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The Effects of Mutations at Position 253 on the Thermostability of the *Bacillus subtilis* 3-Isopropylmalate Dehydrogenase Subunit Interface

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3-Isopropylmalate dehydrogenase (IPMDH) is a dimeric enzyme with a strongly hydrophobic core that is composed of residues from four α-helices. We replaced Glu253, which is found in the hydrophobic core and is part of the subunit interface of the *Bacillus subtilis* (Bs) IPMDH, with several other amino acids to probe. The thermostabilities of the mutants were assessed by measuring the residual enzymatic activities at 40°C after heat treatment and by monitoring changes in ellipticity at 222 nm as the environmental temperature increased incrementally. The results of these studies indicate that, for residues with non-polar side chains, when positioned at residue 253, the thermostabilities of their corresponding mutants correlate positively with the relative hydrophobicities of the side chains. Relative activities of all mutants are lower than that of the wild-type enzyme. For two of the mutants, we directly show that the substitution at position 253 negatively affects Mn^{2+} binding, which is required for catalysis. When a lysine is the position 253 residue, the protein dissociates. The results presented herein increase our understanding of the role played by the BsIPMDH dimer interface on the stability and activity of BsIPMDH.

Key words: dimeric enzyme, hydrophobic core, isopropylmalate dehydrogenase, subunit interaction, thermostability.

Abbreviations: BsIPMDH, *Bacillus subtilis* IPMDH; EcIPMDH, *E. coli* IPMDH; IPM, *d-l*-3-isopropylmalate; IPMDH, 3-isopropylmalate dehydrogenase; TtIPMDH, *Thermus thermophilus* IPMDH.

For many proteins, their biological activities depend on proper subunit associations. It would be advantageous to have a relatively simple model system that could be used to study the nature of protein–protein interactions. While examination of an oligomeric protein's three-dimensional structure identifies the chemical nature of interface residues and the interactions among the residues (*1–5*), such examinations are not sufficient to fully comprehend the mechanisms of interface formation and stability.

3-isopropylmalate dehydrogenase (IPMDH), which is a member of the leucine biosynthetic pathway, is a homodimeric enzyme. IPMDH is a member of a family of dehydrogenases that includes isocitrate dehydrogenase, which also has been well characterized (*6*). The genes for IPMDH isozymes have been cloned from many sources and their sequences determined. The first IPMDH three-dimensional structure reported was that of the extreme thermophile, *Thermus thermophilus* (TtIPMDH) (*7*). Now the three-dimensional structures of IPMDHs from *Escherichia coli* (*8*), *Salmonella typhimurium* (*8*), *Bacillus coagulans* (*9*) and *Thermus ferrooxidans* (*10*) are available. Their structures are very similar to that of TtIPMDH.

The IPMDH subunit interface is part of the interior of a four-helix bundle, for which two α-helices are contributed by each subunit. In general, the subunit interfaces of all structurally characterized IPMDHs are lined mostly with non-polar side chains, which results in very stable dimers. The residues that make up the subunit interface are usually conserved, but Leu246 and Val249 of TtIPMDH are replaced by Glu and Met, respectively, in the IPMDHs of mesophiles such as *E. coli* and *Bacillus subtilis*. When the subunit interface residues Glu256 and Met259 of the *E. coli* IPMDH (EcIPMDH) are substituted for Leu and Val, which are the corresponding residues of TtIPMDH (*i.e.* Leu246, Val249), the thermostability of EcIPMDH increases (*11*).

Recently, we examined the effects of mutations on position 256 alone in *Bacillus subtilis* IPMDH (BsIPMDH) (*12*). The thermostabilities of those mutants inversely correlate with the side chain volumes of the position 256 residues, but do not correlate with the relative hydrophobicities of the side chains. It may be that, for residue 256, hydrophobic packing, rather than hydrophobicity *per se*, is the crucial contributor to subunit interface stability. For the present study, we prepared BsIPMDH mutants by replacing the wild-type Glu253 residue. As noted above, Glu253 corresponds to the subunit interface residue Leu246 of TtIPMDH.

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These mutants were used to probe the forces that stabilize, in part, the subunit interface of BsIPMDH.

MATERIALS AND METHODS

Materials—*d*-*l*-3-Isopropylmalate (IPM) and NAD^+ were purchased from Wako Pure Chemicals and Oriental Yeast Ltd, respectively (Tokyo, Japan). All other reagents were analytical grade.

Construction of Mutant Enzymes—The genes for the site-directed mutants were obtained by PCR-based site-directed mutagenesis (*13*). The plasmid, pET21-c, harbouring the gene for BsIPMDH, was used as the template. Mutations were confirmed by sequence analysis (*14*).

Enzyme Purification—Wild-type and mutant IPMDHs were expressed and purified as described by Hayashi-Iwasaki and Oshima (*15*). Each mutant was overexpressed in *E. coli* BL21 (Δ*leuB*) that contained a recombinant plasmid construct derived from a pET21-c expression vector. After expression, cell suspensions were sonicated and the soluble fractions isolated by centrifugation. To purify the enzymes each supernatant was sequentially chromatographed over (i) Hiprep Q (GE Healthcare Bio-Science), (ii) Phenyl Toyopearl (Toso, Tokyo, Japan) and (iii) Resource Q (GE Healthcare Bio-Science) resins.

Measurement of the Thermostabilities of the Enzymes—The residual activities of the enzymes after heat treatment were measured as described previously (*16*). Enzyme solutions containing 0.4 mg/ml protein, 20 mM potassium phosphate (pH 7.6), 0.5 mM EDTA were incubated at various temperatures for 10 min. Then, the samples were placed on ice for 10 min before being centrifuged at 15000 rpm for 10 min. The residual enzymatic activity associated with each supernatant was measured at 40°C. Thermal denaturation was monitored by recording the $CD_{222\,nm}$ of a sample with a Jasco J-720C spectropolarimeter (*17*). Enzyme solutions used in this analyses were 0.2 mg/ml protein, 20 mM potassium phosphate (pH 7.6). The temperature of an enzyme solution was controlled by circulating thermostated water through an insulated cuvette holder and by increasing the temperature at a rate of 1.0°C/min using a programmable temperature controller (Neslab, Thermo Electron Co., Waltham, MA). The temperature of a sample was monitored with a thermocouple.

Enzyme Assay—The residual activities after heat treatment and the specific activities were measured at 40°C by monitoring the reduction of NAD^+ to NADH at 340 nm, using a Beckman DU7400 spectrophotometer, as described previously (*18*). The assay buffer contained 100 mM potassium phosphate (pH 7.6), 1 M KCl, 0.2 mM $MnCl_2$, 0.8 mM IPM and 0.8 mM NAD^+. The kinetic parameters, K_m for IPM and k_{cat}, were calculated using data obtained from steady state experiments that were performed at 40°C with an assay buffer of 100 mM potassium phosphate (pH 7.6), 1 M KCl, 0.2 mM $MnCl_2$, 8 mM NAD^+ and concentrations of IPM, ranging between 5 and 150 μM. To determine the K_m of NAD^+, the concentrations of the coenzyme varied between 50 and 1500 μM, with the IPM concentration held at 0.4 mM. Initial rates were measured as described previously (*18*).

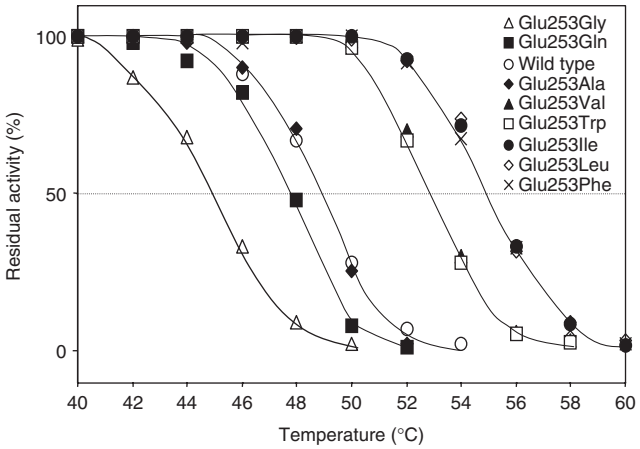

Fig. 1. **The relationship between residual activity and heat treatment for BsIPMDH and its mutants.** After 10 min of heat treatment at the indicated temperatures, enzymatic activities were assayed at 40°C.

RESULTS

Thermal Stabilities of Mutant BsIPMDHs—To examine, in part, the importance of hydrophobicity on interface stability and consequently on thermostability, we prepared mutant BsIPMDHs that had Gly, Gln, Ala, Val, Ile, Leu, Phe or Trp residues in place of the native Glu at position 253. The thermal stabilities of the purified mutant BsIPMDHs were found by measuring the residual activities at 40°C after heat treatment at various temperatures for 10 min as described previously (*12*). All mutant BsIPMDHs and the wild-type enzyme were irreversibly inactivated by the heat treatments. Fig. 1 shows plots of the remaining activities of the enzymes versus the temperatures at which they were heated. The T_h's (T_h is the temperature at which 50% of the baseline activity is lost) for the mutants, Glu253Leu, Glu253Ile and Glu253Phe are about six degrees higher than that of the wild-type enzyme. The thermostabilities of Glu253Val and Glu253Trp increase by about four degrees. The T_h of Glu253Ala is approximately same to the wild-type and that of Glu253Gln is slightly lower than the wild-type T_h; whereas, for Glu253Gly, it is about four degrees lower.

Thermal denaturation of the proteins was monitored by measuring the $CD_{222\,nm}$ as the temperature of an enzyme solution increased (Fig. 2). The thermal denaturation process was also irreversible for all enzymes. Denaturation of the enzymes occurs as a single-phase process. T_m's (the temperature at which 50% of the original secondary structure is lost) of the mutant BsIPMDHs were compared with that of the wild-type enzyme. Glu253Val and Glu253Trp have T_m values that are about four degrees greater than that of wild-type BsIPMDH; while Glu253Ile, Glu253Leu and Glu253Phe have T_m values that are about six degrees higher. The T_m of Glu253Ala is similar to that of the wild-type. The T_m of Glu253Gln is about two degrees lower and the T_m of Glu253Gly is about four degrees lower. Thus, the T_m of each mutant is similar to the corresponding T_h. In Fig. 3A, a plot of the

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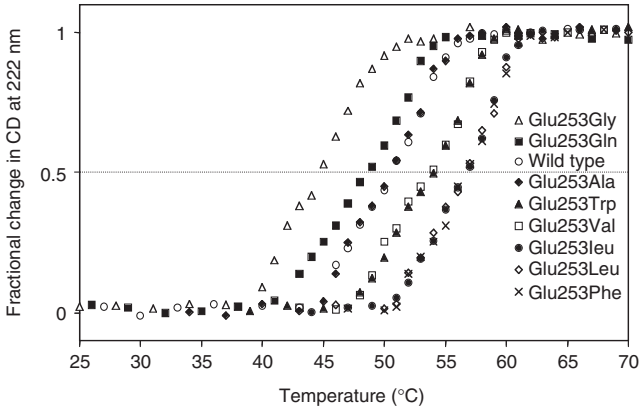

Fig. 2. **Thermal melting profiles for BsIPMDH and its mutants.** Thermal denaturation was monitored by recording the $CD_{222\,nm}$ as the temperature of the protein sample increased. Samples were 0.2 mg/ml protein, 20 mM potassium phosphate, pH 7.6.

T_h for each mutant versus a hydrophobicity score for its position 253 side chain is shown. A linear correlation between thermal stability and hydrophobicity exists for those IPMDHs containing position 253 residues with hydrophobic side chains. The data for the wild-type enzyme and the mutant, Glu253Gln, are outliers. These proteins are more thermostable than would be expected based only on their hydrophobicity scores. Glu253 and Gln253 may have other types of interactions with surrounding residues such as hydrogen bond or Coulombic interaction.

Specific Activities of Mutant BsIPMDHs—IPMDH catalyzes the dehydrogenation and the decarboxylation of IPM in the presence of NAD^+ and a divalent cation, such as Mn^{2+} or Mg^{2+}. The specific activities of mutant BsIPMDHs were measured at 40°C, pH 7.6 in the presence of 0.2 mM $MnCl_2$, 0.8 mM IPM and 0.8 mM NAD^+ as described by (*18*). The specific activities of the mutants are all less than that of the wild-type enzyme (Table 1). The enzymes' kinetic parameters, K_m for NAD^+, K_m for IPM and k_{cat}, are presented in Table 2. The mutants with a different hydrophobic residue (Glu253Ala, Glu253Val, Glu253Leu), with an aromatic residue (Glu253Try) and with a polar uncharged residue (Glu253Gln) were selected for the measurement. The mutants' Michaelis constants, K_m for NAD^+ and K_m for IPM, are slightly larger than that of the wild-type enzyme except for K_m values for NAD^+ of Glu253Val and Glu253Trp, which are slightly smaller than that of the wild-type enzyme. The mutants' catalytic constants, k_{cat}, are less than that of the wild-type enzyme. To examine the effects of mutation on divalent cation (Mn^{2+}) binding, the specific activities of the wild-type enzyme, and two mutants with different side chain volume (Glu253Ala and Glu253Leu) were measured at 40°C in the presence of varying Mn^{2+} concentrations. The increase in the specific activity of the wild-type enzyme plateaus at 0.2 mM Mn^{2+} (Fig. 4), which is the Mn^{2+} concentration routinely used to measure the specific activities of all the BsIPMDHs. Greater concentrations of Mn^{2+} are required to maximize the activities

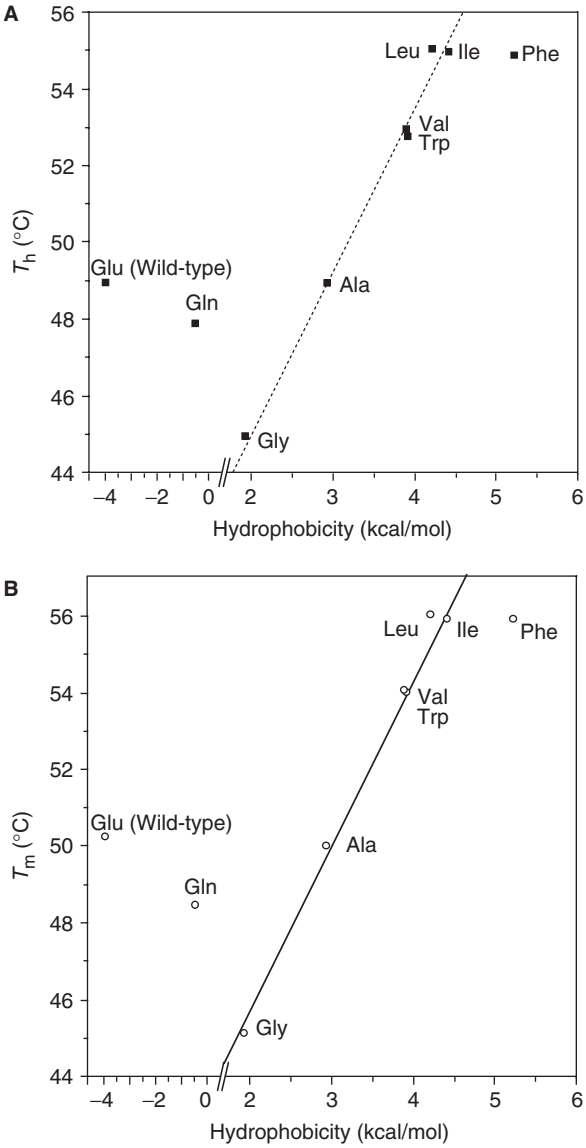

Fig. 3. **The relationship between thermostability and the relative hydrophobicity of the side chain of the position 253 residue for BsIPMDH and its mutants.** The hydrophobicity scale is that of von Heijne and Blomberg (*28*).

of Glu253Ala and Glu253Leu (Fig. 4). Therefore, the presence of Glu253 in the native enzyme probably facilitates Mn^{2+} binding.

Effect of Charge at Position 253—To examine the effect of a positive charge at position 253, we prepared the Glu253Lys mutant. It was overexpressed in *E. coli* BL21 (Δ*leuB*) cells. Because activity could not be detected using the routine assay conditions, *i.e.* pH 7.6, the purification of Glu253Lys was monitored using SDS–PAGE (with a polyacrylamide concentration of 13%). When the pH of the assay solution was increased, the enzymatic activity of Glu253Lys was detectable. At pH 9.0, the activity of Glu253Lys is 9.5%, that of wild-type BsIPMDH at pH 9.0. The CD spectra of Glu253Lys and the wild-type enzyme were recorded at pH 7.6 and pH 9.0 (Fig. 5). The CD spectrum

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of Glu253Lys, approximately between 205 nm and 230 nm, is consistently less negative at pH 7.6 than that of the wild-type enzyme. However, at pH 9.0, not only are the spectra of both proteins nearly indistinguishable, but the spectra also overlap the spectrum of wild-type BsIPMDH at pH 7.6 (Fig. 5). At pH 7.6, the elution time of the dimeric wild-type BsIPMDH and Glu253Gly correspond to a weight of ~75–80 kDa; whereas, that of Glu253Lys corresponds a weight of ~35–40 kDa (Fig. 6). Therefore, at neutral pH, Glu253Lys exists as a monomer, which results in the loss of activity. Gel filtration chromatographs of Glu253Lys and wild-type BsIPMDH were also performed at pH 9.0. At this pH, the elution volumes of Glu253Lys and wild-type BsIPMDH are very similar and are those expected for dimers. Therefore, introduction of a positive charge at position 253 prevents dimerization, which is necessary for enzymatic activity. It is also noted that all other BsIPDHs with a hydrophobic or negatively charged residue at position 253 are expectedly dimers.

DISCUSSION

Although the X-ray crystallographic structure of BsIPMDH has yet to be determined, the three-dimensional structures of its isozymes TtIPMDH and EcIPMDH are known (*7*, *8*). The residues Glu256 of EcIPMDH and Leu246 of TtIPMDH, both of which correspond sequentially to Glu253 of BsIPMDH, occupy similar positions at the subunit interface of their respective enzyme. This interface is found in the interior of a four-helix bundle with two α-helices donated by each subunit and maintained by hydrophobic interactions. The Glu256/Thr230 of one subunit pack against the Glu256/Thr230 of a second subunit in EcIPMDH is shown in Fig. 7. For TtIPMDH, Leu246 from one subunit packs against the Leu246 from the other subunit.

Table 1. **Specific activities of the wild-type and mutant BsIPMDHs at 40°C.**

IPMDHs	Specific activity (unit/mg)	Rate (%)
Wild type	13.9	100
Glu253Gly	5.9	42.5
Glu253Gln	7	50.4
Glu253Ala	4.7	33.8
Glu253Trp	5.2	37.4
Glu253Val	9	64.7
Glu253Ieu	7.4	53.2
Glu253Leu	7.6	54.7
Glu253Phe	7.6	54.7

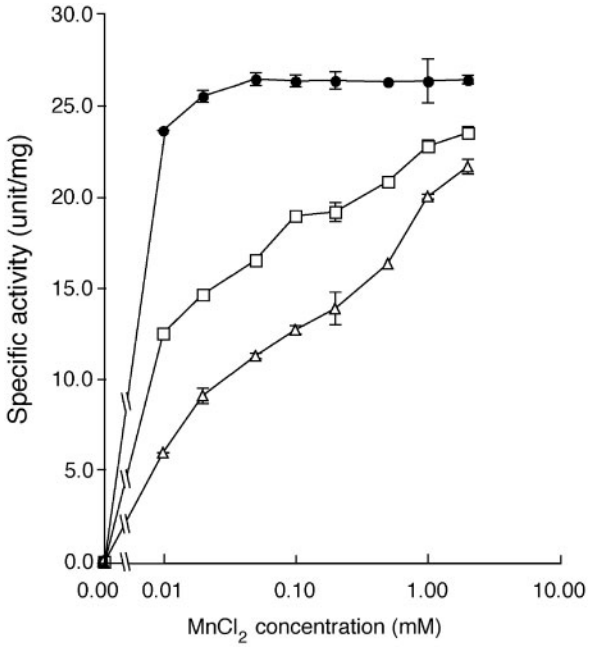

Fig. 4. **The effects of Mn^{2+} concentrations on the specific activities of wild-type BsIPMDH (filled circles), Glu253Ala (open triangles) and Glu253Leu (open squares).** Enzyme activities were measured in 50 mM HEPES buffer, pH 8.0, 1 M KCl, 1 mM IPM and 5 mM NAD^+ in the presence of 0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1 or 2 mM $MnCl_2$ at 40°C.

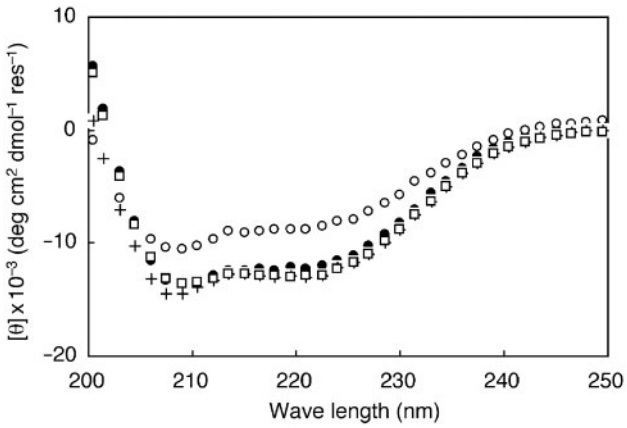

Fig. 5. **CD spectra of wild-type BsIPMDH (filled circles, pH 7.6; crosses, pH 9.0) and Glu253Lys (open circles, pH 7.6; open squares, pH 9.0).** Samples were 0.2 mg/ml protein and either 20 mM potassium phosphate, pH 7.6 or 20 mM $NaHCO_2$, pH 9.0.

Table 2. **Kinetic constants of the wild-type and mutant BsIPMDHs.**

IPMDHs	K_m(μM)		k_{cat}	k_{cat}/K_m	
	IPM	NAD	(s^{-1})	IPM	NAD
Wild type	9.1 ± 0.3	246 ± 39	14.9 ± 1.0	1.65×10^{-3}	6.09×10^{-5}
Glu253Ala	16.0 ± 0.1	425 ± 22	9.5 ± 0.3	5.92×10^{-4}	2.23×10^{-5}
Glu253Leu	12.2 ± 1.6	474 ± 32	13.7 ± 0.3	1.13×10^{-3}	2.90×10^{-5}
Glu253Val	24.4 ± 4.0	171 ± 17	12.4 ± 0.4	5.10×10^{-4}	7.26×10^{-5}
Glu253Gln	18.9 ± 5.1	273 ± 16	9.9 ± 0.1	5.22×10^{-4}	3.62×10^{-5}
Glu253Trp	25.4 ± 0.8	244 ± 4	8.3 ± 0.1	3.26×10^{-4}	3.40×10^{-5}

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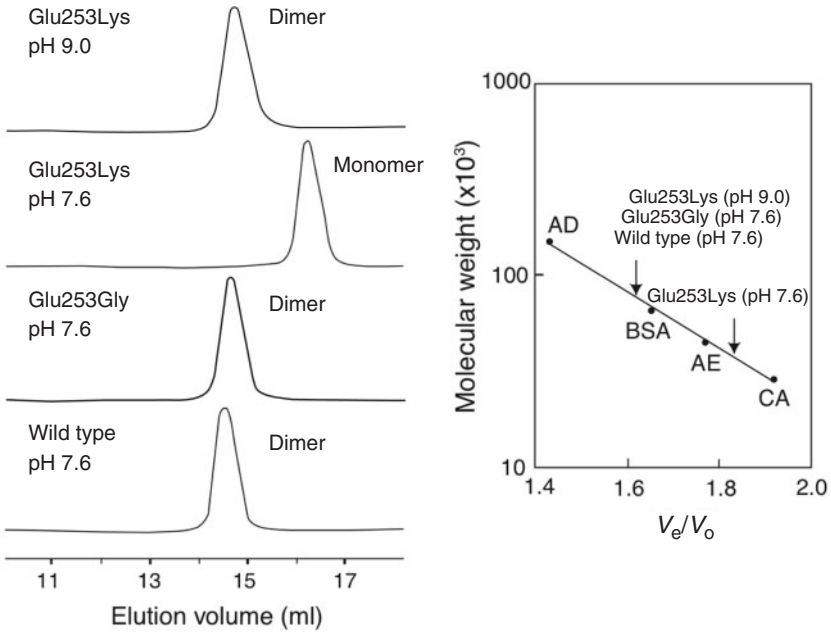

Fig. 6. **Gel filtration of wild-type BsIPMDH, Glu253Gly and Glu253Lys.** A column of Superdex 200 resin with an elution buffer of 20 mM potassium phosphate, pH 7.6, 0.15M NaCl or 50 mM $NaHCO_3$, pH 9.0, 0.15 M NaCl was used. The enzyme concentrations were 0.2 mg/ml. A semi-logarithmic plot of the molecular weight of protein standards versus the normalized elution volume (V_e/V_o) is shown on the right. The protein standards used are: alcohol dehydrogenase (AD, 150 kDa), bovine serum albumin (BSA, 440 kDa), egg albumin (AE, 45 kDa) and carbonic anhydrase (CA, 29 kDa).

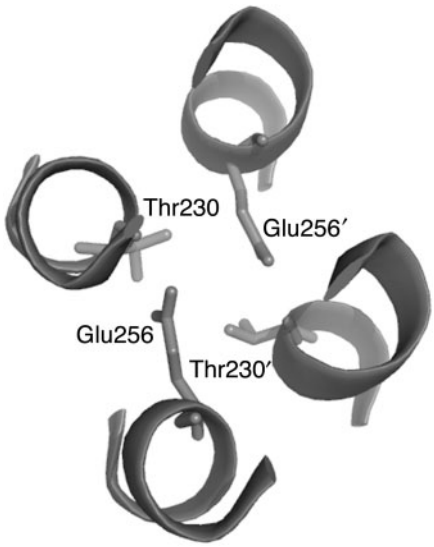

Fig. 7. **The structure of EcIPMDH around the residues corresponding Met253 and Ala227 in BsIPMDH.** Primed residue numbers indicate those from the other subunit.

Whereas, the side chain hydroxyl of Glu256 of EcIPMDH hydrogen bonds to the main-chain carbonyl group of Thr230 (corresponding to Ala227 of BsIPMDH) within the same subunit and is part of the hydrophobic core. Therefore, the Glu256 side chain carboxyl of EcIPMDH may be protonated even at pH 9.5 (*8*). The thermostability of mesophilic EcIPMDH increases when the subunit-interface mutations, Glu256Leu and Met259Val are present (*11*). These residues correspond to Leu246 and Val249 of TtIPMDH, respectively. The BsIPMDH mutants, containing Glu253Leu or Met256Val, are also more thermostable than is the wild-type enzyme (*19*). For this report, the effects of a series of mutations at position 253 on the subunit interface stabilities have been characterized. We conclude that the thermostabilities of BsIPMDH and its mutants depend on how hydrophobic the side chains of the residue at position 253 are (Fig. 3). This conclusion is consistent with the observations that Glu256Leu mutation stabilizes EcIPMDH (*11*) and that BsIPMDH is stabilized by the Glu253Leu mutation (*19*). The stability of the monomeric α-subunit of tryptophan synthase increases linearly with increasing hydrophobicity of residues substituted at position 49 in the protein's core (*20*). Lin *et al.* (*21*) examined the interiors of four-helix bundles that are part of protein cores and/or subunit interfaces. They suggested that possibly the bundles are stabilized by cooperative helical twists, dipole alignments and/or interhelical connections. Prior to this report, there has not been a definitive study relating to hydrophobic interactions and stability at a subunit interface. Now, for the dimeric mutants of BsIPMDH, we find that when the side chain of the position 253 residue can be characterized as hydrophobic in nature, its relative hydrophobicity correlates directly with the thermostability of the mutant.

The presence of a divalent cation, such as Mn^{2+} or Mg^{2+}, is essential for the catalytic activity of IPMDH (*18*). When Glu253 of BsIPMDH is replaced with the mutations prepared for this study, apparently, Mn^{2+} binding is negatively affected, which leads to a decrease in activity. The active site has a binding site for

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a divalent cation. This site is located in a cleft between the two domains and is formed by residues of both subunits. For EcIPMDH, the cation is coordinated by the side chain carboxyls of Asp227, Asp251 and Asp255 (corresponding to Asp224, Asp248 and Asp252 of BsIPMDH). These residues are situated one layer removed from the subunit interface (*8*). Although Glu256 of EcIPMDH (*i.e.* Glu253 of BsIPMDH) is near the cation binding cleft, its side chain does not interact directly with the divalent cation (*8*). Because the decreased activities of the BsIPMDH mutants probably are caused by weaker Mn^{2+} binding, these mutations may cause conformational alterations in their Mn^{2+} binding sites. In accord with this interpretation, Leu246Glu mutant of TtIPMDH showed the Mg^{2+} concentration dependence of catalytic activity similar to wild-type TtIPMDH (Umezawa, A., Ohkuri, T. and Yamagishi, A. unpublished data) the effect of the amino acid residue on activity at this site is conformational rather than Coulombic.

Examination of the X-ray crystallographic structure of EcIPMDH suggests that the side chain of BsIPMDH Glu253 may be protonated even if the pH of the solution is 9.5 (*7*). We examined the effect of introducing a positively charged side chain at position 253 by preparing and studying Glu253Lys. Glu253Lys is monomeric when the pH of its solution is near neutrality. Therefore, it is probable that the introduction of a positive charge is sufficient to overwhelm the forces responsible for BsIPMDH dimerization. Often when an attempt has been made to produce stable monomers derived from a multisubunit protein by mutagenesis the attempt has failed because the mutation also destabilizes the monomeric fold (*22–26*). Because IPMDH dimers are very stable, detection of a monomeric form has not been possible. No monomeric EcIPMDH or TtIPMDH intermediates were detected when the proteins were unfolded (*27*). A monomeric IPMDH, whether present transiently during folding, or stabilized by mutation or by environmental conditions, would be a useful tool for understanding the mechanisms of subunit interactions.

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