Extremophiles (1999) 3:259–262

ORIGINAL PAPER

Koichi Numata · Yoko Hayashi-Iwasaki
Katsuhide Yutani · Tairo Oshima

Studies on interdomain interaction of 3-isopropylmalate dehydrogenase from an extreme thermophile, *Thermus thermophilus*, by constructing chimeric enzymes

Received: September 29, 1998 / Accepted: June 25, 1999

Abstract In our previous study, we showed that a chimeric isopropylmalate dehydrogenase, 2T2M6T, between an extreme thermophile, *Thermus thermophilus*, and a mesophile, *Bacillus subtilis*, isopropylmalate dehydrogenases (the name roughly denotes the primary structure; the first 20% from the N-terminal is coded by the thermophile *leuB* gene, next 20% by mesophile, and the rest by the thermophile gene) denatured in two steps with a stable intermediate, suggesting that in the chimera some of the interdomain interaction was lost by amino acid substitutions in the "2M" part. To identify the residues involved in the interdomain interactions, the first and the second halves of the 2M part of the chimera were substituted with the corresponding sequence of the thermophile enzyme. Both chimeras, 3T1M6T and 2T1M7T, apparently showed one transition in the thermal denaturation without any stable intermediate state, suggesting that the cooperativity of the conformational stability was at least partly restored by the substitutions. The present study also suggested involvement of one or more basic residues in the unusual stability of the thermophile enzyme.

Key words Thermophilic enzymes · 3-Isopropylmalate dehydrogenase · Thermostability · *Thermus thermophilus* · Chimeric proteins · Interdomain interaction · Differential scanning calorimetry

Communicated by K. Horikoshi

K. Numata[1]
Department of Life Science, Tokyo Institute of Technology, Yokohama, Japan

Y. Hayashi-Iwasaki · T. Oshima (✉)
Department of Molecular Biology, Tokyo University of Pharmacy and Life Science, Hachioji, Tokyo 192-0392, Japan
Tel. +81-426-76-7134; Fax +81-426-76-7145
e-mail: toshima@ls.toyaku.ac.jp

K. Yutani
Institute of Protein Research, Osaka University, Suita, Japan

Present address:
[1] Future Project Division Bio Lab., Toyota Motor, Toyota, Japan

Introduction

3-Isopropylmalate dehydrogenase from an extreme thermophile, *Thermus thermophilus*, consists of two identical subunits. The high-resolution X-ray structure of the enzyme has been determined by Imada et al. (1991). The subunit can be divided into two domains. The first domain consists of two segments, 99 residues from the N-terminal and 94 residues from the C-terminal. The second domain consists of 152 residues from 100–251 and is involved in the subunit–subunit interaction. The active site is located in the deep cleft between these two domains.

The enzyme from the thermophile showed only one unfolding phase upon heating. However, a chimeric isopropylmalate dehydrogenase, 2T2M6T, denatured in two steps with a stable intermediate stage (Hayashi-Iwasaki et al. 1996). The chimera was constructed by ligating fragments of the thermophile and *Bacillus subtilis leuB* genes coding for isopropylmalate dehydrogenase, and the name denotes the primary structure of chimera; the first 20% from the N-terminal is coded by the thermophile *leuB* gene, the next 20% is coded by the mesophile gene, and the rest is coded by the thermophile (Fig. 1). The X-ray crystal structure of 2T2M6T has been already solved by Onodera et al. (1994), and the results indicate that the three-dimensional (3-D) structure of 2T2M6T is very close to that of the thermophile enzyme. Calorimetric measurements of a chimeric isopropylmalate dehydrogenase 2T2M6T and its mutant enzymes showed that two structural domains of the chimeric enzyme denatured independently; the first transition temperature is related to the unfolding of the first domain and the second to that of the second domain (Hayashi-Iwasaki et al. 1996). In chimera 2T2M6T, the 2M part coded by the mesophile gene is located in both the first and second domains (Fig. 1B), and some of the interdomain interactions might be abolished. To analyze the roles of side chains in the interdomain interaction and in the thermal unfolding process of the enzyme, two chimeric isopropylmalate dehydrogenases, 2T1M7T and 3T1M6T, were constructed (Fig. 1). In 2T1M7T, the mesophile *leuB*

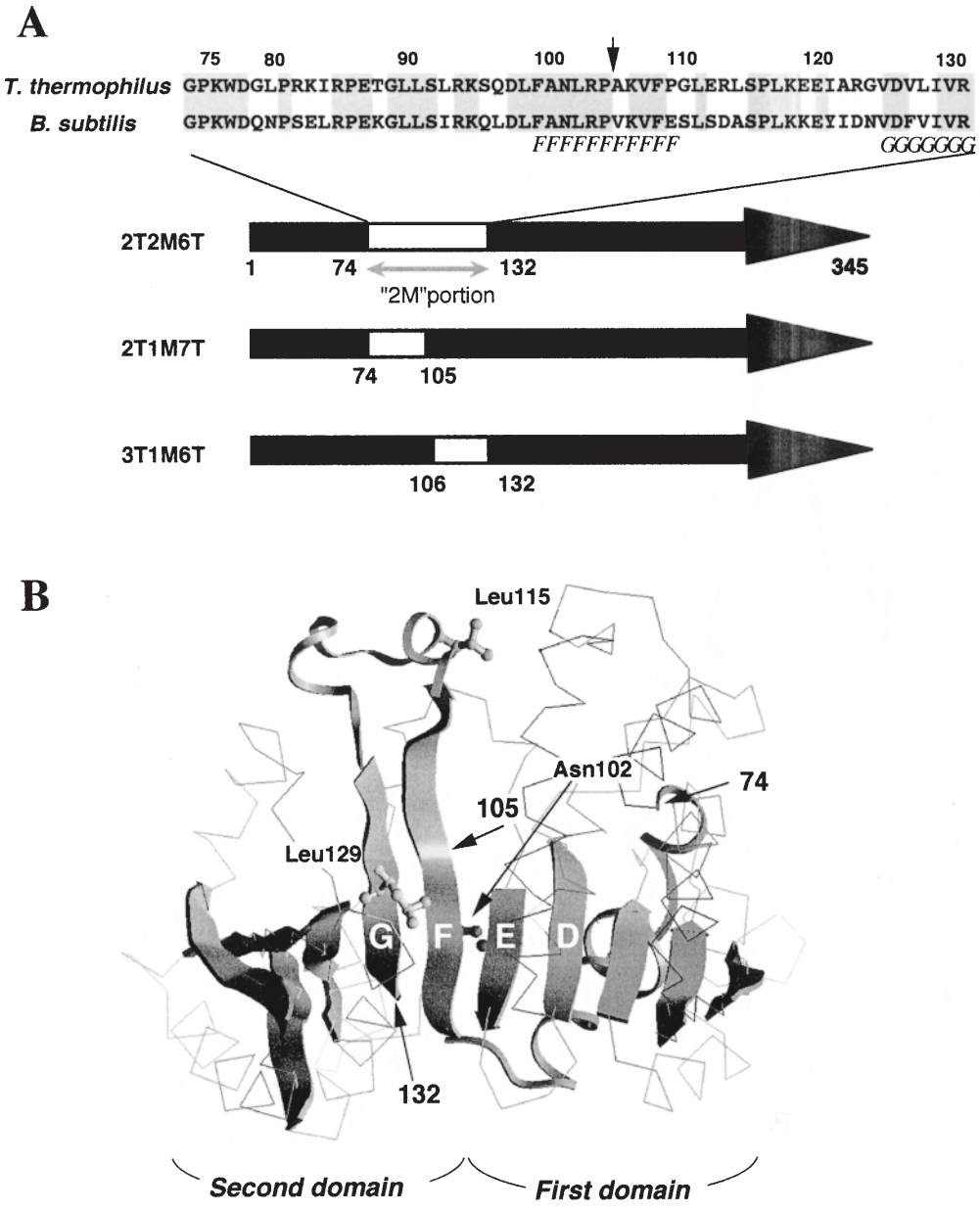

Fig. 1. **A** Sequence comparison of the amino acid residues in the "2M" part of 2T2M6T chimeric isopropylmalate dehydrogenase. The residues in β-sheets F and G are labeled below the sequence. *Arrows* schematically represent the primary sequence of chimeric enzymes. *Closed* and *open* parts are coded by the *Thermus thermophilus* and *Bacillus subtilis leuB* gene, respectivity. **B** Three-dimensional structure of a subunit of *T. thermophilus* isopropylmalate dehydrogenase (drawn based on data reported by Imada et al. 1991). All β-sheets are shown as *arrows*. The 2M part is represented as the *thick ribbons and arrows* (residue 74–132). The boundary of the first and second halves of the 2M part (residue 105) is indicated, and the β-sheets *D*, *E*, *F*, and *G* are labeled (signs are according to Imada et al. 1991). The side chains of Leu 115 and Leu 129, Nδ2 of Asn 102, and O of Ala 260 are shown as a ball-and-stick model (drawn with Insight II, Molecular Simulations Inc.)

gene codes a part of the first domain plus five residues in the second domain (74–105), whereas the mesophile *leuB* gene codes only for a part of the second domain of 3T1M6T (106–132). This article reports the thermal melting profiles of these chimeras.

Materials and methods

Constructions, expressions, and purifications of chimeric enzymes used in the present study are described in a previous paper (Numata et al. 1995). The concentration of the enzyme was estimated using a molar absorbance coefficient of 30400 at 280 nm (Yamada et al. 1990). The solution was filtrated and degassed, and the pH value was confirmed before each measurement. The enzyme activity was assayed as described by Numata et al. (1995).

Calorimetric measurements were carried out with a scanning microcalorimeter, DASM-4 (Bureau of Biological Instrumentation of Academy of Science of the USSR), under an extra pressure of 2 atm. The experimental conditions have been described previously (Hayashi-Iwasaki et al. 1996). Enzyme concentration was 0.8–1.0 mg/ml at pH 10.2. The scan rate was 1.0°/min. The data were transferred to a personal computer (NEC) and analyzed using a computer program, Origin (MicroCal, Northampton, MA).

Circular dichroic (CD) measurements were carried out with a J-500C spectropolarimeter (Jasco, Tokyo, Japan). The 2-mm cell (protein concentration, 0.1 mg/ml at pH 7.6)

was used for the present study. The temperature of the enzyme solution in the cell was controlled with a HAAKE circulating bath and a programmable temperature controller. The temperatures in the cell were monitored with a thermocouple. The scan rate was 0.5°/min.

Results and discussion

Both chimeric enzymes, 2T1M7T and 3T1M6T, are functionarily active and their enzymatic properties resemble those of the wild-type enzyme from the extreme thermophile as described in a previous report (Numata et al. 1995).

The results of the calorimetric measurements are illustrated in Fig. 2 and summarized in Table 1. Only one peak was recorded in the thermograms for *T. thermophilus* isopropylmalate dehydrogenase, 2T1M7T, an 3T1M6T (Fig. 2A–C). In contrast, the differential scanning calorimetry (DSC) profile of 2T2M6T has two distinct peaks with a stable intermediate as reported previously (Fig. 2D; Hayashi-Iwasaki et al. 1996). Although the $\Delta H_{cal}/\Delta H_{vH}$ ratios of the thermal denaturation of these enzymes were approximately 4 (Table 1), which indicates that the thermal denaturation of these enzymes did not follow an ideal all-or-none manner (Privalov et al. 1981), the results strongly suggest that the substitutions of amino acid residues of either the first or second half of "2M" part of 2T2M6T recovered the interdomain interactions and the cooperativity of the conformational stability.

The thermal denaturation profiles obtained by CD spectropolarimeter are shown in Fig. 3. Under the applied conditions, no aggregation was observed during the heat treatment. Compared with the thermal denaturation of 2T2M6T, both 2T1M7T and 3T1M6T denatured without any stable intermediate, supporting the conclusion obtained from the DSC measurements that the interdomain interactions are restored by the substitutions of either the first or the second half of the 2M part of 2T2M6T.

In the X-ray crystal structures of both the thermophile and 2T2M6T enzymes, the first half of the 2M part contains

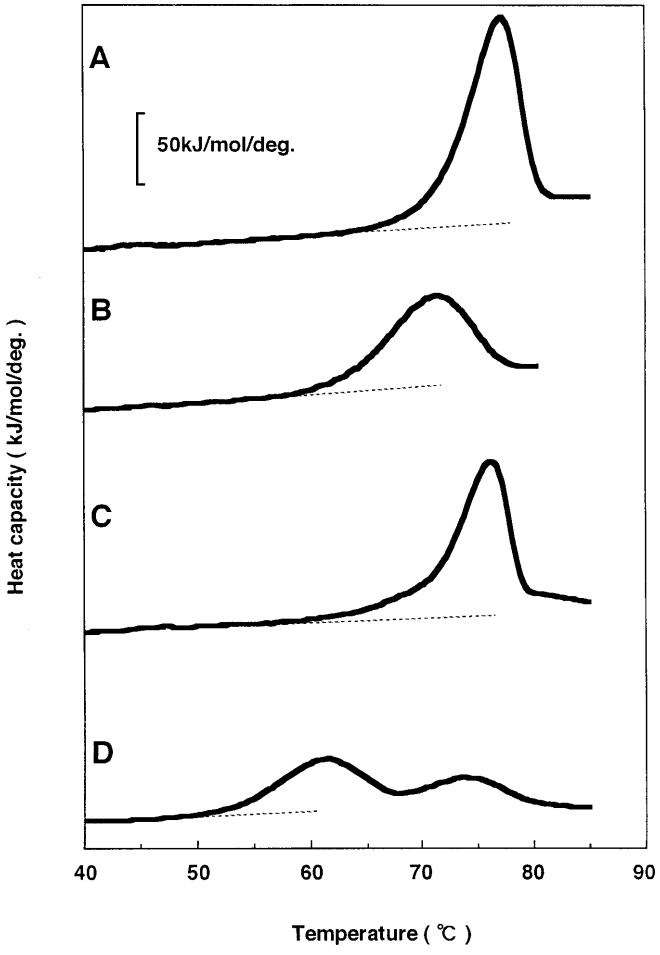

Fig. 2A–D. Differential scanning calorimetry (DSC) melting profiles of the chimeric and *T. thermophilus* isopropylmalate dehydrogenases in 20 mM $NaHCO_3$-NaOH buffer, pH 10.2. **(A)** *T. thermophilus* IPMDH; **(B)** 3T1M6T; **(C)** 2T1M7T; **(D)** 2T2M6T

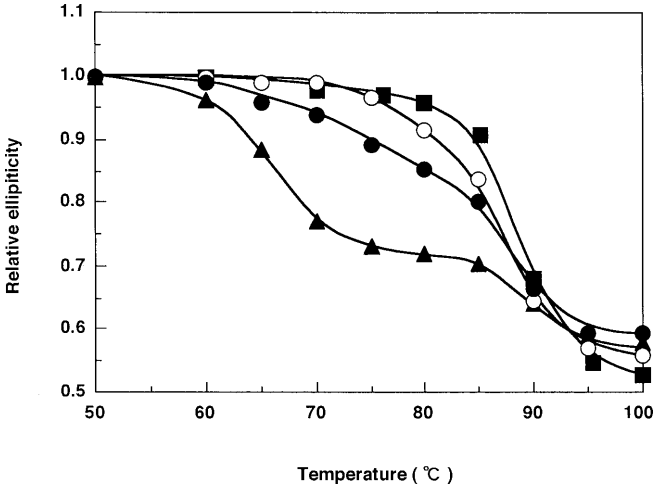

Fig. 3. Thermal unfoldings of the chimeric and *T. thermophilus* isopropylmalate dehydrogenases monitored by circular dichroism (CD). Ellipticity at 222 nm in 20 mM potassium phosphate buffer, pH 7.6, was recorded. Relative ellipticity at 222 nm, normalized by the value of the native state of each protein, is plotted against temperature. *Closed rectangles*, *T. thermophilus* isopropylmalate dehydrogenase; *open circles*, 3T1M6T; *closed circles*, 2T1M7T; *closed triangles*, 2T2M6T

Table 1. Thermodynamic parameters of the unfolding for isopropylmalate dehydrogenases

Abbreviated enzyme name	Tp[a] (K)	ΔH_{cal}[b] (kcal/mol)	ΔH_{vH}[c] (kcal/mol)	$\Delta H_{cal}/\Delta H_{vH}$
10T	350.0	845	183	4.6
2T1M7T	348.9	694	187	3.7
3T1M6T	344.3	543	110	4.9

Note: These values were obtained from temperature dependence of partial heat capacity of these isopropylmalate dehydrogenases (IPMDHs) shown in Fig. 2

[a] Peak temperature

[b] Calorimetric enthalpy

[c] van't Hoff enthalpy

helix d of the first domain and the N-terminal half of the β-sheet F of the second domain (see Fig. 1A,B) (Imada et al. 1991; Onodera et al. 1994). It has been pointed out in our previous paper that one of the hydrogen bonds between the β-sheets E of the first domain and F of the second domain (i.e., Nδ2 of Asn 102 to O of Ala 260; Fig. 1B) is missing in 2T2M6T, as compared with the structure of the thermophile enzyme (Hayashi-Iwasaki et al. 1996). 3T1M6T has the thermophile sequence in this region, which should allow restoration of the hydrogen bond between the first and the second domains, and therefore shows cooperative denaturation (Figs. 2, 3).

On the other hand, the second half of the 2M part includes the C-terminal half of the β-sheet F and the whole of sheet G of the second domain (Fig. 1B). It has already been suggested in the previous report that Leu-129 in the β-sheets F (Fig. 1B) of the thermophile IPMDH (corresponding to Val in 2T2M6T) may be responsible for the packing of the interdomain hydrophobic core of the thermophile IPMDH (Hayashi-Iwasaki et al. 1996). In addition, another interdomain hydrophobic cluster is also found around the turn region between sheets F and G. Leu-115 of the thermophile enzyme is located at the turn region and is involved in the formation of the hydrophobic cluster (Fig. 1B). However, this residue is substituted by a less hydrophobic side chain, Ala, in 2T2M6T. Thus, 2T1M7T, which has the thermophile sequence at the region, restores the cooperativity of the thermal denaturation, probably because of improvement of the interdomain hydrophobic packing. Taken together, the results reported herein suggest that both the hydrophobic interaction and the hydrogen bonds between domains contribute to the stability of the thermophile IPMDH.

Inspection of the denaturation temperatures of 3T1M6T and 2T1M7T suggests that 2T1M7T is more stable than 3T1M6T in the DSC profiles, while the opposite result was obtained with the CD profiles (Figs. 2, 3). It should be noted in this connection that the thermal denaturation temperatures of all the enzymes recorded by CD are different from those by DSC measurements because in scanning calorimetry the protein concentration used (0.8–1 mg/ml) was fairly high (for CD measurements, we used 0.1 mg/ml) and an alkaline buffer was used to prevent the formation of insoluble aggregates of the denatured protein. Interestingly, the denaturation temperature of 3T1M6T in alkaline pH is much lower than that of *T. thermophilus* enzyme under the same conditions (DSC; Fig. 2), but is close to that of the thermophile enzyme under the neutral environment (CD; Fig. 3). These results suggest that one or more basic residue(s), which are protonated in the neutral pH used for the CD measurement, in the region between residues 74 and 105, are involved in electrostatic interactions, which are important for the extreme stability of the thermophile enzyme. The sequence comparison of this region shown in Fig. 1B indicates that the plausible candidates are Arg82 and Lys83.

In conclusion, the interdomain interactions abolished in 2T2M6T seemed to be, at least partly, resorted both in 2T1M7T and in 3T1M6T, and these chimeras showed only one phase in the thermal denaturation without any apparent intermediates in both neutral and alkaline environments. It is likely that the residues in the first and the second halves of the 2M part are equally important for the domain–domain interaction of the thermophile enzyme. The present study also suggested the importance of basic residues (Arg82 or Lys83) for the unusual stability of the *T. thermophilus* isopropylmalate dehydrogenase, which will be tested by site-directed mutagenesis in our future studies.

Acknowledgments We thank Drs. F. Arisaka (Tokyo Institute of Technology) and S. Kidokoro (Sagami Chemical Research Center) for valuable discussion.

References

Hayashi-Iwasaki Y, Numata K, Yamagishi Y, Yutani K, Sakurai M, Tanaka N, Oshima T (1996) A stable intermediate in the thermal unfolding process of a chimeric 3-isopropylmalate dehydrogenase between a thermophilic and a mesophilic enzymes. Protein Sci 5:511–516

Imada K, Sato M, Tanaka N, Katsube Y, Matsuura Y, Oshima T (1991) Three-dimensional structure of a highly thermostable enzyme, 3-isopropylmalate dehydrogenase of *Thermus thermophilus* at 2.2 Å resolution. J Mol Biol 222:725–738

Naumata K, Muro M, Akutsu N, Nosoh Y, Yamagishi A, Oshima T (1995) Thermal stability of chimeric isopropylmalate dehydrogenase genes constructed from a thermophile and a mesophile. Protein Eng 8:39–43

Onodera K, Sakurai M, Tanaka N, Numata K, Yamagishi A, Oshima T, Sato M, Katsube Y (1994) Three-dimensional structure of chimeric enzymes between *Bacillus subtilis* and *Thermus thermophilus* 3-isopropylmalate dehydrogenases. Protein Eng 7:453–459

Privalov PL, Mateo PL, Khechinashvili NK (1981) Comparative thermodynamic study of pepsinogen and pepsin structure. J Mol Biol 152:445–464

Yamada T, Akutsu N, Miyazaki K, Kakinuma K, Yoshida M, Oshima T (1990) Purification, catalytic properties, and thermal stability of *threo*-Ds-3-isopropylmalate dehydrogenase coded by *leuB* gene from an extreme thermophile, *Thermus thermophilus* strain HB8. J Biochem (Tokyo) 108:449–456