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Effect of polar side chains at position 172 on thermal stability of 3-isopropylmalate dehydrogenase from *Thermus thermophilus*

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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 Ala[172] 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 the formation of extra electrostatic interactions and/or improvement of hydrophobic packing of the interior core.
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Key words: 3-Isopropylmalate dehydrogenase; Site-directed mutagenesis; Thermal stability; Electrostatic interaction; Hydrophobic packing

1. 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 example, by introducing disulfide bonds or metal binding site to reduce the difference in entropy between native and denatured states [1–4]; changing specific residues to stabilize the dipoles of α-helices [5]; improving intramolecular hydrophobic packing [6]; or reducing the solvent accessible hydrophobic surface area [7]. However, these strategies are only applicable to the proteins of which 3-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 [8]. The unusually thermostable enzyme of the thermophile IPMDH has been purified and characterized [9]. The 3-dimensional structure of the enzyme has been determined at 2.2 Å (1 Å = 0.1 nm) resolution by X-ray diffraction [10], 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 [11,12]. In these previous studies, we found that single amino acid replacements of Ala[172] 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 Ala[172] for Val and for 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 et al., unpublished]; 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 [13].

In this study, we constructed additional mutant enzymes of *T. thermophilus* IPMDH by site-directed mutagenesis, in which Ala[172] was replaced with polar residues. In contrast to our expectation, three mutant enzymes, in which Ala[172] 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.

2. Material and methods

2.1. 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, ΔtrpE5, 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. [14]. Oligonucleotides used for generating mutations were 5′-GCT TCC TCG CCT CTT CGA AGG CCA CCC G-3′ for substituting Ala[172] to Glu, 5′-CTT CCT GCG CTT GCG CGC ATC CTC AAA GGC CA-3′ for substituting Ala[172] to Asp, 5′-GCT TCC TCG CCT GTT CGA AGG CCA CCC G-3′ for substituting Ala[172] to Gln and 5′-CTT CCT GCG CTT GCG CGC GTT CTC AAA GGC CAC-3′ for substituting Ala[172] to Asn. Mutations in the gene were confirmed by nucleotide sequencing [15]. Expression and purification of the wild-type and mutant IPMDHs were done as described previously [9]. 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 Ala[172] is substituted for Glu, Asp, Gln and Asn, respectively.

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2.2. Thermal stability measurement

The stability against thermal inactivation of the purified enzymes was determined as described previously [11].

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 $NaHCO_3$ (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.

2.3. 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 55°C as described previously [11].

2.4. 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 [16], 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 et al. (unpublished).

3. Results and discussion

Previously, we successfully stabilized the thermophilic IPMDH by replacing Ala^{172} with more hydrophobic residues [11,12]. According to the 3-dimensional structure of the wild-type enzyme determined by X-ray crystallography [10], the Ala^{172} 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 Lys^{175} and His^{300} are surrounding the methyl side chain of Ala^{172}. Therefore, we examined the effect of introducing acidic residues at 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 Fig. 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 Fig. 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.

Table 1
Kinetic parameters of IPMDHs[a]

Variant	K_m		k_{cat}
	D-3-IPM (μM)	NAD (μM)	(s^{-1})[b]
Wild-type	22.1 (±2.5)	116 (±26)	107 (± 8)
A172D	16.9 (±1.8)	76 (±17)	100 (±13)
A172E	25.7 (±0.5)	100 (±22)	117 (± 2)
A172N	23.4 (±0.5)	105 (±30)	117 (± 2)
A172Q	26.7 (±2.2)	94 (±10)	111 (± 2)

[a]Each value is the mean of three independent determinations.
[b]k_{cat} values are expressed as reaction per dimer.

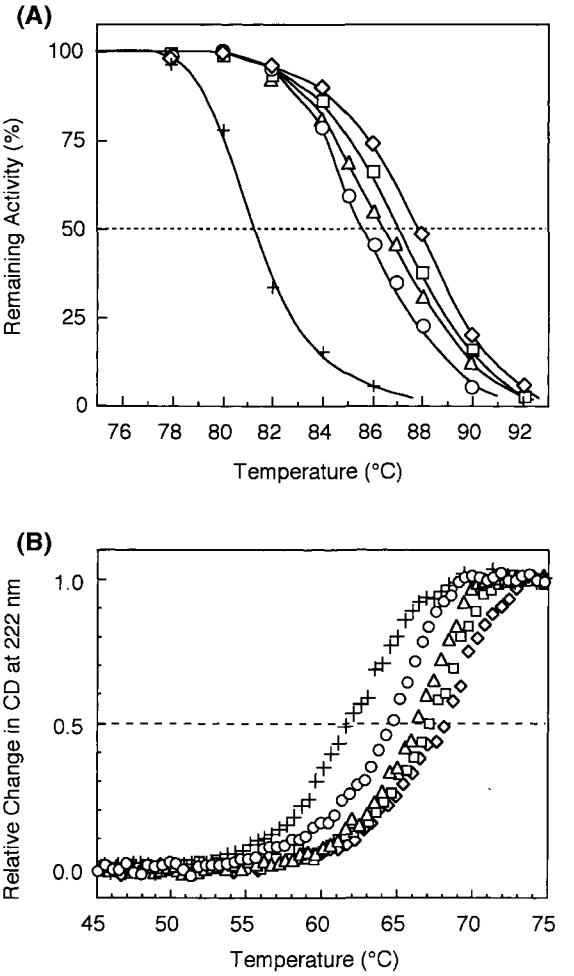

Fig. 1. Thermal stability of *T. thermophilus* IPMDHs. A: Remaining activities of IPMDHs after heat treatment. Each enzyme was diluted to 0.1 mg/ml with 20 mM potassium phosphate, pH 7.6, containing 0.5 mM EDTA and incubated at the indicated temperature for 10 min. The remaining activities were expressed 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 Section 2. The melting curves were normalized with respect to the baselines of the native and denatured states. ○, wild-type; +, A172D; △, A172E; □, A172N; ◇, A172Q.

The thermal denaturation processes of the wild-type and 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. Fig. 1B indicates that the mutations of $Ala^{172} \rightarrow$ Glu, Asn and Gln increase T_m by 1.5°C, 2.5°C and 3.3°C, respectively, while $Ala^{172} \rightarrow$ 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 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-

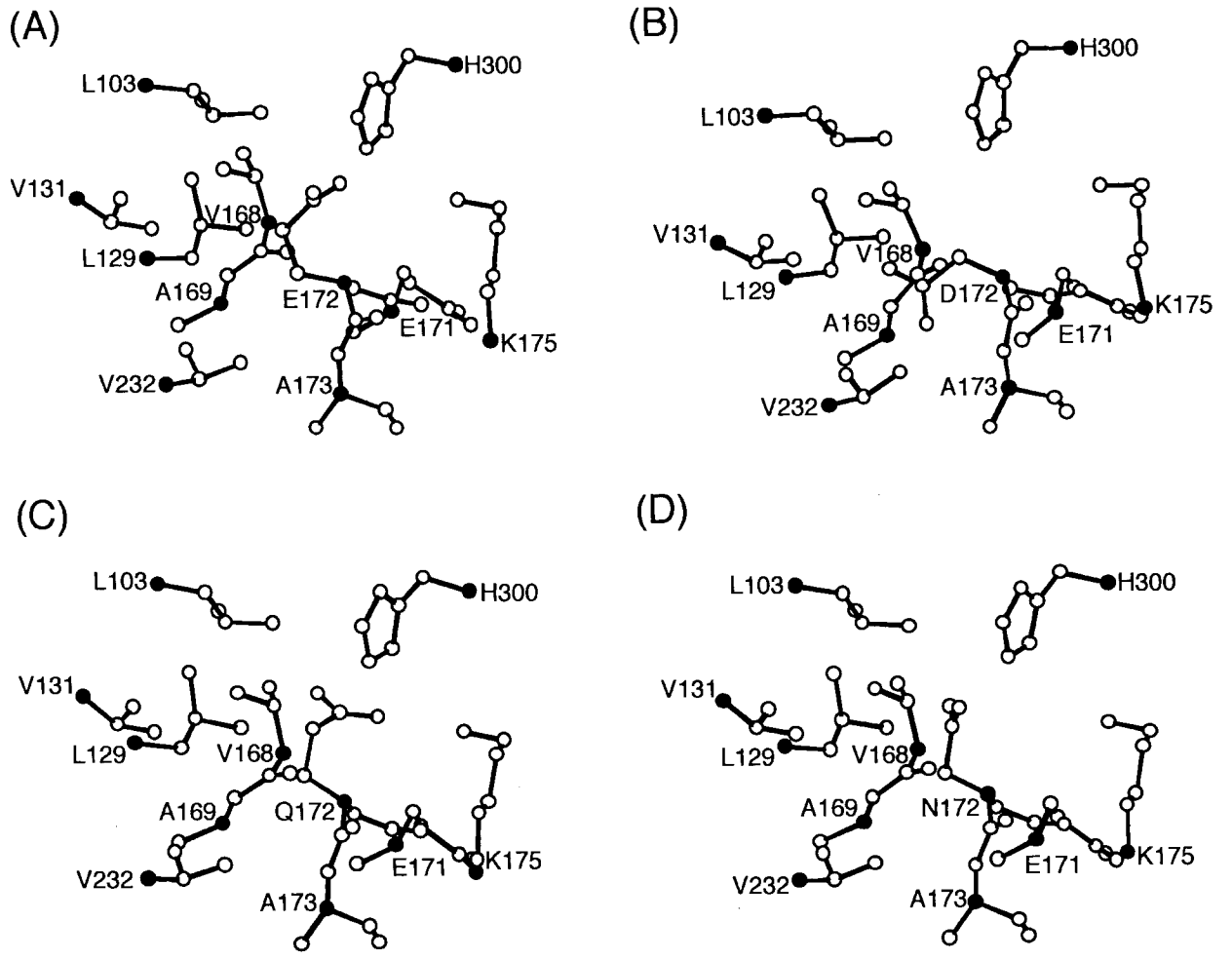

Fig. 2. Modeled structures around mutation site of A172E (A), A172D (B), A172Q (C) and A172N (D). The α-carbons were represented with solid circles. Amino acid residues are designated by the one-letter code and numbers represent the positions of the amino acid residues.

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 $Ala^{172} \rightarrow Glu$, Asn and Gln enhanced the stability of the thermophilic enzyme without significant change in the enzymatic activity and that the mutation $Ala^{172} \rightarrow Asp$ slightly improved the affinity both for D-3-IPM and for NAD at the cost of decrease in thermostability.

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 et al. The detailed models will be published elsewhere. 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 Glu^{172} side chain fills up the hydrophobic core with its two methylene groups and its terminal carboxylate group points to His^{300}, possibly forming a salt bridge or a hydrogen bond with the side chain of His^{300} (Fig. 2A). Thereby, A172E might acquire the increased thermostability.

In the proposed structures of A172Q and A172N, the side chains of Gln^{172} and Asn^{172} similarly interact with the surrounding residues (Fig. 2C,D). Their methylene groups might be involved in the hydrophobic interaction, and their side chain carbonyl groups form hydrogen bonds with His^{300} and/or Lys^{175}. In addition, their side chain amides are close to the main chain carbonyl of Val^{168} 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 Gln^{172} fills up the hydrophobic core with two methylene groups whereas the amide group of Asn^{172} buried in the hydrophobic core as well as the β-methylene group. In addition, the longer side chain of glutamine makes its amide group possible to locate more suitable position to interact with Lys^{175}. In accord with this observation, the A172Q mutant is more thermostable than the A172N mutant. In the modeled structures of A172Q and A172E, the amide group of Gln^{172} is hydrogen-bonding with the main chain carbonyl of Val^{168} whereas there is no hydrogen bond between the side chain of Glu^{172} and Val^{168}. This may explain the reason 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 Asp^{172} is located in the hydrophobic core and thereby aspartic acid is not preferred in this position (Fig. 2B).

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 [17–20]. 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. [21] 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 Ala^{172}, which was completely buried in the hydrophobic core, with other hydrophobic residues such as Val, Ile, Leu and Phe [11,12]. We speculated that the substitution of Ala^{172} with polar amino acid residues might reduce the stability of the enzyme. To our surprise, the substitutions of Ala^{172} 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 Val^{172} 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 et al., unpublished]. Second, mechanism was found in the mutant with Leu^{172}; 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 [13]. In addition, the conformation change might improve the domain–domain interaction through formation of additional hydrogen bonds between the domains [13]. 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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