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Ancestral Residues Stabilizing 3-Isopropylmalate Dehydrogenase of an Extreme Thermophile: Experimental Evidence Supporting the Thermophilic Common Ancestor Hypothesis

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Ancestral amino acid residues were inferred for 3-isopropylmalate dehydrogenase (IPMDH), and were introduced into the enzyme of an extreme thermophile, *Sulfolobus* sp. strain 7. The thermostability of the mutant enzymes was compared with that of the wild type enzyme. At least five of the seven mutants tested showed higher thermal stability than the wild type IPMDH. The results are compatible with the hyperthermophilic universal ancestor hypothesis. The results also provide a new method for designing thermostable enzymes. The method only relies on the first dimensional structures of homologous enzymes that can be obtained from genetic databases.

Key words: archaeon (archaebacterium), common ancestor, 3-isopropylmalate dehydrogenase, protein stability, *Sulfolobus*.

The antiquity of thermophilic microorganisms has been suggested by Woese (*1*). He pointed out that thermophilic organisms can be found in every phyla of bacteria and archaea. The deepest branching organisms are thermophilic in both archaea and bacteria in the phylogenetic tree, and these organisms tend to have shorter branch lengths. Several other authors have also discussed the thermophilic origin and/or thermophilic common ancestor of the extant life on the earth (*2–4*). We have named the universal ancestor or the last and most recent common ancestor Commonote (*4*). However, Miller and Lazcano have criticized the thermophilic origin of life based on the instability of biological compounds (*5*). The thermophilic nature of the universal common ancestor has also been criticized by Forterre (*6*). He pointed out the possibility that thermophilic organisms may have been selected after the separation of bacteria and archaea from the universal ancestor. Galtier *et al.* recently estimated the G + C content of the rRNA sequence of the universal ancestor and suggested that the result is not compatible with the hypothesis of a hyperthermophilic common ancestor (*7*). In this study we construct mutant enzymes with ancestral amino acid residues and analyze the thermal stability of the mutant enzymes. If the universal ancestor was thermophilic, an enzyme with ancestral residues is expected to exhibit higher thermal stability than that of the wild type enzyme.

MATERIALS AND METHODS

Phylogenetic Analysis—The amino acid sequences used in the present phylogenetic analyses were obtained from the GenBank Sequence Database. The sequences were aligned using the program package CLUSTAL W (*8*), and subsequently adjusted manually on the basis of the secondary and tertiary structural information obtained on X-ray crystallographic analysis of *Thermus thermophilus* 3-isopropylmalate dehydrogenase (IPMDH) (*9*) and *Escherichia coli* isocitrate dehydrogenase (ICDH) (*10*), as performed previously (*11*). To improve the reliability, we selected and used three highly conserved regions including the substrate and coenzyme binding sites (*11*, *12*). Phylogenetic analysis was carried out with a program package, PHYLIP 3.56c (*13*). A composite tree of IPMDH and ICDH was constructed by means of the neighbor joining method (*14*) with evolutionary distance values calculated with the PAM matrix. The tree was also inferred by the parsimony method. The ancestral sequence of IPMDH and ICDH was inferred by means of the PROTPARS program in the package. The statistical significance of the branching of the tree was examined by the bootstrap method (*15*) with 1,000 repetitions.

Construction of Mutant Genes—DNA manipulations were carried out following the methods described in the literature (*16*). A DNA fragment containing the *Sulfolobus* sp. strain 7 *leuB* gene encoding IPMDH in plasmid pE7-SB6 (*11*) was digested with *Nde*I and *Eco*RI, and then recloned into plasmid pET21c (Novagen, USA). The genes of mutant-a, -b, -b′, -b″, -c, and -d were obtained by site directed mutagenesis using the oligonucleotide primers TTTGCTGGTCTTAAGTTGGCATAAAGATCATAAATTTGTC, TGCAAAGTTTAGCGCTACTCTTGCTATTCTCTC, AGTTTAGCCCTACGCTCGCGATTCTCTCAGAAGC, AATGCAAAGTTTAGCGCTACTTTTGCTATTC, TCCAGCAATGTCCGGAGCACTACCGTGTACTG, and TCATACATTCTCTCGAGCATCATACTTAC, respectively, by the method of Kunkel and Roberts (*17*). The mutants were selected by analyzing the introduced recognition site of restriction enzymes,

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Abbreviations: ICDH, isocitrate dehydrogenase; IPMDH, 3-isopropylmalate dehydrogenase.

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which are underlined, and confirmed by sequence analysis. Mutant-abcd was obtained by ligating the 5'-fragment of mutant-a and the 3'-fragment of mutant-bcd, which was obtained with primers-b, -c, and -d simultaneously, at a *Sma*I site.

Overexpression and Purification of Sulfolobus sp. Strain 7 IPMDHs—The wild type and mutant *leuB* genes were overexpressed in *E. coli* MA153 or *E. coli* BL21-Codon Plus (DE3)-RIL (Stratagene), and then purified as described by Suzuki *et al.* (*11*). The purity of the recombinant enzyme was confirmed by SDS polyacrylamide gel electrophoresis. The quantity of protein was estimated with a BCA Protein Assay Reagent Kit (Pierce, Illinois, USA) using bovine serum albumin as a standard.

Thermostability Analysis of IPMDHs—The IPMDHs were dissolved (0.25 mg/ml) in 20 mM K phosphate buffer, pH 7.0, containing 0.5 mM EDTA. The enzymes were incubated at 99°C and chilled in ice water for 5 min, and then centrifuged (13,000 rpm, 20 min) to remove aggregated protein. The remaining activity in the supernatant was measured in 50 mM CHES buffer, pH 9.5, which contained 200 mM KCl, 1 mM NAD^+, 0.4 mM IPM, and 5 mM $MgCl_2$, by monitoring the production of NADH at 340 nm at 75°C (*11*). Denaturation curves for the enzymes were obtained by recording the change in circular dichroism at 222 nm with a Spectropolarimeter J-720 (Jasco, Tokyo), and analyzed as described in the literature (*18*). The temperature was increased at the rate of 0.5°C/min.

RESULTS AND DISCUSSION

We used IPMDH to test the hyperthermophilic common ancestor hypothesis. IPMDH is the enzyme involved in the leucine biosynthetic pathway and catalyses the oxidative decarboxylation from 3-isopropylmalate to 2-oxoisocaproate. The catalytic reaction of IPMDH is similar to that of another enzyme, ICDH, which catalyzes the oxidative decarboxylation from isocitrate to 2-oxoglutarate in the TCA cycle. The three-dimensional structures as well as first dimensional ones of these two enzymes are similar to each other (*19*). These two enzymes probably originated from a common ancestral enzyme.

Multiple alignment of relatively conserved parts of these enzymes is shown in Fig. 1A. A composite evolutionary tree of these two enzymes was constructed by the neighbor joining method using the sequence shown in Fig. 1A and depicted in Fig. 1B. The bootstrap values are relatively low, especially in the ICDH tree, because of the limited se-

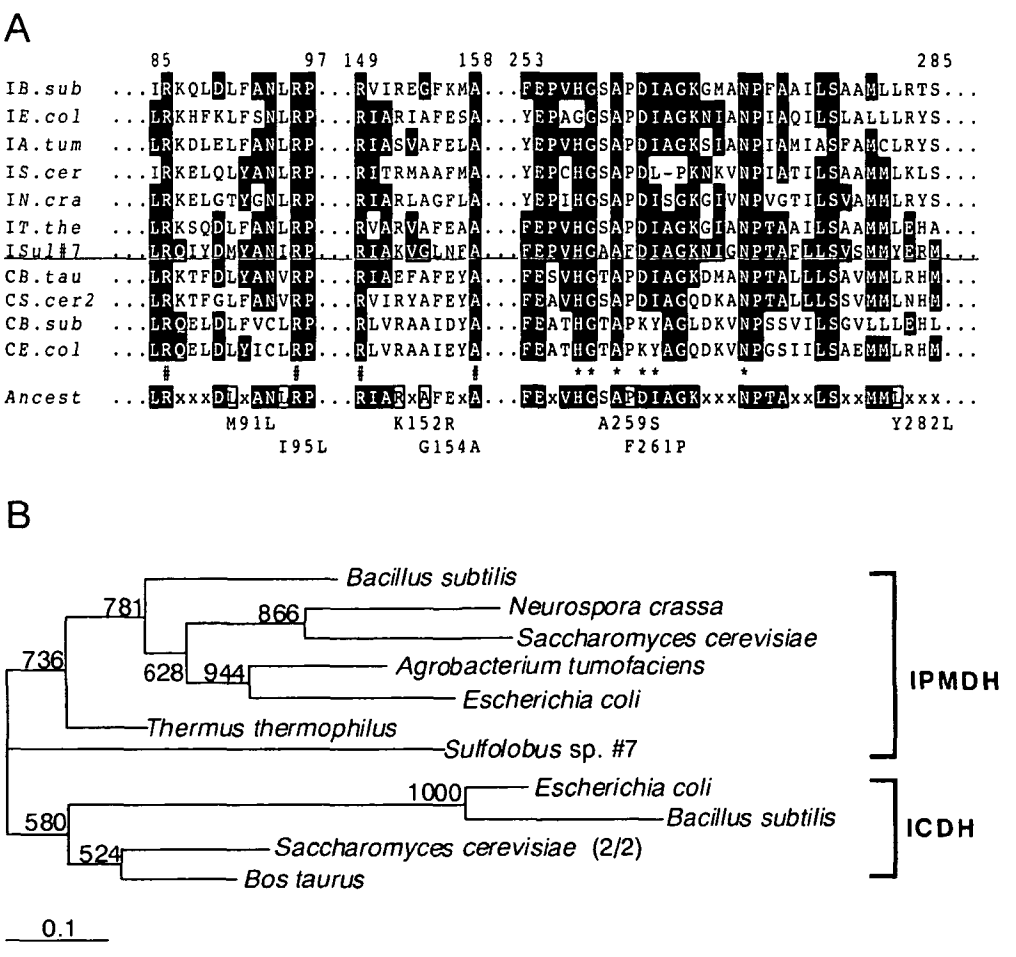

Fig. 1. **(A) Multiple alignment of IPMDHs and ICDHs of selected organisms.** *Sulfolobus* sp. strain 7 IPMDH (337 residues) was aligned with IPMDHs and ICDHs of several sources, three regions (85–97, 149–158, and 253–285 of *Sulfolobus* sp. strain 7 IPMDH numbering) being selected and shown in this figure. Residues identical to ones in the *Sulfolobus* sp. strain 7 IPMDH sequence are boxed and inverted. Residues denoted by # interact with the malate moiety of the substrate (IPM in IPMDH and isocitrate in ICDH). Residues binding with the adenine-ribose portion of NAD are indicated by asterisks. Abbreviations of IPMDHs: IB.sub, *Bacillus subtilis*; IE.col, *Escherichia coli*; IA.tum, *Agrobacterium tumefaciens*; IS.cer, *Saccharomyces cerevisiae*; IN.cra, *Neurospora crassa*; IT.the, *Thermus thermophilus*; ISul#7, *Sulfolobus* sp. strain 7. Abbreviations of ICDHs: CB.tau, *Bos taurus* ICDH (3/4); CS.cer2, *Saccharomyces cerevisiae* NAD-dependent ICDH (IDH2); CB.sub, *Bacillus subtilis* ICDH; CE.col, *Escherichia coli* ICDH. The bottom sequence is the ancestral sequence estimated by parsimony. The letters x indicate the sites where ancestral residues could not be uniquely estimated. Seven residues of *Sulfolobus* sp. strain 7 IPMDH substituted in the present study are indicated below the ancestral sequence. **(B) A composite phylogenetic tree of IPMDHs and ICDHs of selected organisms.** The sequences shown in Fig. 1 were used. The tree was estimated by the neighbor joining method. The values indicate bootstrap confidence. The standard bar shows the number of substitutions per site.

quence data used to construct the tree. However, the tree topology was similar to that of those constructed from longer sequence data by both the neighbor joining method and the maximal parsimony method reported previously (*11*), and is also similar to that of the tree constructed from rRNA sequences (*1*).

Inference of ancestral enzymes and amino acid residues has been performed to analyze the evolutionary history of enzymes (*20–23*). Ancestral enzymes of ancestral ancestors of modern game birds (*20*) and artiodactyla (*21*) have been constructed and analyzed. Ancestral residues have also been used to modify the coenzyme specificities of ICDH and IPMDH (*23*). Ancestral amino acid residues in relatively conserved regions at the far-left node of the tree were inferred by parsimony and are shown in Fig. 1A. The node indicates the state before the separation of *Sulfolobus* and thus archaebacterial and eubacterial IPMDHs. By using the composite phylogenetic tree of the two enzymes, it becomes possible to infer ancestral residues at the node. The ancestral residue at site 259 was not estimated uniquely and was inferred to be serine, threonine, or alanine. Because both threonine and serine have a hydroxyl group, the ancestral residue is expected to have been either serine or threonine. Because serine is conserved in IPMDHs, and the size of serine is similar to that of alanine, we selected serine at site 259.

The ancestral residues tend to be conserved in the sequence of the enzymes used for inference of the phylogenetic tree. Never the less, residues are randomly substituted with other residues in each sequence during the evolution from the common ancestor. The effect of ancestral residues introduced into the contemporary enzyme was examined by site-directed mutagenesis. To examine the effect of ancestral residues, we used IPMDH of *Sulfolobus* sp. strain 7, which is the most stable IPMDH that has been studied (*11*). *Sulfolobus* sp. strain 7 is the archaeon that grows optimally at 80°C. Most of the ancestral residues are conserved in *Sulfolobus* sp. strain 7 IPMDH. However, some ancestral residues were not conserved in *Sulfolobus* sp. strain 7 IPMDH because of the mutations that have accumulated during evolution. These residues were selected and the ancestral residues were introduced into *Sulfolobus* sp. strain 7 IPMDH by site-directed mutagenesis. Because the two residues in the vicinity may have some mutual interactions, these mutations were introduced into the same mutant. The ancestral residues we have selected are those robust as to species sampling and did not vary in modified trees. However, the inferred residues at sites 155 and 156 depended on the species used for the phylogenetic analysis, thus we did not adopt these residues.

We have constructed mutants, a, b, c, and d: The respective mutants have mutations M91L/I95L, K152R/G154A, A259S/F261P, and Y282L, respectively. One of the two mutations of mutant-b, K152R/G154A, was introduced separately in mutants-b′ (K152R) and b″ (G154A). All of the 7 mutations were introduced into mutant-abcd. The mutants were overexpressed in *Escherichia coli* and then purified. Because the heat denaturation of *Sulfolobus* sp. strain 7 IPMDH is irreversible, the heat inactivation rate and denaturation process, monitored by circular dichroism, have been estimated. Figure 2 shows the time courses of denaturation of the mutants and the wild type enzymes monitored by measuring the residual activity after incubation at 99°C. Most mutants and the wild type enzymes showed first order kinetics of heat denaturation. However, mutants-b′, -c, and -abcd showed deviation from first order kinetics. The half denaturation time or apparent half denaturation time (for mutants-b′, -c, and -abcd) was estimated from Fig. 2 and is listed in Table I. Most mutant enzymes showed larger half denaturation times than the wild type enzyme, except mutant-d, which showed a similar $t_{1/2}$ value to that of the wild type enzyme. Though the kinetics of denaturation of mutants-b′, -c, and -abcd were not first order, higher remaining activity than that of the wild type enzyme was observed with a longer denaturation period in Fig. 2.

Denaturation of the enzymes was also analyzed by measuring circular dichroism at 222 nm, which represents the secondary structure content of enzymes (Fig. 3). The enzymes were unfolded and lost their secondary structure during the increase in the temperature. The half denaturation temperature was estimated and is listed in Table I. Among the seven mutant enzymes with ancestral amino acid residues, at least five (mutants-a, -b, b″, -c, and -abcd) acquired higher thermal stability than the wild type enzyme.

The response of structural alterations on the thermal stability of proteins to genetic mutations is context-dependent and difficult to predict with any confidence (*24*). When random mutagenesis was performed for a mesophilic enzyme with a melting temperature of 52°C, less than one-third of the mutant enzymes gained thermal stability (*25*). Because we started with an enzyme with a much higher denaturation temperature (96°C), the fraction of random

TABLE I. **Specific activity and thermal stability of the wild type and mutants of *Sulfolobus* sp. strain 7 3-isopropylmalate dehydrogenase.**

	Specific activity (U/mg)[a]	Apparent $t_{1/2}$ (min)[b]	T_m (°C)[c]
Wild type	16.8 ± 0.2	3.67 (1.0)[d]	96.0 (0.0)[e]
Mutant-a (M91L/I95L)	15.6 ± 0.6	5.52 (1.5)	99.2 (+3.2)
Mutant-b (K152R/G154A)	16.9 ± 1.1	6.56 (1.8)	97.2 (+1.2)
Mutant-b′ (K152R)	18.5 ± 1.3	5.72 (1.6)	95.6 (−0.4)
Mutant-b″ (G154A)	15.7 ± 1.3	7.13 (1.9)	97.9 (+1.9)
Mutant-c (A259S/F261P)	9.5 ± 0.1	4.79 (1.3)	97.1 (+1.1)
Mutant-d (Y282L)	17.8 ± 0.4	3.79 (1.0)	94.7 (−1.3)
Mutant-abcd (M91L/I95L/K152R/G154A/A259S/F261P/Y282L)	2.0 ± 0.1	6.50 (1.8)	96.4 (+0.4)

[a]Activity indicates the specific activity of the purified enzyme. M ± SEM. [b]$t_{1/2}$ indicates the apparent half denaturation time at 99°C estimated from Fig. 2. [c]T_m represents the apparent half denaturation temperature estimated from the denaturation profile monitored by circular dichroism at 222 nm (Fig. 3). The figures in parentheses indicate relative values normalized as to the value for the wild type enzyme (d), or the difference from the value for the wild type (e). Triplicate measurements of T_m of mutant-b showed the deviation ± 0.2°C. The accuracy of T_m of the other IPMDHs is expected to be similar.

mutant enzymes with higher thermal stability than the wild type is expected to be much less than that observed in the experiment with the mesophilic enzyme. In contrast to the expectation based on the results of random mutagenesis experiments, 5 or 6 mutants out of the 7 tested gained thermal stability: 3 pairs out of 3 pairs and one single mutation supported higher thermal stability. Accordingly, the tendency of stabilization induced by ancestral residues is clear. The results suggest that Commonote, the organism that possessed the ancestral IPMDH, could grow at a temperature higher than the growth temperature of *Sulfolobus*, *i.e.* 80°C, and support the thermophilic common ancestor hypothesis.

The ancestral amino acid residues we tested in this study are rather conserved among IPMDHs. Organisms included in the phylogenetic analysis have lower growth temperatures than *Sulfolobus* sp. strain 7. The most thermostable IPMDH among the rest, *Thermus thermophilus* IPMDH, has an about 10°C lower T_m than that of *Sulfolobus* sp. strain 7 IPMDH (*11*, *26*). If the amino acid residues were conserved in less thermophilic organisms at particular sites because of the requirement of the mesophilicity of the enzyme, the results would have been opposite. The current results imply that the conservation of these residues in less thermophilic organisms is inherited from the ancestral enzyme.

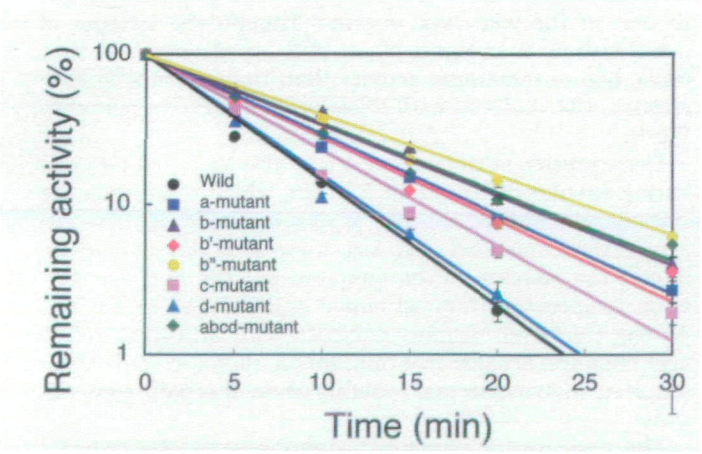

Fig. 2. **Heat inactivation of the wild type and mutants of *Sulfolobus* sp. strain 7 IPMDH.** Residual activity after treatment at 99°C was estimated. Symbols: black, wild type; blue, mutant-a; purple, -b; red, -b′; yellow, -b″; pale purple, -c; light blue, -d; green, -abcd. The data shown are averages of 3 to 6 measurements. Error bars show SEM.

Mutant-abcd showed an intermediate increase in stability among the mutants tested. In other words, little additivity was observed for the stabilization effect of these mutations. From the structural point of view, residues that interact with each other tend to show low additivity. Because these mutations are located at the vicinity of one another, the residues may interact with one another. From the phylogenetic point of view, each enzyme of modern species has both adaptive mutations and random mutations. All of the mutations may not be related to the adaptation toward lower temperatures in *Sulfolobus* sp. strain 7 IPMDH.

The catalytic activity of the enzyme was not significantly affected by the mutations. However, mutant-abcd showed significantly lower catalytic activity than the wild type enzyme. Because the mutations tested in this experiment are in the vicinity of the substrate and cofactor-binding site, combination of the mutations may have affected the binding and/or catalytic function.

The current results are incompatible with the results reported by Galtier *et al.* (*7*). They estimated the G+C content of rRNA of the universal ancestor based on a nonhomogeneous nonstationary model in which the equilibrium G + C content that would be reached after infinitely long evolution is allowed to vary between branches. Their results were incompatible with the hypothesis of a hyperthermophilic common ancestor (*7*). However, based on their estimation, the rate of the G + C content change in thermophilic organisms must be higher than that in nonthermophilic organisms, because the rRNA of thermophilic organisms has a high G + C content. This tendency seems to be contradictory as to the relative rate of evolution of rRNA: The rate of base-substitution of the rRNA sequence of a thermophilic species, which is represented by the shorter branch lengths of thermophilic species in the phylogenic tree, is lower than that of a nonthermophilic species (*1*). Though they tested the model using simulated data, the estimation of the G + C content of the common ancestor may depend on the model used. In our study, ancestral residues were inferred by parsimony without any assumption of the evolutionary process. The ancestral residues tested were robust as to species sampling and tree topology.

It is important to distinguish the origin of life and the

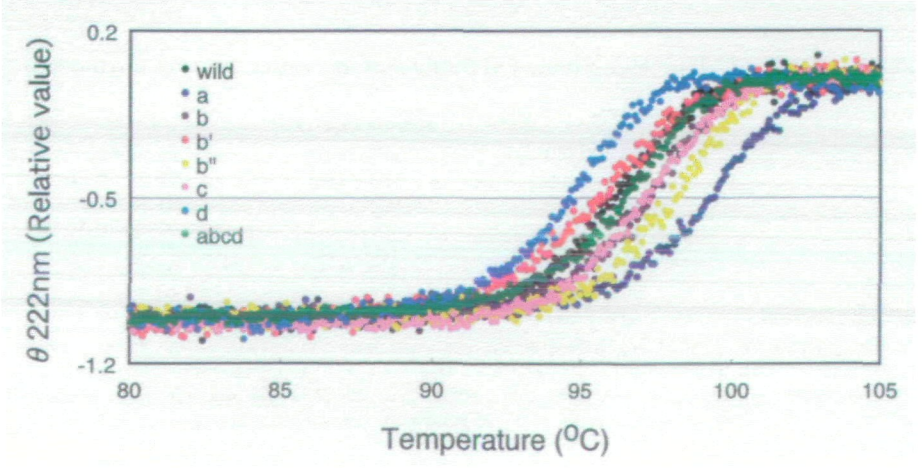

Fig. 3. **Denaturation curve of the wild type and mutants of *Sulfolobus* sp. strain 7 IPMDH estimated by circular dichroism at 222 nm.** Symbols: black, wild type; blue, mutant-a; purple, -b; red, -b′; yellow, -b″; pale purple, -c; light blue, -d; green, -abcd.

common ancestor Commonote. Though current results support the hyperthermophilic common ancestor hypothesis, the most recent common ancestor Commonote could be selected after the evolution from a nonhyperthermophilic ancestor, as discussed by Forterre (*6*) and Gogarten-Boekels *et al.* (*27*). The latter authors proposed that the thermophilic ancestor may have been selected on elevation of the surface temperature of the earth due to meteorite bombardment (*27*).

The three-dimensional structure of *T. thermophilus* IPMDH has been reported (*19*). Because the residues of interest are in the conserved region, it is possible to locate the corresponding residues in the three-dimensional structure of *T. thermophilus* IPMDH. All of the ancestral residues tested are conserved in *T. thermophilus* IPMDH. IPMDH is a dimer molecule. Each subunit consists of two structural domains (Fig. 4). The mutations in mutant-a, M91(99 of *T. thermophilus* numbering)L and I95(103)L are located at the interface of structural domains II and I. We previously found mutations at the domain–domain interface in a stabilized mutant of *T. thermophilus* IPMDH (A172V). The mutation was expected to improve the hydrophobic packing at the domain–domain interface, resulting in an increase in thermal stability (*26*). Mutations M91L and I95L may have a similar effect. Mutations in mutant-b, K152R and G154A, are located on helix-e in domain II. Though K152(167)R is located at the hydrophilic surface and the effect of the mutation is not clear, G154(169)A points to the hydrophobic core. The latter mutation may have improved the thermal stability by improving the hydrophobic interaction (*28*, *29*). Mutations in mutant-c, A259(275)S and F261(277)P, are located on a loop at the surface of domain I. These mutations may have improved the thermal stability through the introduction of a hydrogen bond and stabilization of the loop. A similar effect of a mutation on another loop of *B. subtilis* IPMDH has been reported (*30*). The mutation in mutant-d Y282(298)L is located on the surface loop and the side chain points to the hydrophobic core. The mutation rather decreased the stability of the enzyme. Though it is possible to rationalize some of the molecular mechanism of stabilization, current knowledge on the stabilization of proteins based on the three-dimensional structure is not sufficient to predict a correct design and so it was not used when designing mutations. Because the target residues are conserved in less thermostable IPMDHs, they were rather expected to decrease the thermal stability.

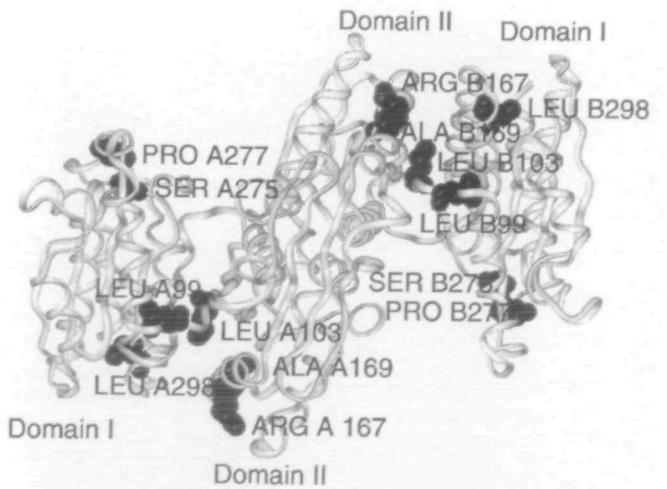

Fig. 4. **A molecular model structure showing the ancestral residues introduced in this experiment.** The residues are shown as a space-filled model on the strand model of *T. thermophilus* IPMDH. The model is depicted using an Insight II program.

The current study provides a new method for designing thermostable proteins. The conventional methods need three-dimensional structural information for the design of mutations to improve the protein stability (*24*). Alternatively, a screening system for a random library is required to obtain a stabilized mutant enzyme (*25*, *26*). The method reported here only relies on the first dimensional structure of homologous proteins, which may be available in genetic databases. The ancestral residues were inferred from the amino acid sequences of the homologous proteins based on the phylogenetic tree and parsimony. The sequences of both thermophilic and mesophilic organisms can be used to infer ancestral residues. This is in contrast to the conventional methods, in which thermophilic proteins are often used as models for designing thermostable proteins. However, it is desirable to use the sequence of archaebacteria in addition to the sequence of eubacteria, for adequate inference of ancestral residues of the proteins in Commonote. Construction of a composite tree of twin enzymes, as performed in this study, also enabled us to infer ancestral residues that may have been possessed by the common ancestor before the separation of eubacteria and archaebacteria. Recent progress of genome projects on eubacteria and archaea serves as the resources for the construction of such a phylogenetic tree and inferring ancestral sequences.

In the present study, inferred ancestral residues were introduced into the thermophilic enzyme. The designed enzymes showed increased stability, though only slightly when the stability was estimated by CD measurement, and significantly when the stability was estimated by measuring the residual activity. However, it is not necessary to use a thermophilic enzyme as the starting material for the construction of mutant enzymes. On the contrary, it is expected that the introduction of ancestral residues into a mesophilic enzyme will also improve the thermal stability of the mesophilic enzyme. Experiments to examine this expectation are in progress.

We examined the ancestral residues in the vicinity of the substrate and cofactor-binding sites, because the homology was high and it was easy to infer ancestral residues in these regions. The effect of ancestral residues in other regions will be examined in the future.

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