested that changing residues at the interface between structural units to improve the domain-domain or subunit-subunit interaction makes an important contribution to the increased stabilities of these enzymes.

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Acknowledgments

The present work has been performed under the direction of Professor Nobuo Tanaka, Tokyo Institute of Technology, and Professor Tairo Oshima, Tokyo University of Pharmacy and Life Science.

I wish to express my sincere gratitude to Prof. Tairo Oshima and Prof. Nobuo Tanaka for suggesting this project and constant guidance throughout the course of this study.

I also gratefully acknowledge to Drs. Akihiko Yamagishi, Hideaki Moriyama and Masatada Tamakoshi for valuable advice and discussion.

Special thanks are going to many co-workers; Drs. Masahiro Sakurai and Chunxu Qu for the X-ray analyses of the mutant enzymes, Drs. Kentaro Miyazaki, Yoko Hayashi-Iwasaki, Shin-ichi Yokobori, Ms. Chie Motono, Mrs. Takashi Kotsuka, Jitsutaro Kawaguchi and Masaaki Tomuro for helpful discussion.

Finally, I gratefully thank to all co-workers in Tanaka laboratory and in Oshima laboratory.

List of Publications

The component of this thesis is composed of the following papers;

Chapter 2

Kotsuka, T., Akanuma, S., Tomuro, M., Yamagishi, A. and Oshima, T. (1996) Further stabilization of 3-isopropylmalate dehydrogenase of an extreme thermophile, *Thermus thermophilus*, by a suppressor mutation method. *J. Bacteriol.* **178**, 723-727.

Chapter 3

Akanuma, S., Yamagishi, A. and Oshima, T. (1995) Thermostabilization of 3-isopropylmalate dehydrogenase from an extreme thermophile, *Thermus thermophilus*, by introducing a bulky side chain at position 172. *Protein Eng.* **8**, Supple. 4.

Akanuma, S., Qu, C., Yamagishi, A., Tanaka, N. and Oshima, T. Multiple mutations at a single position increasing thermal stability of 3-isopropylmalate dehydrogenase. in preparation.

Chapter 4

Akanuma, S., Qu, C., Yamagishi, A., Tanaka, N. and Oshima, T. (1997) Effect of polar side chains at position 172 on thermal stability of 3-isopropylmalate dehydrogenase from *Thermus thermophilus*. *FEBS Lett.* **410**, 141-144.

Chapter 5

Akanuma, S., Yamagishi, A., Tanaka, N. and Oshima, T. (1996) Spontaneous tandem sequence duplications reverse the thermal stability of carboxyl-terminal modified 3-isopropylmalate dehydrogenase. *J. Bacteriol.* **178**, 6300-6304.

Chapter 6

Akanuma, S., Yamagishi, A., Tanaka, N. and Oshima, T. (1998) Serial increase in the thermal stability of 3-isopropylmalate dehydrogenase from *Bacillus subtilis* by experimental evolution. *Protein Sci.* in press.

Chapter 7

Akanuma, S., Yamagishi, A., Tanaka, N. and Oshima, T. Stepwise increasing thermal stability of 3-isopropylmalate dehydrogenase from *Bacillus subtilis* by random and site-directed mutagenesis. in preparation.

The other supplementary papers;

Takada, T., Akanuma, S., Kotsuka, T., Tamakoshi, M., Yamagishi, A. and Oshima, T. (1993) Recombination-deficient mutants of an extreme thermophile, *Thermus thermophilus*. *Appl. Environ. Microbiol.* **59**, 2737-2739.

Qu, C., Akanuma, S., Moriyama, H., Tanaka, N. and Oshima, T. (1997) A mutation at the interface between domains causes rearrangement of domains in 3-isopropylmalate dehydrogenase. *Protein Eng.* **10**, 45-52.

Qu, C., Akanuma, S., Moriyama, H., Tanaka, N. and Oshima, T. (1997) Design, crystallography and molecular modeling study of mutant enzyme of 3-isopropylmalate dehydrogenase from *Thermus thermophilus*. submitted.