Adaptation of a thermophilic enzyme, 3-isopropylmalate dehydrogenase, to low temperatures

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Random mutagenesis coupled with screening of the active enzyme at a low temperature was applied to isolate cold-adapted mutants of a thermophilic enzyme. Four mutant enzymes with enhanced specific activities (up to 4.1-fold at 40°C) at a moderate temperature were isolated from randomly mutagenized Thermus thermophilus 3-isopropylmalate dehydrogenase. Kinetic analysis revealed two types of cold-adapted mutants, i.e. $k_{\text{cat}}$-improved and $K_m$-improved types. The $k_{\text{cat}}$-improved mutants showed less temperature-dependent catalytic properties, resulting in improvement of $k_{\text{cat}}$ (up to 7.5-fold at 40°C) at lower temperatures with increased $K_m$ values mainly for NAD. The $K_m$-improved enzyme showed higher affinities toward the substrate and the coenzyme without significant change in $k_{\text{cat}}$ at the temperatures investigated (30–70°C). In $k_{\text{cat}}$-improved mutants, replacement of a residue was found near the binding pocket for the adenine portion of NAD. Two of the mutants retained thermal stability indistinguishable from the wild-type enzyme. Extreme thermal stability of the thermophilic enzyme is not necessarily decreased to improve the catalytic function at lower temperatures. The present strategy provides a powerful tool for obtaining active mutant enzymes at lower temperatures. The results also indicate that it is possible to obtain cold-adapted mutant enzymes with high thermal stability.

Keywords: cold-adaptation/3-isopropylmalate dehydrogenase/random mutagenesis/thermal stability/thermophilic enzyme

Abbreviations: IPM, 3-isopropylmalate; IPMDH, 3-isopropylmalate dehydrogenase (EC 1.1.1.85, IPMDH); MNNG, N-methyl-N’-nitro-nitosoguanidine

Introduction

Enzymes isolated from thermophilic microorganisms possess higher thermal stability than do the mesophilic counterparts. Thermostable enzymes also exhibit higher stability to organic solvents, acidic and alkaline pH and detergents. These characteristics are of great advantage for industrial applications (Vieille et al., 1996). One serious drawback in the application of thermophilic enzymes is the low catalytic activity at moderate or lower temperatures. They often show poor activity at low temperatures at which the mesophilic or psychrophilic counterparts show maximum catalytic activity (Somero, 1975; Vihinen, 1987; Varley and Pain, 1991; Meiering et al., 1992; Shoichet et al., 1995; Zavodszky et al., 1998). Random mutagenesis has been utilized to enhance or alter various enzyme characteristics, including enzyme activity (Graham et al., 1993; Krebs and Fierke, 1993; Huang et al., 1996; Vipond and Halford, 1996), thermal stability (Liao et al., 1986; Tamakoshi et al., 1995; Giver et al., 1998), alkaline stability (Commingham and Wells, 1987) and substrate specificity (Olivphant and Struhl, 1989; Yano et al., 1998), and also to restore the catalytic activity of an enzyme damaged by site-directed mutagenesis (Hermes et al., 1990; Giver et al., 1998).

Momose and co-workers have isolated several mutant enzymes of subtilisin with improved catalytic activity at low temperature (Kano et al., 1997; Taguchi et al., 1998, 1999). Lebbink et al. (2000) have isolated mutant enzymes with improved low-temperature catalysis of Pyrococcus furiosus β-glucosidase CelB. Merz et al. (2000) have isolated mutants with improved catalytic activity at low temperature of the trpC gene of Sulfolobus solfataricus. However, detailed analysis of the adaptation mechanism to lower temperature has not yet been reported.

In this paper, we describe the application of random mutagenesis and isolation of cold-adapted mutant enzymes of 3-isopropylmalate dehydrogenase of an extreme thermophile, Thermus thermophilus. IPMDH encoded by leuB gene catalyzes the oxidative decarboxylation of 3-isopropylmalate in the presence of NAD as a cofactor and is an ideal enzyme for industrial applications. A kind gift from Dr C.M. Hamilton (Hamilton et al., 1995). This study, T.thermophilus leuB gene was introduced into a leuB locus of Escherichia coli genome by using a newly constructed integration vector and cold-adapted mutant enzymes with higher activity at 40°C were isolated by screening the random mutant library. The isolated mutant enzymes were then characterized to elucidate the adaptation mechanism of enzymes to lower temperatures.

Materials and methods

Bacterial strains, media and materials

E.coli OM17, a leuB-deficient strain of E.coli JM105, was used in this study. The medium used was either LB broth or M9 minimum medium (Sambrook et al., 1989). Ampicillin (50 μg/ml), chloramphenicol (30 μg/ml), kanamycin (5 μg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG, 4 μg/ml) were added to the media when required. Plasmid pMAK705 with a temperature-sensitive replication origin was a kind gift from Dr C.M. Hamilton (Hamilton et al., 1989).

Construction of an integration vector for leuB locus

A 4.4 kbp HindIII–BanHI fragment including E.coli leu′ABC′ genes (Kirino et al., 1994) was cloned in a plasmid pUC19.
and used as a starting material. Two recognition sites, NdeI and PstI, were introduced at the initiation and near the termination codons of the leuB gene, respectively, by the method of Kunkel (Kunkel and Roberts, 1987) using two synthetic oligonucleotides (5′-CAATATGTAATTCTAGA-CATATGTCAGGTTTTCCTTGTC-3′, 5′-ATTGCCCCGCTATGCTGAGAAGGGGGTG-3′). The E. coli leuB gene bordered by the NdeI and PstI sites was removed from the resulted plasmid and an NdeI–XbaI fragment encoding *T.thermophilus* leuB gene (Tamakoshi *et al.*, 1995) and an XbaI–PstI fragment encoding kanamycin nucleotidyl transferase gene (Matsumura *et al.*, 1984) were inserted between NdeI and PstI sites of the plasmid. To terminate the transcription from an upstream promoter, a trpA transcription-terminator sequence (Sambrook *et al.*, 1989) was synthesized (5′-AGCTTGAGCCCCTTAATGAGCGGGCTTTTTTT-3′, 5′-CGCGAAAAAAGCAGCTGATTAGGCCGGCTCACA-3′) and inserted between HindIII and MluI sites of the fragment. The resulting 5.0-kbp HindIII–BamHI fragment was inserted into the plasmid pMAK705 (Hamilton *et al.*, 1989) previously digested by HindIII and BamHI. The resulting plasmid was named pTS-EKT6 (Figure 1).

**Integration of *T.thermophilus* leuB gene into *E. coli* genome**

Integration of the vector pTS-EKT6 was performed by the method of Hamilton (Hamilton *et al.*, 1989) with some modifications (Figure 1). *E. coli* OM17 harboring the plasmid pTS-EKT6 was cultivated on an LB agar plate containing chloramphenicol at 44°C to isolate the transformants in which the vector is integrated into the genome. The isolation was repeated three times to delete intact vectors completely from the cells. Then, the colonies isolated from the plates were cultivated on LB agar plates supplemented with kanamycin at 30°C to clip out the vector from the genome. The colonies were purified by repeated cultivation. Finally, the clipped-out vector in the cell was removed by incubation of the isolated transformants on LB agar containing kanamycin at 44°C. They were purified by repeated plating and culturing and one of the strains was named TS4.

**Mutagenesis with MNNG and screening of cold-adapted mutant strains**

Mutagenesis with a mutagen, MNNG, was performed according to the method described by Miller (1992). *E. coli* TS4 cells treated with MNNG were incubated on M9 minimum medium agar plates containing kanamycin at 40°C for 3 days. Colonies appeared on the agar plates were purified by repeated plating and culturing and used for the following analyses.

**Sequence analyses**

The leuB genes of the isolated strains were amplified by a polymerase chain reaction with the synthetic primers 5′-ACAACGAAAAACAACAGAAAAC-3′ and 5′-GTCAT-TATTATTGGTCATCCAC-3′. The amplified fragments were digested with NdeI and EcoRV and cloned into the plasmid pNV119 (Tamakoshi *et al.*, 1995) previously digested with NdeI and EcoRV. The sequence of the cloned genes was the Michaelis–Menten equation. Thermodynamic parameters, i.e., activation free energy ($\Delta G^\ddagger$), enthalpy ($\Delta H^\ddagger$) and entropy ($\Delta S^\ddagger$) and van’t Hoff free energy ($\Delta G_{Km}$), enthalpy ($\Delta H_{Km}$) and entropy ($\Delta S_{Km}$), were derived according to the relationships given by Lehrer and Barker (1970).

**Analytical procedures**

The specific activity of IPMDH was measured in 50 mM HEPES buffer (pH 8.0) containing 100 mM KCl, 5.0 mM MgCl$_2$, 2.0 mM NAD and 1.0 mM D-3-isopropylmalate (IPM). The initial velocity of the reaction was determined by spectrophotometrically monitoring the reduction of NAD at 340 nm. The concentration of NADH was calculated by using a molar extinction coefficient of 6330 M$^{-1}$ cm$^{-1}$. Michaelis constants and $k_{cat}$ values were obtained by fitting initial velocity data to the Michaelis–Menten equation. Thermodynamic parameters, i.e., activation free energy ($\Delta G^\ddagger$), enthalpy ($\Delta H^\ddagger$) and entropy ($\Delta S^\ddagger$) and van’t Hoff free energy ($\Delta G_{Km}$), enthalpy ($\Delta H_{Km}$) and entropy ($\Delta S_{Km}$), were derived according to the relationships given by Lehrer and Barker (1970).

Analysis of the irreversible thermal inactivation was performed by the method described previously (Kirino *et al.*, 1994). Enzyme solution (0.2 mg/ml) was incubated at the
Cold-adapted mutants of a thermophilic enzyme

Table I. Mutations and kinetic parameters of the cold-adapted mutant IPMDHs

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Amino acid (nucleotide) substitution</th>
<th>40°C</th>
<th>70°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>K_m (µM)</td>
<td>k_cat (s⁻¹)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IPM⁻¹</td>
<td>NAD</td>
</tr>
<tr>
<td>Wild-type</td>
<td>None</td>
<td>1.25</td>
<td>25.8</td>
</tr>
<tr>
<td>P115</td>
<td>Val151leu(G43A)</td>
<td>2.40 (1.92)</td>
<td>160 (6.20)</td>
</tr>
<tr>
<td>P215</td>
<td>Val126Met (G376A)</td>
<td>1.20 (0.96)</td>
<td>1010 (39.2)</td>
</tr>
<tr>
<td>P315</td>
<td>None</td>
<td>1.30 (1.04)</td>
<td>157 (6.09)</td>
</tr>
<tr>
<td>P415</td>
<td>Asn237Asp (A709G)</td>
<td>1.30 (1.04)</td>
<td>157 (6.09)</td>
</tr>
</tbody>
</table>

*Relative values with respect to that for the wild-type enzyme are indicated in parentheses. Standard errors of the mean were <5% in all of the estimates.

IPM, d-3-isopropylmalate.

Fig. 2. Temperature dependence of specific activity of the wild-type and cold-adapted mutant IPMDHs. The specific activity was determined in the presence of 2 mM NAD and 1 mM d-IPM. The specific activity of all four mutant enzymes increased at 40°C when compared with the wild-type enzyme: wild-type, 6.98 units/µg; P115, 26.5 units/µg; P215, 28.6 units/µg; P415, 19.1 units/µg; and T1155, 8.39 units/µg. P215 exhibited the highest activity among the mutants (4.1-fold at 40°C). Closed circles, wild-type IPMDH; open squares, P115; open circles, P215; crosses, P415; open triangles, T1155.

Results and discussion

Integration of *T.thermophilus* leuB gene into a leuB locus in *E.coli* genome

For the efficient screening of cold-adapted mutant enzymes, it is important to avoid isolation of mutants with undesirable mutations such as the mutations to induce increased copy number of the gene. Therefore, *T.thermophilus* leuB gene was introduced into a leuB locus of *E.coli* genome in this study, since the copy number of the thermophilic leuB gene introduced is expected to be stable in the host genome.

*T.thermophilus* leuB gene and kanamycin nucleotidyl transferase gene were introduced into a leuB locus of *E.coli* genome (Blattner *et al.*, 1997) by using the integration vector pTSKET6 (Figure 1). The vector possessed the 5.0 kbp HindIII–BamHI fragment containing the *E.coli* leuA, *T.thermophilus* leuB, kanamycin nucleotidyl transferase (Km') and *E.coli* leuC' genes. The chloramphenicol acetyl transferase gene (Cm') was used to select transformants harboring the vector. *E.coli* OM17 was transferred with the vector. The vector also has a temperature-sensitive replication origin (Hamilton *et al.*, 1989) that inactivates at 40°C and above, which is useful for removing the vector from the host cells. *E.coli* OM17 harboring the integration vector was cultivated on LB agar plates containing chloramphenicol at 44°C. At this temperature the vector cannot replicate because of the inactivation of the temperature-sensitive replication origin. Consequently, only the cells with the vector integrated into the genome are expected to survive. The integration vector pTS-EKT6 is designed to have sequences identical with *E.coli* genome at 'leuA and leuC' loci. Accordingly, the homologous recombination with *E.coli* genome was expected to occur in one of two regions, i.e. the 1.1 kbp MluI–NdeI and 1.5 kbp PstI–BamHI regions (*1 and *2 in Figure 1). Subsequently, the isolated strains were cultured on LB agar plates containing kanamycin at 30°C. The plasmid replicon on the genome is active at the temperature applied, which inhibits the original replication of the genome (Hamilton *et al.*, 1989). Hence only the strains without the integrated vector are expected to grow efficiently. There are four possible recombinations, indicated by *3, *4, *5 and *6 in Figure 1. The transcription terminator located upstream of the fragment also enables us to select the strain in which Km' and *T.thermophilus* leuB genes are integrated into the genome by using kanamycin. Only the recombination at position *4 or *5 can promote expression of Km' gene and therefore the recombinants of the two types are expected to be selected on kanamycin plates at 30°C. Then, the isolated strains were cultured on a kanamycin plate at 44°C to remove the clipped-out vector from the cell. Thus, by using this vector system, the *T.thermophilus* leuB gene and kanamycin nucleotidyl transferase gene can be substituted for *E.coli* leuB gene on the bacterial genome without monitoring the enzyme activity of the introduced leuB gene. One of the strains obtained was named TS4 and used for the following screening experiments.

Screening of cold-adapted mutant IPMDHs

The *E.coli* TS4 showed cold sensitivity in the leucine-free medium. The strain could grow only at 42°C and above.
Table II. Thermodynamic parameters of the wild-type and cold-adapted mutant IPMDHs at 40°C

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ for IPM (kJ/mol)</th>
<th>$K_m$ for NAD (kJ/mol)</th>
<th>Activation (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Delta G_{m}^\circ$</td>
<td>$\Delta H_{m}^\circ$</td>
<td>$T\Delta S_{m}^\circ$</td>
</tr>
<tr>
<td>Wild-type</td>
<td>-35.4</td>
<td>-58.2</td>
<td>-22.7</td>
</tr>
<tr>
<td>P115</td>
<td>-33.7 (1.7)</td>
<td>-42.7 (15.5)</td>
<td>-9.0 (13.7)</td>
</tr>
<tr>
<td>P215</td>
<td>-35.5 (-0.1)</td>
<td>-64.1 (-5.9)</td>
<td>-28.6 (-5.9)</td>
</tr>
<tr>
<td>P415</td>
<td>-35.3 (0.1)</td>
<td>-58.2 (0.0)</td>
<td>-22.9 (-0.2)</td>
</tr>
<tr>
<td>T1155</td>
<td>-36.0 (-0.6)</td>
<td>-59.9 (-1.7)</td>
<td>-23.9 (-1.2)</td>
</tr>
</tbody>
</table>

*Values in parentheses indicate the difference between parameters of mutant enzyme and that of the wild-type enzyme.*

without leucine. The observed cold sensitivity can be ascribed to the low activity of the *Thermophilus* IPMDH at moderate temperature (Zavodszky et al., 1998). The strains were treated with a mutagen, MNNG and screened on leucine-free M9 minimum medium plate containing kanamycin at 40°C.

About 100 colonies appeared on the plate within 3 days per 7.5×10⁶ surviving cells. The apparent efficiency was 1.2×10⁻⁴. Five strains were randomly selected from the colonies and labeled TS4-P115, -P215, -P315, -P415 and -T1155, respectively. The *Thermophilus leuB* gene of each mutant was amplified by PCR and cloned into a plasmid pNV119 (Tamakoshi et al., 1995). Sequence analyses of the cloned *leuB* genes showed 1–3 base-substitutions per gene, except for TS4-P315 of which *leuB* gene had no mutations (Table I). Four of the six detected substitutions were G:C to A:T transitions, as expected from the effect of the mutagen MNNG (Miller, 1992). Four of the mutant *leuB* genes were overexpressed in *E.coli* and the expressed mutant enzymes, P115, P215, P415 and T1155, were separately purified to homogeneity and characterized.

Temperature dependence of the specific activity of the mutant IPMDHs

Figure 2 depicts the temperature dependence of the apparent specific activity of the mutant enzymes. While T1155 showed a similar temperature dependence to that of the wild-type, P115, P215 and P415 showed the optimum temperatures shifted to lower temperatures. This apparent change of the optimum temperature was caused by the increase in $K_m$ at higher temperatures as shown in the following section. The activity of the three mutants was less temperature dependent than that of the wild-type, resulting in higher activity at temperatures below 60°C.

Kinetic parameters of the cold-adapted mutant IPMDHs

Table I summarizes the kinetic parameters of the wild-type and cold-adapted mutant IPMDHs at 40 and 70°C. Three of the mutant enzymes, P115, P215 and P415, showed improved $k_{cat}$ values at 40°C. The three mutants can be called $k_{cat}$-improved cold-adapted mutants. The $k_{cat}$-improved mutants showed increased $k_{cat}$ values at the cost of the increases in $K_m$ for IPM and/or especially NAD (Table I). This simultaneous increases in $k_{cat}$ and $K_m$ values can also be seen in naturally occurring cold-adapted *A.* lactate dehydrogenases (Fields and Somero, 1998) and elastases (Asgeirsson and Bjarnason, 1993). The kinetic analysis for mutant T1155 revealed the improved binding affinities for both the substrate and NAD (Table I), resulting in an improvement of the catalytic efficiency (1.3- and 1.8-fold for IPM and NAD, respectively, at 40°C). Low-temperature adapted mutants with improved $k_{cat}$ and $K_m$ have been isolated by random mutagenesis (Kano et al., 1997; Taguchi et al., 1998, 1999; Lebbink et al., 2000; Merz et al., 2000) or by directed mutagenesis (Imoto et al., 1994).

It is important to note that NAD is a general cofactor, which...
Cold-adapted mutants of a thermophilic enzyme

Fig. 4. Crystal structure of *T. thermophilus* IPMDH complexed with NAD (Hurley and Dean, 1994). (A) This enzyme is composed of two domains (Imada *et al.*, 1991), domain I (Met1 to Leu99 and Gly252 to Leu345) and domain II (Phe100 to Pro251). An NAD molecule bound in domain I is shown in ball-and-stick representation. The adenine moiety of NAD is depicted with gray spheres. Side chains of the mutated residues in the cold-adapted mutant IPMDHs are also shown in ball-and-stick representation (see also Table I). (B) Interaction of adenine portion of the bound NAD with *T. thermophilus* IPMDH. Hydrophobic interactions are formed with Ile11, Val15, Gly255, Leu254, Ile279 and with the aliphatic portion of Asp326 (Hurley and Dean, 1994). The models were displayed using the program package MOLSCRIPT (Kraulis, 1990).

Fig. 5. Residual activity after heat-treatment (A) and melting profiles (B) of the wild-type and cold-adapted mutant IPMDHs. CD signals were monitored at 222 nm with a scanning rate of 1°C/min. The protein concentration was 0.2 µg/ml in 20 mM potassium phosphate buffer, pH 7.6. Closed circles, wild-type IPMDH; open squares, P115; open circles, P215; crosses, P415; open triangles, T1155.

is used in many biological reactions in *E. coli* cells. Accordingly, the concentration of NAD is expected to be constant and independent of the activity of a specific enzyme. At a constant, moderate NAD concentration, higher *k*\text{cat} and lower *K*\text{m} are expected to improve the rate of the catalytic reaction.

**Changes in the thermodynamic parameters for the catalytic reaction**

The temperature dependence of *k*\text{cat} and *K*\text{m} is shown in Figure 3. The thermodynamic parameters of *k*\text{cat} and *K*\text{m} were derived from the figure and are listed in Table II. It is clear that *k*\text{cat} improved by the decrease in Δ*G*\text{‡} that can be ascribed to the lower activation enthalpy Δ*H*\text{‡} in *k*\text{cat} improved mutants (P115, P215, P415). It is interesting that Δ*G*\text{Km} especially for NAD was significantly increased in the mutants. The increase in Δ*G*\text{Km} for NAD is induced by the increase in Δ*H*\text{Km}, although the increase is partially compensated by the increase in Δ*S*\text{Km}.

Relatively temperature-independent catalysis of these *k*\text{cat} improved mutants can be ascribed to the decreased activation enthalpy. Although there are no reports of thermodynamic parameters for isolated cold-adapted mutant enzymes, a similar observation has been reported for naturally occurring cold-adapted myofibrillar ATP synthases (Johnston and Goldspink, 1975) and lactate dehydrogenases (Low and Somero, 1974).

The evidence suggests that an increase in catalytic activity at low temperature is induced by a decrease in activation enthalpy in this type of cold adaptation. Consequently, the catalysis becomes less temperature dependent, as seen in the exothermic enzymes.

Dean and Koshland (1993) reported that the release of the products is a rate-limiting step for the catalytic reaction in *E. coli* isocitrate dehydrogenase sharing high structural and reaction-mechanical similarities with *T. thermophilus* IPMDH. Because the adenine portion does not change during the catalytic reaction from NAD to NADH and is located far from the nicotinamide ring (see Figure 4B), mutations which lead...
to lower binding affinity for the adenine portion of NAD are expected to improve the release of the catalytic product NADH. Therefore, the changes in the kinetic properties of the $k_{cat}$-improved cold-adapted mutants, such as in $k_{cat}$ and $K_{cat}$ and $\Delta H_{cat}$ for NAD and $\Delta F$, can be interpreted as a result of improving the coenzyme release caused by decreased binding affinities for the oxidized and reduced forms of NAD. However, we have not determined the affinity of the reduced form of the cofactor. The releasing process of the cofactor will be analyzed in the future.

In contrast to the $k_{cat}$-improved mutants, the $K_{cat}$-improved mutant T1155 improved the binding affinities for IPM and NAD by the increase in enthalpy change for the formation of the Michaelis complex (Table II). It should be noted that the activation enthalpy ($\Delta H^*$) and entropy ($\Delta S^*$) were not significantly affected by the mutation. Accordingly, the releasing process of the reduced cofactor was not affected in the mutant.

**Location of the mutation sites in the 3D structure of IPMDH**

Residues substituted in cold-adapted mutants are shown on a ribbon model of *T. thermophilus* IPMDH in Figure 4. *T. thermophilus* IPMDH binds the extended form of NAD (Hurley and Dean, 1994). The enzyme interacts with NAD at the adenine moiety: the adenine portion of NAD is in hydrophobic contact with side chains of Ile11, Val15, Leu254, Gly255 and Ile279 and with the aliphatic portion of Asp326 (Hurley and Dean, 1994). Two mutant enzymes, P115 and P415, possess amino acid substitutions in one of the binding residues, Val15Ile. P415 showed kinetic behavior similar to that of P115, suggesting that the altered kinetic properties in P415 are due at least partially to the substitution Val15Ile. The change of the temperature dependence of the kinetic properties of P115 and P415 mutant enzymes, as well as the low binding affinity toward NAD, can be interpreted as a result of improper fitting of the adenine portion caused by the change of the binding pocket. This interpretation agrees with the results of the kinetic analysis of lactate dehydrogenases of naturally cold-adapted organisms, in which a less temperature-independent catalytic function was ascribed to the decreasing number of weak interactions in the enzyme–substrate complex (Borgmann et al., 1975).

Another $k_{cat}$-improved mutant, P215, showed similar kinetic behavior to P115 and P415 (Table I). The substitution is found at Val126 and the site is located in domain II. Crystal structural analysis of the mutant Val126Met revealed that the mutation induced the movement of Leu254 that interacts with the adenine ring (R. Hirose, personal communication).

The mutant T1155 improved the catalytic function by a substitution of Ser92 with phenylalanine. As shown in Figure 4A, the residue is located in helix-d from Pro86 through Lys94 in the active site (Kadono et al., 1995; Imada et al., 1998). The substitution of Ser92 seems to induce a geometric change of the binding residues for the substrates. However, the detailed mechanism for the increased affinity of the $K_{cat}$-improved mutants with the coenzyme is not clear.

**Thermal stability of the cold-adapted mutant IPMDHs**

The thermal stability of the enzymes was investigated by two methods. Irreversible thermal inactivation was analyzed by measuring residual activity after heat treatment (Figure 5A). Half inactivation temperatures ($T_{1/2}$) were 87°C (wild-type), 85°C (P115), 87°C (P215), 82°C (P415) and 87°C (T1155). Temperature-dependent unfolding of the secondary structure was followed by monitoring changes in circular dichroism (CD) at 222 nm (Figure 5B). Half denaturation temperatures ($T_{1/2}$) monitored by CD were 87, 85, 87, 80 and 87°C for wild-type, P115, P215, P415 and T1155 enzymes, respectively and were similar to those estimated from the residual activity measurement. These two analyses revealed that two of the cold-adapted mutant IPMDHs, P215 ($k_{cat}$-improved type) and T1155 ($K_{cat}$-improved type), retained the original thermal stability, whereas P115 and P415 IPMDHs slightly lost stability. A $k_{cat}$-improved mutant with the same thermal stability as the wild-type has been reported for *P. furiosus* ß-glucosidase (Lebbink et al., 2000). A $K_{cat}$-improved mutant with the same thermal stability as the original enzyme has been reported for subtilisin (Kano et al., 1997). These results, including ours, suggest that activity can be improved without loss of thermal stability, supporting the idea that thermal stability and activity at moderate temperature are partially independent factors (Imoto et al., 1994; Akamata et al., 1998; Giver et al., 1998).

**Conclusions**

Cold-adapted mutants were efficiently isolated from a thermophilic enzyme by laboratory evolution. The laboratory evolution allowed us to identify mutation sites to improve catalytic activity without lowering thermal stability. The strategy described here provides a general way of creating active enzymes at lower temperatures with high thermal stability.

**Acknowledgements**

We are grateful to Dr. C.M. Hamilton (University of Georgia, USA) for providing us with the temperature-sensitive plasmid pMAK705. We appreciate the suggestions and advice of Dr. M. Iwakura (National Institute of Bioscience and Human Technology, Japan) during the preparation of the manuscript. The study was financially supported by grants from the Ministry of Education, Culture and Sports and the Ministry of International Trade and Industry, Japan.

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