Thermostabilization of a chimeric enzyme by residue substitutions: four amino acid residues in loop regions are responsible for the thermostability of *Thermus thermophilus* isopropylmalate dehydrogenase

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Abstract

A chimeric 3-isopropylmalate dehydrogenase, named 2T2M6T, made of parts from an extreme thermophile, *Thermus thermophilus*, and a mesophile, *Bacillus subtilis*, was found to be considerably more labile than the *T. thermophilus* wild-type isopropylmalate dehydrogenase. In order to identify the molecular basis of the thermal stability of the *T. thermophilus* isopropylmalate dehydrogenase, 11 amino acid residues in the mesophilic portion of the chimera were substituted by the corresponding residues of the *T. thermophilus* enzyme, and the effects of the side chain substitutions were analyzed by comparing the reaction rate of irreversible heat denaturation and catalytic parameters of the mutant chimeras with those of the original chimera, 2T2M6T. Four single-site mutants were successfully stabilized without any loss of the catalytic function. All these four sites are located in loop regions of the enzyme. Our results strongly suggest the importance of these loop structures to the extreme stability of the *T. thermophilus* isopropylmalate dehydrogenase. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: 3-Isopropylmalate dehydrogenase; Chimera; Protein stability; Site-directed mutagenesis; *Thermus thermophilus*

1. Introduction

Elucidation of the molecular basis of high thermostability of the proteins produced by extreme thermophiles contributes not only to the comprehensive understanding of how a protein keeps its native conformation, but also to the development of enzyme applications in industry. To identify the key factors responsible for the increased thermostability, an initial attempt was made to conduct the multiple amino acid sequence comparisons of homologous enzymes isolated from various organisms with different growth temperatures (e.g., [1]). Extensive structural comparisons lead to the conclusions that the overall conformation of each structure is very similar to that of its counterpart proteins from mesophilic organisms, and only the slight differences in structure, including small deletions and/or insertions, make thermophilic proteins more stable than their mesophilic counterparts [2–5].

In order to understand the roles of particular side
chains in proteins, specific sites of some proteins were substituted by other amino acids [6-9]. This raises the possibility that even a single amino acid substitution may increase the thermostability of a protein to a considerable extent, although a chance to increase the protein stability significantly depends on the overall structure of a protein and local environments of side chain residues. Thus, the systematic mutational analysis of proteins with the known three-dimensional (3-D) structures is essential to find out the correlation between the structure and the stability of proteins.

Analysis of chimeras between thermophile and mesophile enzymes provides another promising way to elucidate stability-structure relationships of a thermophilic enzyme [10,11]. We have previously constructed and analyzed a series of chimeric threeo-Ds-3-isopropylmalate dehydrogenase (IPMDH, EC 1.1.1.85) by ligating parts of the genes of the extreme thermophile Thermus thermophilus strain HB8 [12-15] and the mesophile Bacillus subtilis [16]. These chimeric IPMDHs showed catalytic properties similar to those of the T. thermophilus wild-type IPMDH, while the stability of each chimeric enzyme was approximately proportional to the fraction of the thermophilic sequence [17]. The 3-D structures of the T. thermophilus and two of the chimeric IPMDHs have been solved by high-resolution X-ray structural analysis [4,18] and the overall structures of these enzymes have been shown to be very similar to each other.

In this work, one of the chimeric IPMDHs designated as 2T2M6T was chosen to investigate the molecular basis of the thermostability of T. thermophilus IPMDH. The name, 2T2M6T, represents approximately the primary sequence, roughly 20% from the N-terminus of T. thermophilus, the next 20% from B. subtilis, and the rest from the T. thermophilus sequence (Fig. 1A). The B. subtilis portion of the chimeric IPMDH, i.e., the '2M' portion, is located at the hinge region between two domains, and consists of a helix, two β-strand segments and three loops connecting these secondary structures (Fig. 1B). Excluding the identical amino acid residues, the chimeric IPMDH has only 22 amino acid residues different from the T. thermophilus wild-type enzyme out of 345 residues, although the stability of the chimeric IPMDH is considerably lower than the thermophilic wild-type enzyme; in terms of the half-denaturation temperature, $T_D$ (the temperature at which half of the initial activity was irreversibly lost after 10 min), the $T_D$ values for the wild-type and the chimeric enzyme are 83°C and 65°C, respectively [17]. Thus, the chimeric enzyme 2T2M6T is an ideal exercise for improvement of thermostability of a protein, since the stability of the chimeric IPMDH may be improved by substituting some of these residues by the corresponding residues of the T. thermophilus enzyme. In the present study, we addressed to what extent a single amino acid residue contributes to the stability of the T. thermophilus IPMDH and at which part the most effective residue(s) is located. Eleven mutant chimeras having a single amino acid replacement in the 2M portion by the corresponding residue of the T. thermophilus enzyme were constructed, and the effects of the side chain substitutions were analyzed by comparing the thermostability of the mutant chimeras with the original chimera, 2T2M6T.

2. Materials and methods

2.1. Reagents

threeo-Ds,Ls-3-Isopropylmalate was purchased from Wako Pure Chemicals (Tokyo), and NAD was from Oriental Yeast. All reagents were of reagent grade.

2.2. Bacterial strains and plasmids

A bacterial strain, Escherichia coli MV1490 (Δ(lac-proAB), Δ(syl-recA)306::Tn10, supE, thi+, [F+, lacF(+), lacZAM15, proAB, traD36]), was used as the host in site-directed mutagenesis and in sequencing. A leuB strain E. coli J1A221 [F−, hsrR, ΔtrpE5, leuB6, lacY, recA1, λ−] was used as the host in expressing recombinant IPMDHs. Recombinant DNA experiments were performed according to Sambrook et al. [19]. DNA sequencing was carried out using a DNA sequencer (Applied Biosystems 373A) based on the dideoxy chain termination method [20].

2.3. Site-directed mutagenesis

Site-directed mutagenesis was performed according
to Kunkel et al. [21]. The template DNA used in site-directed mutagenesis was prepared from the recombinant plasmid pCHL226 carrying the chimeric *len*B gene coding for 2T2M6T IPMDH [17]. The oligonucleotides were synthesized with an Applied Biosystems Model 381A DNA synthesizer. The oligonucleotides used were 5'-T CAG TTC CGA AGG CAG CTG ATC CCA TTT TCT-3' for the mutant chimera N80L (asparagine at 80 was replaced with leucine), 5'-G TCT CAG TTC CCT AGG ATT TTG AT-3' for S82R, 5'-T GAG CAG CCC TGT TTC CCG TCT-3' for K88T, 5'-AAA CAC ATC AAG CGA TTT TCT GAT GCT-3' for Q96S, 5'-GTG AAG GTA TTT CCA AGC TTT CCT GAC-3' for E110P, 5'-GTC AGA AAG GCC TCC AAA TAC CTT-3' for S111G, 5'-AGC GTC AGA AAG GCC TGG AAA TAC CTT CAC AGG-3' for E110P/S111G, 5'-AGG CGA AGC GTC TTT ACG GCT TCT AAA-3' for S113E, 5'-TTC TTT TTT CAA AGG ACT TAA GTC AGA AAG GCT TTC A-3' for A115L, 5'-AAC GAA ATC AAC ACC ATC TAT ATA TT-3' for N125G, 5'-AC GAT AAC GAC GTC AAC ATT ATC TA-3' for F128V, and 5'-ACG AAC GAT AAG GAA ATC AAC ATT-3' for V129L. The mutations were ascertained by DNA sequencing.

2.4. Enzyme purification and analytical methods

Purification of mutant chimeras, N80L, S82R, A115L, N125G, and S82R/N125G was performed as reported in the previous paper [17]. The purified enzymes were stored as precipitant in 60% saturated ammonium sulfate solution at 4°C until use. The precipitated enzyme in ammonium sulfate solution was collected by ultracentrifugation, dissolved in 20 mM potassium phosphate buffer, pH 7.6, containing 0.5 mM EDTA, and dialyzed against the same buffer before use. Protein concentration was determined using a BCA protein assay kit (Pierce) with bovine serum albumin as a standard. The enzyme activity was measured as described previously [17,22]. The assay mixture contained 1.0 M KCl, 0.2 mM MnCl₂, 0.8 mM NAD⁺, 0.4 mM *D*<sub>s</sub>-L-3-isopropylmalate in 100 mM *N*-cyclohexyl-2-aminoethanesulfonic acid buffer, pH 9.0, or 100 mM potassium phosphate buffer, pH 7.6.

We reported the analysis of the thermal unfolding process of the *T. thermophilus* wild-type and the chimeric IPMDHs by CD spectroscopy and differential scanning microcalorimetry previously [23]. These unfolding are irreversible and kinetically controlled process, thus the kinetic analysis is applied for detailed, quantitative comparison. The rate constants of the irreversible thermal denaturation at various temperatures are determined by the following method. The enzymes (0.2 mg/ml) in 20 mM potassium phosphate buffer, pH 7.6, containing 0.5 mM EDTA, were incubated at each temperature. After appropriate intervals, the enzyme was immediately chilled in an ice bath, and the rate of the irreversible denaturation was estimated from the decrease of the remaining activity measured at 50°C and at pH 7.6. The obtained rate constants (*kₐ*) at various temperatures were analyzed based on the Arrhenius equation:

\[ \ln kₐ = A - Eₐ/RT \]

where *A* is a constant, *Eₐ* is the activation energy of the irreversible denaturation, *R* is the molar gas constant, and *T* is the absolute temperature. The activation parameters (the change of activation enthalpy (*ΔH*<sup>a</sup>), entropy (*ΔS*<sup>a</sup>), and free energy (*ΔG*<sup>a</sup>)) of the irreversible denaturation process were determined from the following equations based on the transition state theory:

\[ ΔH^a = Eₐ - RT \]

\[ ΔS^a = (A - \ln(k_b T/h) + 1)R \]

\[ ΔG^a = ΔH^a - TΔS^a \]

where *k*<sub>b</sub> is the Boltzmann constant and *h* is the Planck constant.

Alternatively, the thermal denaturation profiles were conventionally obtained using the remaining activity data after heat treatment at various temperatures for 10 min. The half-denaturation temperature (*Tₜ*) was estimated from the profile. In this work, *Tₘ* was used as an empirical parameter to rank thermostabilities of the IPMDHs, although it is not the same as *Tₚ*, the midpoint temperature of the unfolding transition, because of the irreversibility of the protein. However, the obtained profiles were well correlated with the structural unfolding profiles monitored by CD spectroscopy for the original chimera and *T. thermophilus* IPMDH, indicating that
the inactivation of the enzyme function is considerably related to the structural unfolding of the proteins (e.g., [24]). Thus, the obtained profiles were considered to be well correlated with the thermodynamic stability of the protein.

2.5. Analysis on the computer

Screening of hydrophobic core residues of the wild-type enzyme was done by the program ‘SCROIL’ [25]. To generate the refined atomic coordinate, the program ‘X-PLOR’ [26] was used. The electrostatic interaction was calculated and visualized by the program ‘GRASP’ [27].

3. Results

The amino acid sequence of the mesophilic portion, i.e., the 2M portion, of the chimeric IPMDH, 2T2M6T, was compared with that of the corresponding region of T. thermophilus wild-type IPMDH. Among 59 amino acid residues (75–133) in the 2M portion of the chimeric enzyme, 37 residues are identical with the sequence of the thermophilic wild-type enzyme, and the remaining 22 residues are different. We selected 11 amino acid residues out of 22 residues considering the effects of (1) tightening the hydrophobic packing, (2) electrostatic interactions and (3) the removal of steric hindrance. These three effects are referred to be major determinants of the protein stability in some literature (e.g., [28]). These sites are indicated in Fig. 1B and summarized in Table 1. We constructed 11 mutants of 2T2M6T IPMDH each of which has a single amino acid replacement with the corresponding residue of T. thermophilus enzyme at each site described above. The resulting 11 mutant chimeric enzymes were expressed in E. coli JA221.

As the first approach, each mutant chimera was partially purified by heat treatment (for 10 min at 50°C) and subjected to stability analysis. In the present study, the half-denaturation temperature ($T_{1/2}$) of each mutant was measured and compared (see Section 2). The results of the 11 mutant chimeras are summarized in Table 1. Among these, the thermostabilities of four mutants (N80L, S82R, A115L, and N125G) were found to be improved as compared with the original chimera, 2T2M6T. The other seven mutations did not improve the stability. However, it is hard to speculate about the molecular reasons; the expected contribution of the substituted side chain may be canceled by the steric conflict with neighboring side chains.

These four mutants were purified to homogeneity and subjected to further analyses. Table 2 summarizes $K_m$ and $k_{cat}$ values for these four mutants under standard assay conditions at 50°C. No significant difference was found for $k_{cat}$ values of these enzymes, while N80L showed slightly higher affinity, and S82R showed approx. 2 times higher affinity for D-IPM than that of the original chimeric enzyme. Thus,
Table 1
List of the mutation sites and the thermal denaturation temperatures of the mutant chimeric IPMDHs

<table>
<thead>
<tr>
<th>Location</th>
<th>Substituted amino acid residues (abbreviated name of mutant)</th>
<th>$T_h$ ($\Delta T_h$) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loop</td>
<td>Asn80→Leu (N80L)‡</td>
<td>67 (+2)</td>
</tr>
<tr>
<td>Loop</td>
<td>Ala115→Leu (A115L)§</td>
<td>71 (+6)</td>
</tr>
<tr>
<td>β-Strand</td>
<td>Val129→Leu (V129L)‡</td>
<td>65 (0)</td>
</tr>
<tr>
<td>Loop</td>
<td>Ser82→Arg (S82R)‡</td>
<td>71 (+6)</td>
</tr>
<tr>
<td>α-Helix</td>
<td>Lys88→Thr (K88T)‡</td>
<td>62 (–3)</td>
</tr>
<tr>
<td>Loop</td>
<td>Ser113→Glu (S113G)‡</td>
<td>65 (0)</td>
</tr>
<tr>
<td>Type II β-turn</td>
<td>Ser111→Gly (S111G)‡</td>
<td>65 (0)</td>
</tr>
<tr>
<td>Loop</td>
<td>Asn125→Gly (N125G)‡</td>
<td>68 (+3)</td>
</tr>
<tr>
<td>Loop</td>
<td>Glu96→Ser (Q96S)‡</td>
<td>65 (0)</td>
</tr>
<tr>
<td>β-Strand</td>
<td>Phe128→Val (F128V)‡</td>
<td>65 (0)</td>
</tr>
<tr>
<td>Type II β-turn</td>
<td>Glu110→Pro (E110P)‡</td>
<td>65 (0)</td>
</tr>
<tr>
<td>Type II β-turn</td>
<td>Gin110→Pro/Ser111→Gly (E110P/S111G)§</td>
<td>62 (–3)</td>
</tr>
</tbody>
</table>

† Error was estimated to be ±1°C.
‡ $T_h$ differences between each mutant and the original chimera, 2T2M6T ($T_h$ = 65°C).
§ The potential effect of this substitution is tightening of hydrophobic interactions.
¶ The potential effect of this substitution is change of electrostatic interaction.
# The potential effect of this substitution is removal of steric hindrance by canceling the abnormal torsion angle upon introducing glycine residue to the particular site.
* The potential effect of this substitution is removal of steric hindrance by removing a bulky residue.
* A proline residue is introduced to this particular site (see Section 4).

these mutant chimeras were improved in their thermal stability without any loss of catalytic function.

The thermal stabilities of the four mutants are analyzed in detail as described in Section 2. The thermal denaturation profiles of these purified mutant enzymes are shown in Fig. 2. The $T_h$ values obtained from Fig. 2 and the activation parameters of the irreversible denaturation reaction estimated from the Arrhenius plots are summarized in Table 2. The activation enthalpies of all the mutants were increased compared to the original chimera, suggesting an enthalpic effect on the thermal stability of the enzyme. However, no positive correlation was found between $\Delta H^\circ$ and $\Delta T_h$ or $\Delta G^\circ$. The activation Gibbs free energy changes ($\Delta G^\circ$) are in the range of 1–4 kJ/mol per single mutation.

For an attempt, we constructed a double-site-mutated chimera (S82R/N125G, see Fig. 1B), and this mutation showed a cumulative effect on the stability as compared with the case of the individual single point mutation (Table 3). In order to confirm the

Fig. 2. Thermal stability of mutant chimeric IPMDHs. Enzyme (0.2 mg/ml) was treated at various temperatures for 10 min, immediately chilled on ice, and the remaining activity measured at 50°C. •, T. thermophillus; ○, S82R; ■, A115L; ●, N125G; Δ, N80L; ▲, 2T2M6T, □, SP2R/N125G.

Table 2
Kinetic parameters of T. thermophillus IPMDH and the mutant chimeras at 50°C

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPM</td>
<td>32</td>
<td>369</td>
</tr>
<tr>
<td>NAD</td>
<td>12</td>
<td>318</td>
</tr>
<tr>
<td>S82R</td>
<td>15</td>
<td>257</td>
</tr>
<tr>
<td>N80L</td>
<td>19</td>
<td>378</td>
</tr>
<tr>
<td>N125G</td>
<td>21</td>
<td>295</td>
</tr>
<tr>
<td>2T2M6T</td>
<td>135</td>
<td>149</td>
</tr>
<tr>
<td>SP2R/N125G</td>
<td>215</td>
<td>161</td>
</tr>
</tbody>
</table>

Table 3
Kinetic parameters of T. thermophillus IPMDH and the mutant chimeras at 50°C

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ (µM)</th>
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</thead>
<tbody>
<tr>
<td>IPM</td>
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<td>S82R</td>
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<td>257</td>
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<td>N80L</td>
<td>19</td>
<td>378</td>
</tr>
<tr>
<td>N125G</td>
<td>21</td>
<td>295</td>
</tr>
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<td>2T2M6T</td>
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<td>215</td>
<td>161</td>
</tr>
</tbody>
</table>

Fig. 2. Thermal stability of mutant chimeric IPMDHs. Enzyme (0.2 mg/ml) was treated at various temperatures for 10 min, immediately chilled on ice, and the remaining activity measured at 50°C. •, T. thermophillus; ○, S82R; ■, A115L; ●, N125G; Δ, N80L; ▲, 2T2M6T, □, SP2R/N125G.
additivity of each mutation, it is necessary to construct triple or quadruple mutants by combination with the other mutation(s) to the double-mutated enzyme and measure their stability to heat. This is the subject of a future study.

4. Discussion

4.1. Tightening the hydrophobic packing (N80L, A115L, and V129L)

It has been proposed that imperfect packing may be a frequent cause of limited stability and improvement of internal packing increases the stability of a protein (e.g., [29]). On the other hand, the difficulty of this kind of stabilization has also been suggested because of a limited number of appropriate cavities [30].

According to the hydrophobic core analysis of the 3-D structure of the thermophilic enzyme by the program 'SCROIL' [25], the two residues involved in hydrophobic cores of the enzyme (Leu80 and Leu115 of the thermophilic enzyme; Fig. 1B) were found to be replaced by less or smaller hydrophobic residues (Asn and Ala, respectively) in the chimeric enzyme. There is a cavity in the vicinity of the side chain of Ala115 (Fig. 3) in 2T2M6T. Thus, the substitution by Leu at this site most likely strengthens the hydrophobic interaction, conferring an extra stability to the chimeric enzyme. Asn80 in 2T2M6T (Leu in T. thermophilus IPMDH) is also located in the hydrophobic core, and surrounded by Ile46, Trp77 and Pro86. Thus, the hydrophobic residue is favorable at this site, leading to the stabilization of

Table 3
The change of the activation parameters of the thermal denaturation of T. thermophilus IPMDH and the mutant chimeras at 328 K

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$T_k^0$ ($^\circ$C)</th>
<th>$\Delta H^\circ$ ($\Delta H^\circ^b$) (kJ/mol)</th>
<th>$\Delta S^\circ$ ($\Delta S^\circ^b$) (J/mol/deg)</th>
<th>$\Delta G^\circ$ ($\Delta G^\circ^b$) (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. thermophilus</td>
<td>83 (18)</td>
<td>338 (138)</td>
<td>644 (357)</td>
<td>127 (19)</td>
</tr>
<tr>
<td>S82R/N125G</td>
<td>78 (13)</td>
<td>281 (81)</td>
<td>505 (218)</td>
<td>115 (9)</td>
</tr>
<tr>
<td>S82R</td>
<td>73 (8)</td>
<td>256 (56)</td>
<td>442 (155)</td>
<td>111 (4)</td>
</tr>
<tr>
<td>A115L</td>
<td>72 (7)</td>
<td>235 (35)</td>
<td>380 (93)</td>
<td>110 (3)</td>
</tr>
<tr>
<td>N125G</td>
<td>68 (3)</td>
<td>233 (33)</td>
<td>381 (94)</td>
<td>108 (2)</td>
</tr>
<tr>
<td>N80L</td>
<td>67 (2)</td>
<td>236 (37)</td>
<td>394 (107)</td>
<td>107 (1)</td>
</tr>
<tr>
<td>2T2M6T</td>
<td>65</td>
<td>200</td>
<td>287</td>
<td>106</td>
</tr>
</tbody>
</table>

*Half-inactivation temperatures were estimated from the data shown in Fig. 2.

*The values in parentheses are the difference from the corresponding parameters of the original chimera, 2T2M6T.
2T2M6T. N80L and A115L successfully increased the half-denaturation temperature, \( T_d \), of the chimeric enzyme by 2 and 7°C, respectively (Table 3). Our results confirm that tightening of the hydrophobic interactions is one of the most powerful strategies for protein stabilization.

We have shown previously that the interdomain interactions are important to the extreme thermostability of \( T. \) thermophilus IPMDH based on the biophysical analysis of the thermal unfolding process [23,31]. It has also been suggested that Leu115 and Leu129 of \( T. \) thermophilus IPMDH are major candidates involved in the interdomain hydrophobic interaction which is responsible for the stability of the enzyme (Fig. 1B). The results obtained here (Tables 1 and 3) suggest that Leu115 is involved in the interdomain interactions which contribute to the stabilization of the less stable domain, domain 1. On the other hand, the stability of V129L was not improved (Table 1), probably due to the less hydrophobic environment around Leu129. The program SCROIL did not recognize Leu129 as a residue involved in a hydrophobic core in the \( T. \) thermophilus enzyme, in agreement with the experimental results.

4.2. Electrostatic interactions (S82R, K88T, and S113E)

The structural analyses showed that some thermophilic proteins have a large number of ion pairs and networks compared with those of the counterparts from mesophilic organisms (e.g., [3]). On the other hand, the effects of electrostatic interactions between solvent-exposed residues on the surface are weaker and thought to contribute little to protein stability because of the solvent screening and the entropic cost of the interacting partners [30].

Three hydrophilic residues, Arg82, Thr88, and Glu113, in \( T. \) thermophilus IPMDH are replaced by Ser, Lys, and Ser in the chimeric enzyme, respectively (Fig. 1B). The mutation of S113E did not alter the \( T_d \) value of the chimeric enzyme, and that of K88T reduced the \( T_d \) value, suggesting the difficulty of the detection of the effective electrostatic interactions. In the case of Glu113, the hydrogen bond is present between the \( \text{Oe} \) of Glu113 and the main chain nitrogen of Gly111 in the \( T. \) thermophilus enzyme, both of which are replaced by Ser in the chimera.

Fig. 4. Surface potential map around the 82nd residue of IPMDHs. 10T, \( T. \) thermophilus IPMDH [4]; S82R, the mutant chimera [32]; 2T2M6T, the original chimeric enzyme [18]. White bold line indicates the main chain of the enzyme and the number indicates that of each amino acid residue. Dotted area represents the molecular surface, and surface potential is described by color. Red, −50 kcal/mol; white, 0 kcal/mol; blue, 50 kcal/mol.
The substitution of Ser82 by Arg increased the half-denaturation temperature by 8°C. Because this particular residue is fully exposed to the solvent and has no direct contact with any negatively charged groups in this vicinity [32], the substitution was suspected to change the charge distribution at the surface. The analysis of the surface charge distribution of the 3-D structure of 2T2M6T and the mutant chimera [32] showed the existence of a strong negative charge in the vicinity of Ser82 of the chimeric enzyme. This negative charge around site 82 is at least partially neutralized by the substitution with Arg82 and the surface charge distribution of the chimera approached that of the T. thermophilus wild-type enzyme by this substitution (Fig. 4). Thus, the neutralization of charges on the molecule surface gives a profound effect on the thermal stability of the enzyme.

During the preparation of this manuscript, a similar result was reported for ribonuclease T1 [33], which shows an increase in protein stability by reversing the charge of a surface residue.

4.3. Removal of steric hindrance and introduction of proline residues (S111G, N125G, Q96S, F128V, and E110P)

It has been pointed out that removal of structural stress may largely affect protein stability [28]. The two glycine residues in the loop region of the thermophilic enzyme (Gly111 and Gly125) were found to be structurally unfavorable ones in the chimeric enzyme (Ser111 and Asn125). The substitution of Asn125 by Gly increased the $T_{m}$ by 3°C of the chimeric protein. The torsion angles ($\phi$, $\psi$) of Gly125 in the T. thermophilus enzyme are 89.90 and 4.02, respectively [4], and such abnormal torsion angles would not be taken by other residues than Gly. We suggest that the elimination of $\beta$-carbon by the substitution with Gly released the steric stress in the chimeric enzyme, and thus improved the thermal stability (see Tables 1 and 3). On the other hand, the introduction of a Gly residue at the position of Ser111 did not affect the stability (Table 1).

The bulky residues, Q96 and F128 in the chimeric IPMDH, were considered to be destabilizing factors. However, the stability of Q96S and F128V was not improved (Table 1), suggesting tolerance to amino acid substitutions at these portions of the enzyme.

The importance of proline residues to conformational stability has been mentioned for some proteins [34–37]. We constructed E110P and E110P/S111G, considering the combined effect of proline and the adjacent glycine residues at this site. However, neither mutation improved the stability (Table 1).

4.4. Conclusion

In this study, four sites, Leu80, Arg82, Leu115, and Gly125, at the mesophilic portion of the chimera, where replacement restored the thermal stability of the chimeric enzyme, were identified. It should be noted that all four residues are located at loops, suggesting the importance of a loop structure for the extreme stability of the thermophile protein. These sites are apart from one another, and different types of interaction affect the stability. Hence, a cumulative effect contributed by individual amino acids in different loops (see S82R/N125G in Table 3) is expected in thermophile proteins.

Acknowledgements

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