Crystal structures of mutants of *Thermus thermophilus* IPMDH adapted to low temperatures

**Introduction**

A thermophilic enzyme is stable and fully active at elevated temperatures. Because the activity of such an enzyme depends on the flexibility of each molecule that comprises it (Vihinen, 1987), improved conformational rigidity is indispensable for enhancing the stability of an enzyme against heat denaturation (Zavodszyk et al., 1998). Many comparisons between thermostable and thermostable enzymes have been reported in order to elucidate the relationship between their structure and thermostability. These studies have shown that the replacements of enzyme residues result in several stabilizing effects (hydrogen bonds, salt bridges, hydrophobic interactions, etc.). Although these effects stiffen the structure of thermophilic enzymes, they also prevent the enzymes from fitting into a substrate at low temperatures. In other words, the cold-adapted enzymes have highly flexible structures which provide enhanced abilities to undergo conformational changes during catalysis at the cost of thermal stability (Margesin and Schinner, 1994; Feller and Gerday, 1997).

The enzyme 3-isopropylmalate dehydrogenase (IPMDH; EC 1.1.1.85) in the leucine biosynthesis pathway catalyzes the dehydrogenation and decarboxylation of 3-isopropylmalate (IPM). Usually, the enzyme is assembled from two identical subunits, each of which is composed of two domains. The active site is located in a cleft between two domains. The enzyme allows an induced fit upon binding of the substrate IPM and coenzyme NAD$^+$ on the active site. The crystal structure of *Thermus thermophilus* IPMDH (TtIPMDH) complexed with NAD$^+$ (Hurley and Dean, 1994; Kadono et al., 1995) shows that a surface loop hangs over the adenine base of the coenzyme. The crystal structure of IPMDH from *Thiobacillus ferroxidans* (TfIPMDH) (Imada et al., 1998) complexed with IPM revealed the rearrangement of the domains so as to form closed conformations.

**Materials and methods**

**Crystalization and data collection**

Each mutant enzyme was purified in the same way as the wild-type enzyme (Yamada et al., 1990). For crystallization, the protein solution was concentrated to 15 mg/ml and mixed with an equivalent volume of a reservoir solution of 1.0–1.5 M ammonium sulfate in sodium phosphate buffer (pH 7.0–7.5). Mutant enzymes were crystallized by the hanging drop vapor diffusion method under conditions similar to those for the wild-type enzyme.

**Keywords:** cold-adapted mutants/3-isopropylmalate dehydrogenase/thermal stability/*Thermus thermophilus* structural analysis

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Fig. 1. Structure of the wild-type TtIPMDH monomer. The loop regions and the binding sites of NAD$^+$ and IPM are shown with mutations sites of the cold-adapted mutants. This figure was drawn with MOLSCRIPT (Kraulis, 1991).

Table I. Kinetic and thermodynamic parameters at 40°C

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>V15I</th>
<th>V126M</th>
<th>S92F</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (IPM) (mM)</td>
<td>1.3</td>
<td>2.4</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td>$K_m$ (NAD$^+$) (mM)</td>
<td>26</td>
<td>160</td>
<td>1000</td>
<td>11</td>
</tr>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>2.4</td>
<td>11</td>
<td>18</td>
<td>2.4</td>
</tr>
<tr>
<td>$k_{cat}/K_m$ (IPM) (s$^{-1}$ mM$^{-1}$)</td>
<td>1.9</td>
<td>4.6</td>
<td>15</td>
<td>2.4</td>
</tr>
<tr>
<td>$k_{cat}/K_m$ (NAD$^+$) (s$^{-1}$ mM$^{-1}$)</td>
<td>0.092</td>
<td>0.069</td>
<td>0.018</td>
<td>0.22</td>
</tr>
</tbody>
</table>

IPM = 3-isopropylmalate (substrate).

wild-type enzyme (Imada et al., 1991). Drops of mixed solutions were sealed over 1 ml of reservoir solution. After 1 week in an incubator at 25°C, the crystals were grown to a size of ~0.4 × 0.2 × 0.2 mm. A series of diffraction images from the crystal sealed in a glass capillary were obtained with the oscillation method on a Rigaku R-AXIS IV X-ray detector at 25°C. The images were converted to intensities using MOSFLM software (Leslie, 1990) and subsequent processing was conducted using CCP4 suite software (Collaborative Computational Project, 1994). The diffraction data are summarized in Table II.

Structure determination

All structures were determined using the molecular replacement method with the program AMoRe (Navaza, 1994). The search model was constructed by removing water molecules from the PDB file of the wild-type enzyme (PDB code: 1IPD). The highest peaks of the rotation and translation functions were selected and rigid body refinements (Castellano and Olivia, 1992) were applied by AMoRe. Mutations were introduced by examining the electron density map (Figure 2) on the display of the program XFIT in the XtalView package (MacRee, 1999). The structure of each mutant was refined using the maximum likelihood refinement of REFMAC (Murshudov et al., 1997) and validated by PROCHECK (Laskowski et al., 1993). Ten percent of the measured reflections were used as free-$R$ test sets (Brügerz, 1992) in each refinement. The refinement statistics are given in Table III.

Modeling

Since the ES complex of TtIPMDH took a closed conformation, the structures of the closed conformation of S92F and the wild-type enzyme were simulated with the programs QUANTA and CHARMm. Each domain of S92F was fitted to the domains of the closed conformation of TtIPMDH. Two hundred steps of the Adopted-basis Newton Raphson minimization (Brooks et al., 1983) were applied after adding hydrogen atoms to the
The overall structure of each mutant enzyme is similar to that of the wild-type enzyme. It is composed of two identical subunits and each subunit forms an open conformation between the two domains. However, minor structural changes were detected in relation to the adaptation to lower temperatures.

In the structure of V15I, the mutation of Val into Ile fits well into the cavity made by surrounding residues, Ile11, Pro271, Ala290 and His273, most of which are hydrophobic and contribute to the hydrophobic interaction. Hence, the replacement to Ile15 narrowed the NAD\(^+\) binding cleft by 0.7 Å by forcing His273 upward. These facts imply that NAD\(^+\) binding might be weakened; indeed the \(K_m\) value for NAD\(^+\) increased about 6-fold compared with that of the wild-type enzyme.

In V126M, the side chain Met126 also fits well into the cavity surrounded by hydrophobic residues, Ala106, Val128, Pro227, Ala247 and Leu250. The flexible loop (250–254th, Figure 1), which is at the interface between the domains, shifts towards the inside of the second domain (Figure 3) by the hydrophobic interaction between Met126 and Leu250. Although in the wild-type enzyme the end of the loop, Leu254, moves towards NAD\(^+\) when IPM binds to the enzyme (Hurley and Dean, 1994; Kadono et al., 1995), the locking of the loop by the replacement to Met126 prevents the move of Leu254 towards NAD\(^+\), which may explain the decrease in affinity for NAD\(^+\), as indicated by the large \(K_m\) (Table I).

In the case of S92F, no significant structural changes were found by the mutation, because the mutated residue is on the molecular surface and does not conflict with the neighboring residues. Although Phe92 is close to the IPM binding site, the kinetic constants indicate that there is a slight change in its affinity for IPM. The free enzyme takes an open conformation with a wide mouth between domains, although the active form of the enzyme is thought to be a closed conformation as found in the crystal structure of the ES complex of TtIPMDH (Imada et al., 1998). The effects of the mutation on the active form were speculated upon by constructing a model structure by energy minimization using CHARMM software. The calculations were carried out for both the wild-type enzyme and S92F. The results suggested that the residues from 138 to 141 (Figure 1) are shifted towards IPM by 1.6 Å (Figure 4), which may be caused by the interaction among aromatic groups, Phe53, Phe41, Phe92, Tyr139 and Phe140. In the model, the binding of IPM seems to be stabilized by Tyr139 approaching IPM.

### Discussion

#### Structural shifts

The kinetic parameters of mutants suggested two different strategies against the lower temperature environment. V15I and V126M are called \(k_{cat}\) improved mutants, because of the larger \(k_{cat}\) values than for the wild-type enzyme. S92F is called \(K_m\) improved mutant whose smaller \(K_m\) value for NAD\(^+\) increases the \(k_{cat}/K_m\) (NAD\(^+\)) value and a high activity is observed. Although in the wild-type enzyme the end of the loop, Leu254, moves towards NAD\(^+\) when IPM binds to the enzyme (Hurley and Dean, 1994; Kadono et al., 1995), the locking of the loop by the replacement to Met126 prevents the move of Leu254 towards NAD\(^+\), which may explain the decrease in affinity for NAD\(^+\), as indicated by the large \(K_m\) (Table I).

In the case of \(k_{cat}\) improved mutants, the replaced residues fill cavities and do not disturb the hydrophobic core. Although it would be expected that increased hydrophobic interaction would stabilize a protein and raise the thermal denaturation temperature (\(T_1\)) in fact the values of \(T_m\) and \(T_1\) are not raised (Table IV). This implies that the thermostabilities of mutants are not improved, because the weakened bonding of NAD\(^+\) contributes in increasing the value of \(k_{cat}\) by immediately releasing NADH.

In the case of \(K_m\) leading mutant, a large hydrophobic residue, Phe92, is exposed to the solvent and a hydrogen bond between Ser92 and Thr88 found in the wild-type enzyme disappeared. These unfavorable structural changes are compensated for by stabilizing the closed conformation. Previous structural research on TtIPMDH and its homologous enzyme isocitrate dehydrogenase (Kadono et al., 1995; Zhang and Koshland, 1995) suggested that the active form adopts a closed conformation between domains in order to bind IPM in the...
crevice of the molecule. The structure shows that the binding of NAD$^+$ is also stabilized in the closed conformation. Accounting for the equilibrium between the open and closed conformations, the mutation may contribute to an increase in the proportion of closed conformations, by which the molecule buries Phe92 within a hydrophobic core. In other words, the enzyme is adapted to low temperature by improvement of the binding of NAD$^+$ and IPM in a closed conformation. The mutation closes the wide mouth between two domains into a closed conformation. However, the thermostability of the mutant enzyme is not improved because the molecule takes an open conformation in solution which is less stable at higher temperature owing to Phe92 protruding into the solvent region.

**Mutation sites**

It is noteworthy that all mutation sites affect the ligand binding sites indirectly without major structural changes. In the case of the cold-adapted mutant of subtilisin (Kano et al., 1997), the mutation site is on the opposite side of the catalytic triad. Most of the mutation sites that stabilize IPMDH are either at the domain interface (Ile172, Kotsuka et al., 1995; Akanuma et al., 1997; Qu et al., 1997) or behind the helix (Ile93, Tamakoshi et al., 1995). These mutation sites are not in the direct vicinity of the binding site. If the mutation occurs in a ligand-binding site, severe distortion of the binding site would occur and alter the binding specificity of the enzyme. For example, mutations on only three NAD$^+$ binding residues of TtIPMDH affected the cofactor binding and decreased the affinity for NAD$^+$ by 1/153 (Chen et al., 1996). In order to improve the function, it would be appropriate to shift the conformations of side chains involved in the active site rather than to replace the side chains. It is obvious that enzymes have evolved with conservation of the homology of ligand-binding sites among species.

**Effects of evolutionary engineering**

Generally, the introduction of hydrophobic interaction is used in thermal stabilization of the enzyme. Since the hydrophobic interaction is entropic (Tanford, 1962), the enzyme becomes more stable at high temperatures if hydrophobic interactions are strengthened. In the case of the buried core of lysozyme (Shih et al., 1995), a correlation between thermostability and hydrophobicity of introduced residues in hydrophobic pockets was found. In contrast, it was reported that the exposed hydrophobic residues contribute to a lower stability of cold-adapted $\alpha$-amylase (Aghajari et al., 1998). In the case of IPMDH, the thermostabilities of mutants with increased hydrophobic introductions were not improved.

In a sequence alignment of eight mesophilic IPMDH (Wallon et al., 1997), isoleucine at position 15 is found for four species and methionine at 126 and phenylalanine at 92 are not found. Therefore, the present mutations are regarded as not being correlated with the primary sequences of the mesophilic enzymes. In another study, the effects of these mutations were found not to be cumulative (M.Yasugi, personal communication). From these facts, the present mutations may differ from the adaptation on Earth.

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**References**


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