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A stable intermediate in the thermal unfolding process of a chimeric 3-isopropylmalate dehydrogenase between a thermophilic and a mesophilic enzymes

YOKO HAYASHI-IWASAKI,[1,5] KOICHI NUMATA,[2] AKIHIKO YAMAGISHI,[3] KATSUHIDE YUTANI,[4] MASAHIRO SAKURAI,[1] NOBUO TANAKA,[1] AND TAIRO OSHIMA[3]

[1] Department of Life Science, Tokyo Institute of Technology, Nagatsuta, Yokohama 226, Japan
[2] Research Division Bio Laboratory, Toyota Motor Corporation, Toyota-Cho, Toyota, Aichi 471, Japan
[3] Department of Molecular Biology, Tokyo University of Pharmacy and Life Science, Hachioji, Tokyo 192-03, Japan
[4] Institute of Protein Research, Osaka University, Yamadaoka, Suita, Osaka 565, Japan

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Abstract

The thermal unfolding process of a chimeric 3-isopropylmalate dehydrogenase made of parts from an extreme thermophile, *Thermus thermophilus*, and a mesophile, *Bacillus subtilis*, enzymes was studied by CD spectrophotometry and differential scanning calorimetry (DSC). The enzyme is a homodimer with a subunit containing two structural domains. The DSC melting profile of the chimeric enzyme in 20 mM $NaHCO_3$, pH 10.4, showed two endothermic peaks, whereas that of the *T. thermophilus* wild-type enzyme had one peak. The CD melting profiles of the chimeric enzyme under the same conditions as the DSC measurement, also indicated biphasic unfolding transition. Concentration dependence of the unfolding profile revealed that the first phase was protein concentration-independent, whereas the second transition was protein concentration-dependent. When cooled after the first transition, the intermediate was isolated, which showed only the second transition upon heating. These results indicated the existence of a stable dimeric intermediate followed by the further unfolding and dissociation in the thermal unfolding of the chimeric enzyme at pH 10–11. Because the portion derived from the mesophilic isopropylmalate dehydrogenase in the chimeric enzyme is located in the hinge region between two domains of the enzyme, it is probably responsible for weakening of the interdomain interaction and causing the decooperativity of two domains. The dimeric form of the intermediate suggested that the first unfolding transition corresponds to the unfolding of domain 1 containing the N- and C-termini of the enzyme, and the second to that of domain 2 containing the subunit interface.

Keywords: chimeric protein; circular dichroism; domain; scanning microcalorimetry; thermal stability; *Thermus thermophilus*

3-Isopropylmalate dehydrogenase (IPMDH, [EC 1.1.1.85]) is one of the enzymes involved in the leucine biosynthetic pathway. IPMDH from an extreme thermophile, *Thermus thermophilus*, is composed of two identical subunits containing 345 amino acids (Yamada et al., 1990). The high-resolution X-ray structural analysis showed that each subunit has two structural domains with parallel α/β motifs (Imada et al., 1991; see Fig. 1A). One domain, containing the N- and C-termini regions (residues 1–99 and 252–345), is designated as domain 1, and the other, containing the subunit interface (residues 100–251), as domain 2.

The amino acid sequence of *T. thermophilus* enzyme is 58% identical to a mesophilic enzyme from *Bacillus subtilis* (Imai et al., 1987), yet the former enzyme is much more thermostable than the latter (Numata et al., 1995). In order to estimate portions that mainly contribute to thermal stability of the thermophilic enzyme, we have previously constructed several chimeric IPMDHs by artificial crossing over between the mesophile, *B. subtilis*, and the thermophile *leuB* genes (Numata et al., 1995). Numata et al. (1995) have shown that the thermal stability of each chimeric enzyme is approximately proportional to the content of the amino acid sequence from the thermophilic enzyme,

Reprint requests to: Yoko Hayashi-Iwasaki, c/o Prof. Tairo Oshima, Department of Molecular Biology, Tokyo University of Pharmacy and Life Science, Hachioji, Tokyo 192-03, Japan; e-mail: yokoh@ls.toyaku.ac.jp.

[5] Present address: Department of Molecular Biology, Tokyo University of Pharmacy and Life Science, Hachioji, Tokyo 192-03, Japan.

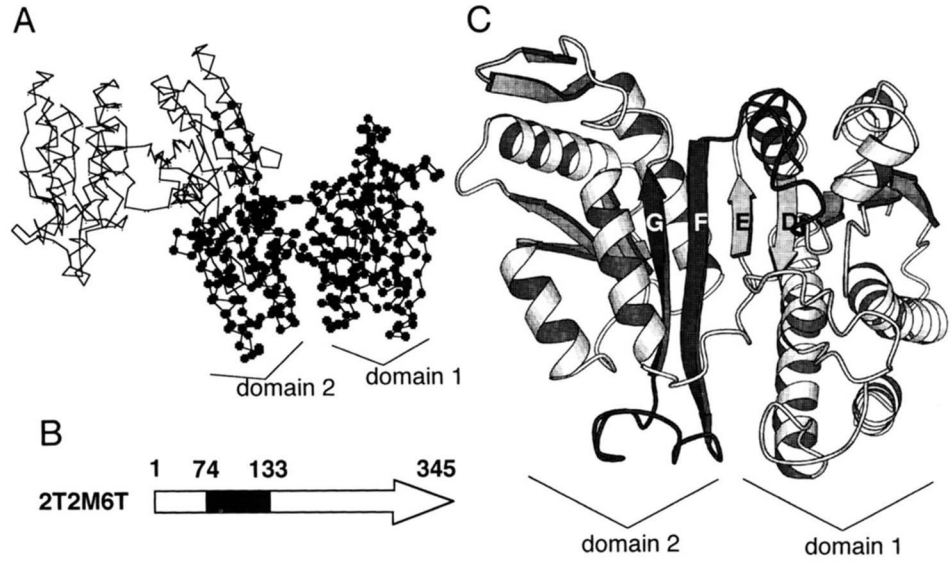

Fig. 1. A: C_α backbone structure of homodimeric *T. thermophilus* IPMDH. Filled circles represent C_α of one subunit of the homodimeric enzyme. Domains 1 (residues 1–99, 252–345) and 2 (residues 100–251) are indicated. **B:** Construction of the chimeric (2T2M6T) IPMDH (arrow). Each number represents an amino acid residue number. White and black portions represent portions derived from the thermophilic and a mesophilic (*B. subtilis*) IPMDHs, respectively. **C:** Ribbon model of one subunit. Drawing was created by the program Molscript (Kraulis, 1991). The bold ribbon represents the C_α of the mesophilic portion (2M portion; residues 75–133) of the chimeric enzyme, 2T2M6T, which is derived from the sequence of *B. subtilis* IPMDH. Domains 1 and 2 are also indicated. Four β-sheets located in the hinge between two domains are labeled with capital letters (D, E, F, G).

and concluded that amino acid residues contributing the thermal stability distribute themselves evenly. However, one of the chimeric enzymes, 2T2M6T, is of particular interest in that it showed considerably lower thermostability than the *T. thermophilus* wild-type enzyme, in spite of 6% difference in their total amino acid sequences (i.e., only 22 residues). In this chimeric enzyme, the substituted portion derived from the mesophilic enzyme (2M) is located around the hinge region that connects the two domains of IPMDH (see Fig. 1B,C).

In this study, we have applied calorimetric and CD measurements to analyze the thermal unfolding processes of the thermophilic IPMDH and the chimeric enzyme, 2T2M6T, and to investigate the effect of the substituted portion on the thermal stability in detail. We detected a partially unfolded dimeric intermediate in the thermal unfolding process of the chimeric enzyme, which is followed by the further unfolding and dissociation. The differences in stability between the wild-type and the chimeric enzymes are discussed in light of the high-resolution X-ray structures of both proteins (Imada et al., 1991; Onodera et al., 1994).

Results

The chimeric IPMDH (2T2M6T) has been constructed previously and characterized by Numata et al. (1995, Fig. 1B). The structural change of the chimeric enzyme upon heating was further investigated by differential scanning calorimetry (DSC) and CD spectroscopy. All the unfolding experiments described were performed at pH above 9.6, because, at pH below 9.6, apparent aggregation of the thermal unfolded protein was noted by the DSC measurement, accompanied by the exothermal process above the peak temperature. Under the conditions in a pH range 10–11, both the wild-type and the chimeric enzymes retain fully their secondary structure contents as judged by CD spectra at least up to 20 h.

DSC measurements

Figure 2A shows the DSC melting profile of the chimeric IPMDH in 20 mM $NaHCO_3$-NaOH buffer, pH 10.4 (solid trace) compared with that of the thermophilic wild-type IPMDH (dashed trace). The melting profiles of the chimeric IPMDH at pH 10–11 had two peaks, whereas the wild-type IPMDH showed only a single peak under the same experimental conditions. The unfolding profiles of both IPMDHs were not reproducible after the first scanning up to 90 °C and cooling down. In addition, the profiles were slightly scan-rate dependent: the peak temperature became higher and the area of the peaks became larger when the scan rate was raised, suggesting that the analysis under the equilibrium assumptions is not suitable in this case. The curve-fitting analyses of the DSC profiles to kinetic models accounting for the irreversible character of the thermal denaturation (for example, Sánchez-Ruiz et al., 1988; Freire et al., 1991) are underway, and the results will be reported elsewhere. When the sample solution of the chimeric enzyme was cooled down in the cell after heating up to the first peak temperature and then the DSC measurement was repeated again, only the second transition was recorded (Fig. 2B). The transition temperature and the peak height of the isolated intermediate were the same as those of the second transition of the chimeric enzyme. This indicates that the intermediate, unchanged upon lowering the temperature, remained in its intermediate form. Likewise, the sample solution in 20 mM $NaHCO_3$ buffer, pH 10.4, heated up to the 62 °C and then cooled down at room temperature, showed no enzy-

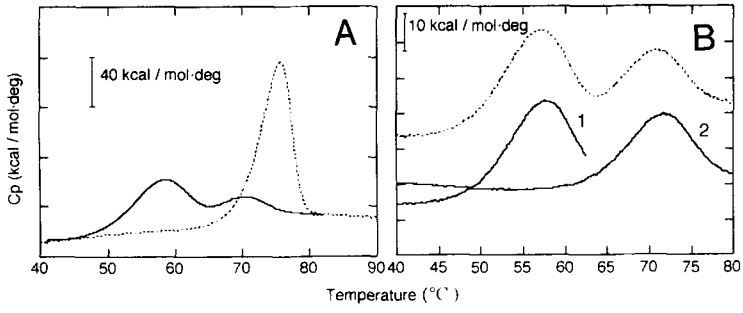

Fig. 2. DSC melting profiles of the chimeric and *T. thermophilus* wild-type IPMDHs in 20 mM $NaHCO_3$-NaOH buffer, pH 10.4. **A:** Solid and dashed lines represent the profiles of the chimeric and *T. thermophilus* wild-type IPMDHs, respectively. **B:** Dashed line represents the profiles of the chimeric enzyme. Solid lines, 1 and 2, represent the profiles of the first run up to 62 °C and the second run, respectively.

matic activities under the standard conditions at pH 7.6 and at 55 °C (see the Materials and methods), indicating that the intermediate is catalytically in an inactive form (data not shown).

Figure 3 shows the dependence of the melting profiles of the chimeric enzyme on the buffer concentration. In the low ion concentration, 1 mM $Na_2B_4O_7$, the thermal unfolding profile of the chimeric enzyme had a single peak, as in the case of the wild-type enzyme, although the peak temperature of the chimeric enzyme was 15° lower than that of the wild-type enzyme at the same pH. The peak temperature of the second transition became higher in higher concentrations of the buffer, whereas that of the first transition was not significantly affected.

CD measurements

Figure 4 shows the changes of the ellipticity at 222 and 268 nm against temperature, measured under the same conditions as shown in Figure 3. The CD profiles showed two-phase unfolding transitions in high concentration of $Na_2B_4O_7$ buffer. Although the first transition remained unchanged, as in the case of the DSC experiments, the second transition shifted toward higher temperature in the concentrated buffers. The presence of the intermediate was most prominent in 20 mM $Na_2B_4O_7$ buffer. Under the conditions, the midpoint temperatures of the first transition (T_{m1}) and the second transition (T_{m2}) obtained from the far-UV CD profile (Fig. 4D) were 56 and 80 °C, respectively. These values are in good agreement with T_{m1} and T_{m2} obtained from the near-UV CD profile (Fig. 4E), and the peak temperatures obtained from the corresponding DSC profile (T_{p1}, 57 °C; T_{p2}, 81 °C; see trace 4 in Fig. 3) within experimental errors. Thus, each transition observed in the DSC profiles was accompanied by destruction of the secondary and tertiary structures. The results of the far-UV CD experiments suggest

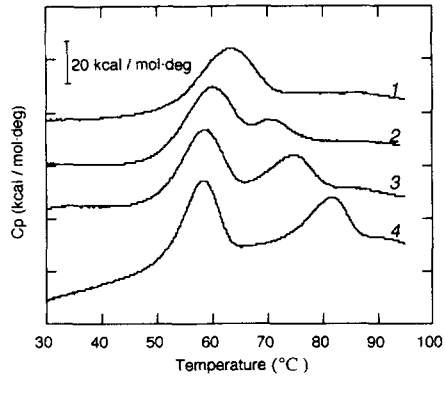

Fig. 3. Effect of the buffer concentration on the DSC profiles of the chimeric enzyme. Lines of 1, 2, 3, and 4 represent the profiles of the chimeric enzyme in 1, 5, 10, and 20 mM $Na_2B_4O_7$-NaOH buffer, pH 10.4, respectively.

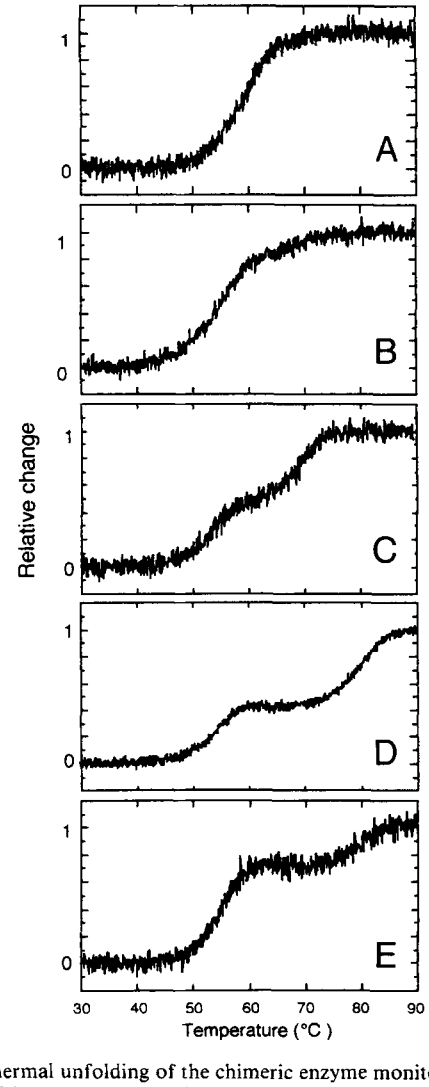

Fig. 4. Thermal unfolding of the chimeric enzyme monitored by CD. **A,B,C,D:** Measured at 222 nm in 1, 5, 10, and 20 mM $Na_2B_4O_7$ buffer, pH 10.4, respectively. **E:** Monitored at 268 nm in 20 mM $Na_2B_4O_7$ buffer, pH 10.4. All profiles were normalized with the baseline of the native and the denatured states.

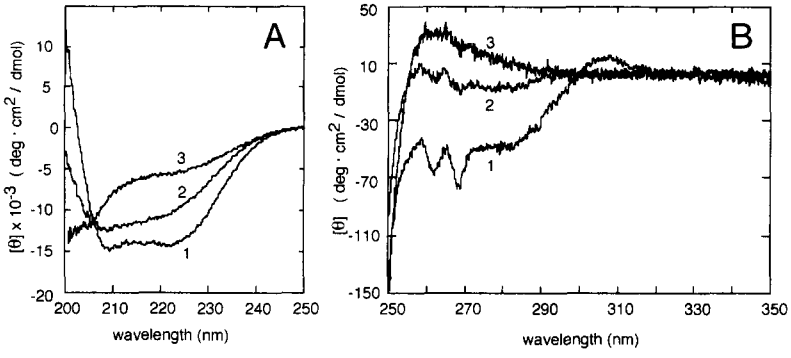

Fig. 5. CD spectra of the native, intermediate, and unfolded states of the chimeric IPMDH. Samples in 20 mM $Na_2B_4O_7$ buffer, pH 10.4, were heated under the same conditions as shown in Figure 4D and E. Spectra of the native (1), intermediate (2), and unfolded (3) states were measured at 25 °C, 62 °C, and 90 °C, respectively. **A:** Far-UV CD spectra were measured in the cell with 0.1-cm pathlength. Protein concentration was 0.18 mg/mL. **B:** Near-UV CD spectra were measured in the cell with 0.5-cm pathlength. Protein concentration was 1.2 mg/mL.

that the intermediate contains about 60% of the secondary structure of the native state. The irreversibility of the intermediate state was also proved by the CD measurements (data not shown). The same experiments as shown in Figure 2B were also done by CD monitored at 222 nm, and the results were the same as those obtained from the DSC experiments: The reheating curves of which the samples once heated up to 61 °C and cooled down showed only the second transition (data not shown).

Figure 5 shows the CD spectra of the native, intermediate, and unfolded states of the chimeric enzyme. The far-UV CD spectra showed that the secondary structure contents of the intermediate state were about half of that of the native state. The near-UV CD spectra of the intermediate state indicated that the tertiary structure of the intermediate was different from either that of the native or that of the unfolded state.

Figure 6 shows the concentration dependence of thermal unfolding of the chimeric enzyme, clearly indicating that the protein concentration affects only the second transition temperature but not the first transition. This suggests the dissociation of the dimeric form of the protein at the second transition.

The first (T_{p1} and T_{m1} for DSC and CD measurements, respectively) and the second (T_{p2} and T_{m2}) transition temperatures are plotted against concentration of sodium ion in Figure 7. T_{p1} and T_{m1} were independent of sodium concentration, whereas T_{p2} and T_{m2} were affected by the concentrations. Upon increment of sodium ion concentration, the temperature of the second transition became higher. Although the temperature of unfolding of the wild-type enzyme was not affected by salt concentrations (data not shown), the intermediate of the chimeric enzyme are stabilized by the sodium ion. Because the pI value of the thermophilic and the chimeric IPMDHs are 4.67 and 4.50, respectively (Numata et al., 1995), both the enzymes are negatively charged at pH around 10. This suggests that cations shield the negatively charged groups of the intermediate molecules and that the repulsion is weakened by the cations. Similar effects of ions on the stability of molecules, such as β-lactamase detected at acidic and alkaline pH, have been reported previously (Goto & Fink, 1989). Although our results cannot exclude a possibility that the stability of the intermediate is related to the chaotropic effect of the ion, $Na_2B_4O_7$ and NaCl had almost the same effects on the stability of the intermediate. Because the radii of the anions do not affect the stability of the intermediate, we can conclude that effects of ions on the hydrophobic interactions are negligible.

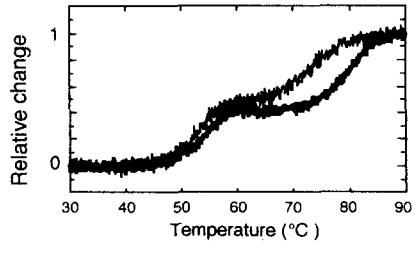

Fig. 6. Effect of the protein concentration on the far-UV (222 nm) CD unfolding profiles of the chimeric IPMDH. Thin and thick lines represent profiles of 0.12 mg/mL and 1.2 mg/mL of the chimeric enzyme in 20 mM $Na_2B_4O_7$ buffer, pH 10.4, respectively.

Discussion

In this study, we found an intermediate state in the thermal unfolding process of the chimeric enzyme by DSC and CD spectroscopy. This intermediate seems to be a dimer, and is subjected to further unfolding and dissociation processes. The high-resolution three-dimensional structure of the chimeric IPMDH

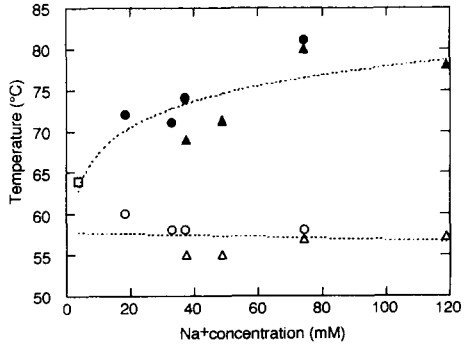

Fig. 7. Temperature dependence of the first and second thermal unfolding transitions of chimeric enzyme on Na^+ concentration. Filled and open circles represent the peak temperature of the first and second transitions, respectively, obtained from the DSC profiles in 5, 10, and 20 mM $Na_2B_4O_7$ and 20 mM $NaHCO_3$ buffer, pH 10.4. Filled and open triangles represent the midpoint temperatures of the first and second transitions, respectively, obtained from the CD profiles in 10 and 20 mM $Na_2B_4O_7$ and 5 mM $Na_2B_4O_7$ containing 30 and 100 mM sodium chloride. Open square represents the peak temperature obtained from the DSC profile in 1 mM $Na_2B_4O_7$, pH 10.4.

has been determined by X-ray diffraction studies (Onodera et al., 1994). In the following, we discuss the nature of this intermediate, considering the structures of the thermophilic and the chimeric IPMDHs (Imada et al., 1991; Onodera et al., 1994).

Three-dimensional structure of the IPMDH shows the presence of the tight hinge region between two domains composed of four antiparallel β-sheets (D, E, F, G; see Fig. 1C), which are parts of 10 β-sheets (A–J) lying over the whole subunit (Imada et al., 1991). A part of this portion, made from two of four β-sheets (F, G) in domain 2, an α-helix (d) in domain 1 and three loops between them, corresponds the mesophilic portion in the chimeric enzyme (residues 75–133). In addition to the distortions, especially in a loop region between F and G sheets (around Ser 111) in the chimeric enzyme (Onodera et al., 1994), we became aware of two other possible factors that may affect the interdomain interactions in the chimeric enzyme: (1) a lack of one of six interdomain hydrogen bonds presents in the thermophilic enzyme (Nδ2 of Asn 102 to O of Ala 260); (2) one substituted residue (Val 129; originally Leu in the thermophilic wild-type enzyme), causing a decrease in the hydrophobicity in the hydrophobic core of the chimeric enzyme, which involves the two β-sheets (F, G) in the hinge (the mesophilic portion). These factors probably cause a loss of cooperativity of the thermal unfolding of two domains in the chimeric IPMDH, and thereby a biphasic transition corresponding to unfolding of two domains.

It has been shown that a decrease in the free energy of interactions between two domains can lower the endothermic peak corresponding to unfolding of a less stable domain (Brandts et al., 1989; Freire et al., 1992). Unfolding of yeast phosphoglycerate kinase shows DSC profiles with only one asymmetric peak, and the transition is a highly cooperative two-state process, whereas the DSC profiles of mutants, with an amino acid replacement in the hinge region, have two distinct peaks corresponding to the unfolding of two domains (Brandts et al., 1989; Bailey et al., 1990). The analysis by Freire et al. (1992) considering an unfolding model involving the free energy of interdomain interaction, has suggested that disruption of several hydrogen bonds and an exposure of the hydrophobic interface to the solvent in the hinge region of the mutant protein caused decooperativity of the unfolding. Recently, Carra et al. (1994) have analyzed several mutants of staphylococcal nuclease containing single point replacements at the active site cleft. The DSC profiles of these mutants have two endothermic peaks at neutral pH resulting from the corresponding two subdomains, whereas the DSC profile of the wild-type protein has only a single endothermic peak. They have concluded that removal of the electrostatic bonds across the cleft by these mutations results in decooperation of the protein structure, leading to a three-state mechanism of denaturation at pH 7.0.

Because the subunit of IPMDH has two structural domains containing similar amounts of the secondary structure, the CD value at 222 nm of the intermediate (about 60% of the ellipticity at 222 nm of the native state) is in line with the assumption that one domain is unfolded, whereas the other remains folded (Figs. 4D, 5). The near-UV CD indicates that the intermediate did not lose all the ordered tertiary structure, because a considerable amount of the ellipticity at 268 nm remained, as shown in Figures 4E and 5. Because the intermediate molecule seemed to be still dimeric as judged by the results of concentration-dependence experiments (Fig. 6) and domain 1 is not involved in the subunit–subunit interaction of the enzyme (Fig. 1A), it is very likely that the intermediate is the molecule that has unfolded domain 1 and folded domain 2 in which the subunit interface is located.

On the other hand, some characteristics of the intermediate, such as its secondary and tertiary structure contents, are similar to those of so-called "molten globule," which is known to be an intermediate state of protein folding and unfolding and has drawn much attention (Ptitsyn, 1992). Although the molten globule states in several different kinds of proteins have been actively discussed in this research field, their nature is still controversial. For example, Mas et al. (1995) have reported a mutant of phosphoglycerate kinase with deletion of the C-terminal 15 amino acid residues. They have suggested that the conformational state of one domain of the mutant may be described as a disordered molten globule and that of the other domain as a native-like molten globule. Consequently, at the present time, we cannot exclude the possibility that the stable intermediate found in the thermal unfolding process of the chimeric IPMDH represents a molten globule. In light of the dimeric and domain structure of IPMDH as discussed above, it would be better to suggest that the one of the two domains of IPMDH may be unfolded in the intermediate state.

In either cases, our results clearly demonstrate the existence of the stable dimeric intermediate, which is markedly stabilized by 10–100 mM of sodium ion, in the thermal unfolding process of the chimeric IPMDH. The amino acid replacements at the hinge region between two domains of the thermophilic enzyme, which were derived from a mesophilic IPMDH from *B. subtilis*, considerably destabilize the native states. These substitutions cause inactivation of the enzyme to occur at lower temperature, prior to fully unfolding of the enzyme.

Materials and methods

Protein preparation

3-Isopropylmalate, a substrate of isopropylmalate dehydrogenase, was kindly provided by Wako Pure Chemicals. All chemicals were of reagent grade. Water was purified by a Milli-Q purification system (Millipore), and was used in all experiments. *T. thermophilus* wild-type and the chimeric (2T2M6T) IPMDHs were produced by *Escherichia coli* JA221 (F^-, hsdR, trpE5, leuB6, lacY, RecA1) carrying recombinant plasmids (Numata et al., 1995). The details of the purification procedures have been reported by Numata et al. (1995). In summary, the *E. coli* cells were disrupted by sonification, and the cell extracts were heated for 10 min at 75 °C for the wild-type enzyme, and at 60 °C for the 2T2M6T enzyme, to remove other proteins. The enzymes were further purified by a Butyl-Toyopearl (Tosoh Corp.) and a Mono-Q (Pharmacia Biotech, Inc.) column chromatographies, and purified enzymes were stored at 4 °C as a suspension in 60% saturated ammonium sulfate in potassium phosphate buffer, pH 7.6, until use. The enzymes were collected by ultracentrifugation from the stock suspension, and resolved in $Na_2B_4O_7$-NaOH or $NaHCO_3$-NaOH buffer, and dialyzed against the same buffer overnight at 4 °C prior to use. The concentration of the enzyme was estimated using a molar absorbance coefficient of 30,400 at 280 nm (Yamada et al., 1990). The solution was filtered and degassed, and pH was adjusted before each measurement.

Enzyme assay

The enzyme activity was determined by measuring the initial rate of increase in the absorbance at 340 nm with a Gilford Response spectrophotometer equipped with a temperature controller. The activity was measured at 60 and 55 °C for the wild-type and the chimeric enzymes, respectively, after a 10-min preincubation. The reaction was initiated by the addition of the enzyme to the standard assay mixture (0.1 M potassium phosphate buffer, pH 7.6, containing 0.2 M KCl, 0.2 mM $MnCl_2$, 40 mM NAD^+, and 40 mM threo-Ds,Ls-3-isopropylmalate).

Calorimetric measurements

Calorimetric measurements were carried out with a scanning microcalorimeter, DASM4, under an extra pressure of 2 atm. Protein concentration was in a range of 0.8–1.2 mg/mL. The scan rate was 1.0°/min for most of the experiments. The data were transferred to an NEC personal computer and analyzed using the computer program Origin (MicroCal, Inc.).

CD measurements

CD measurements were carried out with a JASCO J-500C or J-720C spectropolarimeter. The 0.1- and 0.5-cm cells were used for the far- and near-UV CD measurements, respectively. The temperature of the sample solution in the cell was controlled with a HAAKE circulating bath and a programmable temperature controller. The real temperature was monitored with a thermocouple in the cell. The scan rate was 0.92 deg/min and the concentrations of the enzymes were in a range of 0.8–1.5 mg/mL, except for the concentration-dependence experiments. The thermal unfolding curves of CD at 222 nm and 268 nm were normalized, assuming a linear temperature dependence of the base lines of native and unfolded states.

Acknowledgments

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