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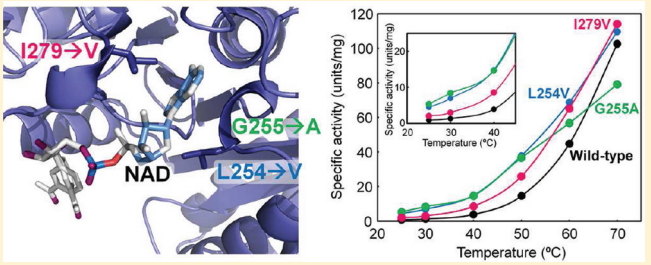
Substitutions of Coenzyme-Binding, Nonpolar Residues Improve the Low-Temperature Activity of Thermophilic Dehydrogenases

Sayaka Hayashi, Satoshi Akanuma, Wakana Onuki, Chihiro Tokunaga, and Akihiko Yamagishi*

Department of Molecular Biology, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan

Supporting Information

ABSTRACT: Although enzymes of thermophilic organisms are often very resistant to thermal denaturation, they are usually less active than their mesophilic or psychrophilic homologues at moderate or low temperatures. To explore the structural features that would improve the activity of a thermophilic enzyme at less than optimal temperatures, we randomly mutated the DNA of single-site mutants of the thermostable *Thermus thermophilus* 3-isopropylmalate dehydrogenase that already had improved low-temperature activity and selected for additional improved low-temperature activity. A mutant (Ile279 → Val) with improved low-temperature activity contained a residue that directly interacts with the adenine of the coenzyme NAD^+, suggesting that modulation of the coenzyme-binding pocket's volume can enhance low-temperature activity. This idea was further supported by a saturation mutagenesis study of the two codons of two other residues that interact with the adenine. Furthermore, a similar type of amino acid substitution also improved the catalytic efficiency of another thermophilic dehydrogenase, *T. thermophilus* lactate dehydrogenase. Steady-state kinetic experiments showed that the mutations all favorably affected the catalytic turnover numbers. Thermal stability measurements demonstrated that the mutants remain very resistant to heat. Calculation of the energetic contributions to catalysis indicated that the increased turnover numbers are the result of destabilized enzyme−substrate−coenzyme complexes. Therefore, small changes in the side chain volumes of coenzyme-binding residues improved the catalytic efficiencies of two thermophilic dehydrogenases while preserving their high thermal stabilities and may be a way to improve low-temperature activities of dehydrogenases in general.



Understanding how homologous proteins from different organisms adapt their functions and tertiary structures to different environments is an important aspect of function−structure studies. Structural comparisons among thermophilic and mesophilic proteins have suggested that various factors, e.g., a reduced number of neutral polar residues, improved hydrophobic interactions and packing density in the core, an increased number of ion pairs and ion pair networks on the protein surface, an increased number of subunits in an oligomeric protein, and an increased number of ion pairs or improved hydrophobic interactions between subunits, might be responsible for the increased stability of thermophilic proteins.[1−10] However, we still do not have a comprehensive understanding of structure−stability relationships because no single structural feature modulates the stabilities of thermophilic proteins and because the contribution by each factor is usually small and often strongly depends on its structural context. A similar uncertainty exists concerning how proteins physically adapt to lower temperatures.

Increasing numbers of low-temperature-adapted proteins are being isolated from psychrophilic organisms, and these proteins are generally less thermally stable and have greater catalytic activities at low temperatures than their mesophilic and thermophilic homologues.[11−13] Several researchers have proposed that the physical and catalytic properties of psychrophilic proteins are a consequence of conformational flexibility in regions involved in catalysis.[14−16] However, the structural origin(s) of such flexibility has not been delineated. Moreover, structural comparisons of psychrophilic and mesophilic or mesophilic and thermophilic homologous proteins have not elucidated a structural feature(s) that can be correlated with low-temperature adaptation of enzyme activity.[16]

Characterization of mutated enzymes with increased low-temperature activities can be used to identify the structural properties that improve catalytic activity at lower than optimal temperatures and could be applied to industrial applications that use thermophilic enzymes. Because robust biocatalysts are necessary for most industrial applications, thermophilic enzymes are attractive tools.[5,17−19] However, a major drawback to their use is the fact that they are generally much less active at moderate temperatures even though their thermal stability is an asset. Therefore, improving the low-temperature activity of thermophilic enzymes offers an economically beneficial tool for industrial processes.[20] Many attempts have been made to

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improve the low-temperature activities of mesophilic and thermophilic enzymes.[21−28] Early studies clearly demonstrated that one or a small number of mutations can improve a protein's low-temperature activity. However, catalytic adaptation to lower temperatures is often the consequence of many different types of subtle conformational changes, and no predictive rules, based on physical and/or chemical guidelines, have been established.

In our laboratory, efforts have been made to improve the low-temperature catalytic activity of 3-isopropylmalate dehydrogenase (IPMDH) from the extreme thermophile *Thermus thermophilus*.[29−32] The enzyme is the product of *leuB* and participates in leucine biosynthesis. The catalytic properties and thermal stability of *T. thermophilus* IPMDH are well-characterized.[33] Its three-dimensional crystal structure has been determined at 2.2 Å resolution[34] and shows the enzyme to be a homodimer, with subunits of 345 amino acid residues. A number of *T. thermophilus* IPMDH mutants that are catalytically more active than the wild-type enzyme at 30 or 40 °C have been isolated from a library composed of randomly mutated IPMDH genes.[29,30] These mutants had an improved $K_m^{NAD^+}$ value for the coenzyme, nicotinamide adenine dinucleotide (NAD^+), or an increased k_{cat} value. The mutations are distributed throughout its tertiary structure, and therefore, no conclusions concerning structural features that modulate low-temperature activity could be made.

For the study reported herein, to identify a general rule(s) that can be used to guide the design of low-temperature-adapted mutants of thermostable enzymes, we randomly mutated five single-site *T. thermophilus* IPMDH mutants that had been shown to have improved low-temperature activity and then selected for mutants with additionally improved low-temperature activity. The results suggested that a small change in the side chain volume of a residue with a nonpolar side chain (I279) that interacts with the adenine of NAD^+ was responsible for the additional improvement in the low-temperature activity. This hypothesis was supported by saturation mutagenesis of the codons of two residues that also interact with the adenine. Moreover, a similar mutation improved the catalytic activity of the thermophilic *T. thermophilus* lactate dehydrogenase (LDH) at 25 °C. Thus, modulation of the coenzyme-binding site volume may be a generic means of controlling the low-temperature activity of thermophilic dehydrogenases.

In this report, the mutants denoted R85C and I279V are those in which Arg85 was replaced with a cysteine and Ile279 was replaced with a valine, respectively. Mutants that contain two or more mutations are denoted with a slash (e.g., R85C/I279V).

EXPERIMENTAL PROCEDURES

Random Mutagenesis by Error-Prone Polymerase Chain Reaction (PCR). The genes encoding the *T. thermophilus* IPMDH mutants that contained a G12 → S, K21 → T, R85 → C, S248 → T, or A335 → V substitution were each cloned into the *Xba*I−*Eco*RI site of a pUC19 plasmid and then used as a template mixture for a slightly modified version of error-prone PCR[35] that also used primers T7P (5′-TAATACGACTCACTATAGGG-3′) and T7T (5′-CTAGTTATTGCTCAGCGGT-3′), Gene *Taq* DNA polymerase (Nippon Gene), and 0.3 mM $MnCl_2$. The time−temperature program was as follows: step 1, 1 min at 95 °C; step 2, 30 s at 95 °C, 1 min at 53 °C, and 5 min at 72 °C (step 2 was repeated 30 times); and step 3, 20 min at 72 °C. For five arbitrarily selected mutated genes, a total of 21 base substitutions were found, which suggested that, on average, 4.2 base substitutions were present in each gene. The mutated genes were digested with *Xba*I and *Eco*RI and then each ligated into a pUC19 plasmid. The ligates were each amplified in *Escherichia coli* JM109, and then the plasmids were recovered (denoted library 1).

Selection. *leuB*-deficient *E. coli* strain OM17, which was constructed from *E. coli* JM105 via deletion of its chromosomal *leuB*,[29] was transformed with library 1. The transformed cells were plated onto agar containing M9 minimal medium and 50 μg/mL ampicillin. After 2 days at 25 °C, colonies that contained a library 1 plasmid and grew faster than OM17 colonies that contained the corresponding parent plasmid were selected.

Site-Directed Mutagenesis. Site-directed mutagenesis of *leuB* was conducted using the splicing-by-overlap-extension PCR method.[36] For amplification, the PCR mixture contained 1× PCR buffer for KOD-plus polymerization (Toyobo), 1 mM $MgSO_4$, dNTPs (0.2 mM each), synthetic oligonucleotides (0.2 μM each), and 1.0 unit of KOD-plus DNA polymerase. The time−temperature program was as follows: step 1, 95 °C for 3 min; step 2, 95 °C for 30 s; step 3, 55 °C for 30 s; step 4, 68 °C for 1 min (steps 2−4 were repeated 25 times). After amplification, the PCR product was digested with *Nde*I and *Bam*HI (New England Biolabs) and then cloned into the *Nde*I−*Bam*HI site of pET21c. The genes encoding LDH A75G and V99I were constructed in a similar manner.

Saturation Mutagenesis and Selection. The codons for L254 and G255 in wild-type *leuB* were replaced with NNS (N = A, C, G, or T; S = C or G) using the splicing-by-overlap-extension PCR method. The PCR conditions were the same as those used for site-directed mutagenesis (see above). The amplified, mutated genes were digested with *Xba*I and *Eco*RI and then each ligated into a pUC19 plasmid. The ligates were then amplified in *E. coli* JM109, after which the plasmids were recovered (denoted library 2). For selection, *leuB*-deficient *E. coli* OM17 was transformed with library 2 and then plated onto agar containing M9 minimal medium, 50 μg/mL ampicillin, and 5 μM isopropyl thio-β-D-galactopyranoside. The plates were incubated at 25 °C, and colonies that grew faster than the OM17 strain harboring the *T. thermophilus* wild-type *leuB* gene were selected after 2 days.

Enzyme Purification. To prepare and purify the *T. thermophilus* wild-type and mutant IPMDHs, we cultivated *E. coli* MA153 inoculums each harboring one of the expression plasmids in LB medium supplemented with ampicillin (150 μg/mL). After overnight cultivation at 37 °C, cells were harvested and disrupted by sonication. The soluble fractions were each isolated after centrifugation at 60000*g* for 20 min. The supernatants were individually heated at 70 °C for 20 min and centrifuged at 60000*g* for 20 min. To purify the enzymes, the supernatants were each successively chromatographed through HiTrapQ (GE Healthcare Bioscience) and butyl-Toyopearl (Tosoh). To prepare and purify the *T. thermophilus* wild-type and mutant LDHs, we individually cultivated *E. coli* Rosetta2 (DE3) inoculums each harboring one of the expression plasmids in LB medium supplemented with ampicillin (150 μg/mL). After overnight cultivation at 37 °C, cells were harvested and disrupted by sonication. The soluble fractions were each isolated after centrifugation at 60000*g* for 20 min. The supernatants were individually heated at 75 °C for 15 min and centrifuged at 60000*g* for 20 min. To purify each enzyme, its respective supernatant was successively chromatographed through

HiTrapQ (GE Healthcare Bioscience), butyl-Toyopearl (Tosoh), and ResourceQ (GE Healthcare Bioscience). The homogeneities of the enzymes were assessed using sodium dodecyl sulfate−polyacrylamide gel electrophoresis and found to be >95%.

Analytical Methods. Protein concentrations were determined using the A_{280} values of protein-containing solutions as described by Pace and colleagues,[37] who followed the procedure of Gill and von Hippel.[38] Extinction coefficients of 30400 M^{-1} cm^{-1} (IPMDH) and 25900 M^{-1} cm^{-1} (LDH) were used.

IPMDH activities were determined by measuring the absorbance at 340 nm, which corresponded to production of NADH, a product of catalysis.[33] The assay buffer consisted of 50 mM HEPES (pH 8.0), 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM D-3-isopropylmalate (D-3-IPM, the substrate), and 5.0 mM NAD^+. One enzyme unit equaled 1 μmol of NADH formed per minute.[33]

The values of $K_m^{\text{D-3-IPM}}$ and k_{cat} were determined using steady-state kinetic data with assay buffers of 50 mM HEPES (pH 8.0), 100 mM KCl, 5 mM $MgCl_2$, 5 mM NAD^+, and 2−30 μM D-3-IPM. To determine the $K_m^{NAD^+}$ value, the coenzyme concentration was varied between 10 and 2500 μM, while the D-3-IPM concentration was fixed at 0.2 mM. To determine the kinetic parameters of LDHs, the decrease in absorbance at 340 nm was measured. The assay buffer consisted of 50 mM HEPES (pH 7.5), 3.5 mM $MgCl_2$, 2.0 mM sodium pyruvate, and 10−200 μM NADH. The kinetic constants were obtained by nonlinear least-squares fitting of the steady-state velocity data to the Michaelis−Menten equation using the Enzyme Kinetics module of SigmaPlot (Systat Software, Richmond, CA).

To determine the dissociation constants (K_d) for the NADH−IPMDH complexes, fluorescence titration experiments were performed at 25 °C with 2 mL of enzyme (0.5 μM) in 50 mM HEPES (pH 8.0), with 5 mM $MgCl_2$. Aliquots (1−4 μL) of an NADH solution (2 mM) were individually added to a protein solution, and then the mixture was stirred for 1 min to ensure equilibration prior to the collection of data. The decrease in the intrinsic tryptophan fluorescence intensity at 340 nm was measured after each NADH addition (excitation at 295 nm). The fluorescence data were corrected for the inner-filter effect of NADH by monitoring the fluorescence emission of *N*-acetyltryptophanamide.[39] The fluorescence intensities and protein concentrations were also corrected for dilution effects. The K_d values were calculated by nonlinear least-squares fitting of the corrected fluorescence data to a ligand binding equation that assumed a single binding site for NAD(H) per protein subunit[40] using SigmaPlot. K_d values for binding of NAD^+ to the wild-type and mutant LDHs were obtained indirectly from competition experiments. The quenching of the intrinsic tryptophan fluorescence upon incremental addition of NADH was measured at 25 °C with 2 mL of protein (1.0 μM) in 50 mM HEPES (pH 7.5) and 0−10 μM NAD. After the fluorescence data had been corrected for the inner-filter effect of NADH and for dilution, K_d^{NADH} and $K_d^{NADH}{}_{(apparent)}$ values were calculated by nonlinear least-squares fitting to the ligand binding equation described above.[40] $K_d^{NAD^+}$ values were then calculated using the following equation:[41]

$$K_d^{NADH}{}_{(apparent)} = K_d^{NADH}(1 + [NAD]/K_d^{NAD^+})$$

Protein thermal denaturations were monitored using the changes in ellipticity at 222 nm, as described previously.[23]

RESULTS

Random Mutagenesis by Error-Prone PCR Followed by Genetic Selection. We previously used random mutagenesis and phenotypic selection to isolate five *T. thermophilus* IPMDHs that each contained a single-site amino acid substitution that improved the enzyme's low-temperature activity.[30] These IPMDH mutants each contained one of the following mutations: G12 → S, K21 → T, R85 → C, S248 → T, or A335 → V. The G12 → S, K21 → T, and A335 → V mutations moderately improved the K_m value for NAD^+ and the k_{cat} value at 30 °C. The R85 → C and S248 → T mutations increased the k_{cat} value 6.9- and 5.7-fold at 30 °C but improved k_{cat} only 1.4- and 1.3-fold at 60 °C, respectively.[30] The presence of one mutation sometimes negatively impacted the positive effect induced by a second mutation. Indeed, our previous study showed that the presence of several beneficial mutations in the same mutant, each of which had improved the catalytic efficiency of the corresponding single-site mutant, did not additively enhance the catalytic activity of the multisite mutant.[31] Therefore, we did not synthesize a mutant that contained all five mutations. Instead, to identify additional beneficial mutations, the genes containing the G12 → S, K21 → T, R85 → C, S248 → T, or A335 → V mutation were pooled, and the mixture was then subjected to error-prone PCR. The resulting randomly mutated IPMDH genes were then each cloned into a pUC19 plasmid, and the ligates were each transformed into *E. coli* JM109. The plasmids were recovered from 2.8×10^4 colonies (library 1). Because the IPMDH gene is 1035 nucleotides in length and the mutation rate was 4.2 base substitutions per gene, library 1 probably covered all possible nucleotide substitutions within the IPMDH gene. Library 1 was used for the subsequent selection experiment despite the relatively small total number of mutated genes. *leuB*-deficient *E. coli* strain OM17, which was constructed from *E. coli* JM105 via deletion of its chromosomal *leuB*,[29] was transformed with library 1 and was grown at 25 °C on M9 minimal medium agar. Five colonies that grew faster than those carrying a plasmid containing one of the original five mutated genes were selected after a 24 h incubation at 25 °C. The faster-growing colonies were assumed to each contain a mutated parent gene that encoded an IPMDH mutant with further improved activity. Plasmid DNAs were recovered from the selected *E. coli* OM17 transformants. The isolated plasmids were then transformed into *E. coli* OM17 inoculums that were cultured on agar containing M9 minimal medium at 25 °C. Only one transformant grew more rapidly than *E. coli* OM17, which contained the parent gene encoding R85C. The plasmid DNA was isolated from the former colony, and the gene was sequenced and found to contain four mutations. One of the mutations corresponded to R85C, and the other three mutations corresponded to K76 → E, V168 → E, and I279 → V substitutions.

Purification of the IPMDH Mutants and Activity Assays. To assess the individual contribution of the three amino acid substitutions, K76 → E, V168 → E, and I279 → V, to the improved activity of the four-mutation mutant, we synthesized three IPMDH genes that contained codons encoding the R85 → C substitution and a K76 → E, V168 → E, or I279 → V substitution using site-directed mutagenesis. To measure the in vitro activities of the IPMDH variants, the mutated genes were first expressed in *E. coli* MA153, which lacked chromosomal *leuB*.[23] Then, the IPMDH mutants were

Table 1. Kinetic Constants for Wild-Type and Mutant *T. thermophilus* IPMDHs at 30 °C

	$K_m^{\text{D-3-IPM}}$ (μM)[a]	$K_m^{\text{NAD}^+}$ (μM)[a]	k_{cat} (s^{-1})[a]	$k_{cat}/K_m^{\text{D-3-IPM}}$ (s^{-1} μM^{-1})	$k_{cat}/K_m^{\text{NAD}^+}$ (s^{-1} μM^{-1})
wild type	0.7 ± 0.4	7.1 ± 0.9	1.1 ± 0.0	1.5	0.15
R85C	27 ± 3	200 ± 20	5.4 ± 0.2	0.20	0.027
R85C/I279V	45 ± 5	630 ± 30	6.9 ± 0.1	0.15	0.011

[a] K_m and k_{cat} were determined using steady-state kinetic data obtained at 30 °C with an assay solution of 50 mM HEPES (pH 8.0), 100 mM KCl, 5 mM $MgCl_2$, and various concentrations of D-3-IPM and NAD^+. The values and standard errors of the kinetic constants were obtained by nonlinear least-squares fitting of the steady-state velocity data to the Michaelis–Menten equation.

Table 2. Kinetic Constants for Wild-Type and Mutant *T. thermophilus* IPMDHs

enzyme	$K_m^{\text{D-3-IPM}}$ (μM)[a]	$K_m^{\text{NAD}^+}$ (μM)[a]	k_{cat} (s^{-1})[a]	$k_{cat}/K_m^{\text{D-3-IPM}}$ (s^{-1} μM^{-1})	$k_{cat}/K_m^{\text{NAD}^+}$ (s^{-1} μM^{-1})
			25 °C		
wild type	nd[b]	6.3 ± 0.8	0.57 ± 0.01	nd[b]	0.092
L254V	nd[b]	110 ± 10	3.0 ± 0.1	nd[b]	0.027
G255A	nd[b]	210 ± 20	3.6 ± 0.1	nd[b]	0.017
I279V	nd[b]	33 ± 2	1.5 ± 0.0	nd[b]	0.044
			40 °C		
wild type	1.8 ± 0.3	34 ± 5	2.7 ± 0.1	1.5	0.078
L254V	2.0 ± 0.4	220 ± 20	9.2 ± 0.3	4.7	0.042
G255A	3.0 ± 0.7	240 ± 20	7.9 ± 0.3	2.7	0.032
I279V	2.0 ± 0.3	67 ± 6	5.9 ± 0.1	3.0	0.087
			70 °C		
wild type	2.3 ± 0.5	250 ± 20	51 ± 1	22	0.21
L254V	2.0 ± 0.6	620 ± 50	52 ± 2	25	0.083
G255A	3.7 ± 0.7	1200 ± 200	47 ± 4	13	0.041
I279V	3.7 ± 0.6	820 ± 20	82 ± 1	22	0.10

[a] K_m and k_{cat} were determined using steady-state kinetic data obtained at 25, 40, or 70 °C with an assay solution of 50 mM HEPES (pH 8.0), 100 mM KCl, 5 mM $MgCl_2$, and various concentrations of D-3-IPM and NAD^+. The values and standard errors of the kinetic constants were obtained by nonlinear least-squares fitting of the steady-state velocity data to the Michaelis–Menten equation. [b] K_m values for D-3-IPM at 25 °C could not be determined because appropriately designed steady-state kinetic experiments would have included reactions that used very low D-3-IPM concentrations, and at such concentrations, the reactions would have been over quickly, precluding accurate velocity measurements.

purified and their specific activities determined at 30 °C. Only R85C/I279V had an improved specific activity (11 ± 0 units/mg) in comparison with that of R85C (9.5 ± 0.3 units/mg) at 30 °C. The specific activities of the other variants (5.8 ± 0.1 units/mg for R85C/K76E and 7.4 ± 0.2 units/mg for R85C/V168E) were smaller than that of R85C. To compare the kinetic parameters of R85C/I279V with those of the wild-type enzyme and R85C, we determined their K_m and k_{cat} values using steady-state kinetic data obtained at 30 °C (Table 1). For R85C/I279V, its K_m values for both D-3-IPM and NAD^+ are larger than those for wild-type IPMDH and R85C, but it has a k_{cat} value larger than those of wild-type IPMDH and R85C. Thus, the I279 → V substitution improved the low-temperature activity of R85C by increasing the turnover number by ~28%. It is not known if the increase in k_{cat} by the I279 → V substitution positively affected the growth rate of the K76E/R85C/V168E/I279V-containing *E. coli* OM17 transformant on the selective medium. Possibly, the faster growth of the auxotrophic *E. coli* host was a result of a change in the expression level of the mutated IPMDH. However, more importantly, introduction of the I279 → V substitution into wild-type IPMDH resulted in 2.5- and 2.2-fold increases in k_{cat} at 25 and 40 °C, respectively (see below and Table 2).

Saturation Substitution of Residues That Interact with the Adenine of NAD. Miyazaki and Oshima suggested that Ile279 is the residue that is mainly responsible for IPMDH coenzyme specificity.[42] In the *T. thermophilus* IPMDH crystal structure,[43] Ile279 is located in the NAD^+-binding pocket and directly interacts with the adenine of NAD^+ (Figure 1). Val15 also interacts with the adenine. We previously found that the V15 → I substitution improved the turnover number by 4.2-fold at 40 °C.[29] We therefore hypothesized that, in general, mutation of a residue that interacts with the adenine of NAD^+ would enhance the low-temperature activity of *T. thermophilus* IPMDH. Leu254 and Gly255 also interact with the adenine (Figure 1). We therefore subjected the wild-type IPMDH gene to saturation mutagenesis at the codons corresponding to

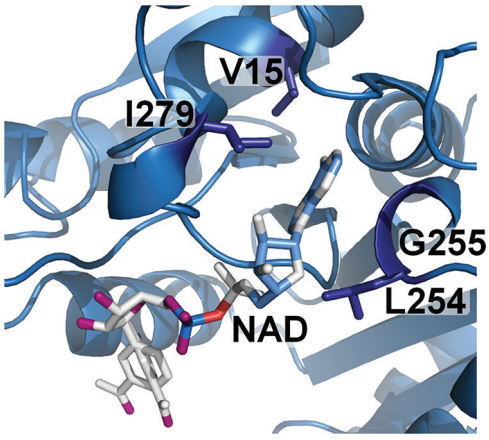

Figure 1. Active site of *T. thermophilus* IPMDH with NAD^+ bound.[43] The four nonpolar residues that interact with the adenine of NAD^+ are identified. This figure was drawn using PyMOL (http://www.pymol.org).

positions 254 and 255 using the NNS oligonucleotides (see Experimental Procedures). The mutated genes (library 2) were then individually cloned into pUC19 plasmids, and the ligates were each amplified in *E. coli* JM109. For selection, *leuB*-deficient *E. coli* OM17 was transformed with library 2 and then incubated on agar containing M9 minimal medium. Colonies that grew faster than the OM17 strain harboring the *T. thermophilus* wild-type *leuB*-containing plasmid were selected 2 days later. Plasmids were recovered from the selected colonies, and the mutated genes were sequenced. As summarized in Table 3, 15 of 26 isolated mutated genes contained either the

Table 3. Number of Transformed and Selected Colonies That Contained a Mutated IPMDH Gene with a Single or Double Amino Acid Substitution That Were Identified as Fast-Growing Colonies

amino acid substitution	no. of selected colonies
L254V	9
L254M	3
L254P	1
G255A	6
G255S	3
G255Y	1
L254I/G255A	1
L254I/G255S	1
L254M/G255S	1
total	26

L254 → V or the G255 → A mutation. Mutations corresponding to L254 → M and G255 → S substitutions were also found in several of the selected clones (Table 3).

Specific Activities as a Function of Temperature. To assess the effects of the mutations of residues involved in NAD^+ binding in vitro, L254V, G255A, I279V, and wild-type IPMDH were expressed in *E. coli* MA153 and purified to homogeneity. Figure 2A depicts their specific activities as a function of temperature, which were measured using almost saturating amounts of D-3-IPM (0.2 mM) and NAD^+ (5.0 mM). For all IPMDHs, their specific activities increased as the temperature increased, and the specific activities of the mutants are 2.4−6.1-fold greater than that of wild-type IPMDH at 25 °C. At 70 °C, L254V and I279V have specific activities similar to that of wild-type IPMDH, whereas G255A exhibited a slightly decreased specific activity. Activation energies (E_a) for the specific activities were calculated from the slopes of Arrhenius plots for temperatures between 25 and 70 °C (Figure 2B). Although I279V had an only slightly reduced E_a value (79 kJ/mol) compared with that of wild-type IPMDH (93 kJ/mol), L254V and G255A had substantially reduced E_a values (62 and 52 kJ/mol, respectively). Thus, the improvements in the low-temperature activities for this thermophilic enzyme are at least partially related to the smaller E_a values, which is a phenomenon that has also often been found for naturally occurring, cold-adapted enzymes.[44−46]

Kinetic Parameters. The kinetic parameters of wild-type IPMDH, L254V, G255A, and I279V were calculated using steady-state kinetic data obtained at 25, 40, and 70 °C (Table 2). The $K_m^{D\text{-}3\text{-}IPM}$ values for the three mutants at 40 and 70 °C were, within the margin of error, the same as or slightly larger than that of wild-type IPMDH. Although the $K_m^{NAD^+}$ values for the mutants were significantly larger than that of wild-type IPMDH, the mutants

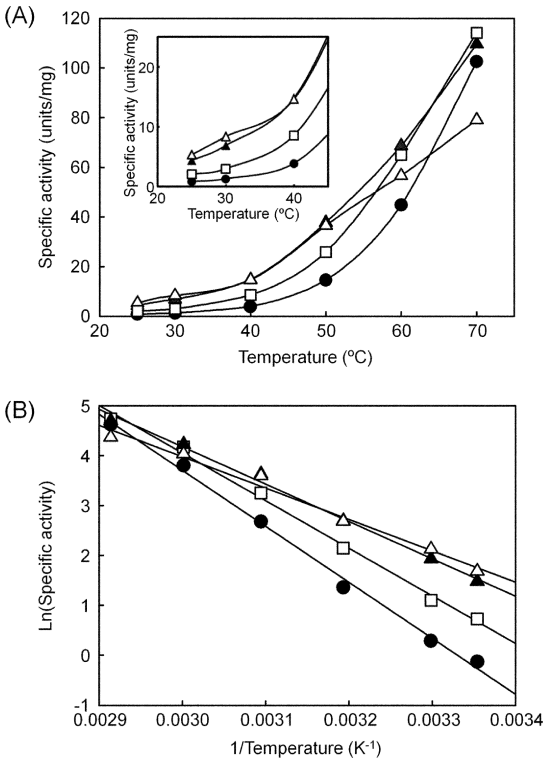

Figure 2. Specific activities of wild-type and mutant *T. thermophilus* IPMDHs. The assay solution consisted of 50 mM HEPES (pH 8.0), 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM D-3-IPM, 5 mM NAD^+, and 2 μg/mL (25−40 °C) or 0.2 μg/mL (50−70 °C) protein. Each value is the average of three replicas. The specific activities of all mutants are greater than that of wild-type IPMDH below 40 °C (inset). (B) Arrhenius plots for the specific activities of wild-type and mutant IPMDHs. Activation energies (E_a) were calculated from the slopes of the plots: wild type, 93 ± 3 kJ/mol; L254V, 62 ± 2 kJ/mol; G255A, 52 ± 3 kJ/mol; I279V, 79 ± 3 kJ/mol. Symbols: (●) *T. thermophilus* IPMDH, (▲) L254V, (△) G255A, and (□) I279V.

had significantly improved k_{cat} values at 25 °C. G255A has the most unfavorable $K_m^{NAD^+}$ but also has the most improved k_{cat} value at 25 °C. A similar relationship was found for the k_{cat} values at 40 °C, although the extent to which the k_{cat} values of the mutants increased was smaller than at 25 °C. Moreover, at 70 °C, only the k_{cat} value for I279V was greater than that of wild-type IPMDH. Therefore, the mutations adapted the catalytic activity of *T. thermophilus* IPMDH to lower temperatures. All mutated IPMDHs have decreased $k_{cat}/K_m^{NAD^+}$ values at 25 °C. Thus, the low-temperature adaptation of the mutated IPMDHs reflects an increased value for the turnover number accompanied by anincrease in the $K_m^{NAD^+}$ value.

Dissociation Constants for NADH. The K_d values for the reaction product NADH were determined by measuring the decrease in the intrinsic tryptophan fluorescence of the wild-type and mutant IPMDHs by titration with NADH. As summarized in Table 4, the K_d^{NADH} values for the two mutants, L254V and G255A, are similar to that of wild-type IPMDH. Therefore, the affinity of NADH for L254V and G255A probably does not affect the increased k_{cat} values found for these mutants. In contrast, a significantly increased K_d^{NADH} value was observed for I279V, suggesting that the weakened interaction between NADH and I279V could contribute to the enhanced catalytic efficiency of I279V.

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Table 4. K_d Values for Dissociation of NAD(H) from Wild-Type and Mutant *T. thermophilus* Dehydrogenases at 25 °C

	K_d^{NADH} (μM)[a]	$K_d^{NAD^+}$ (μM)[b]
	IPMDH	
wild type	46 ± 3	nd[c]
L254V	47 ± 1	nd[c]
G255A	31 ± 0	nd[c]
I279V	310 ± 50	nd[c]
	LDH	
wild type	1.1 ± 0.0	0.70 ± 0.04
A75G	1.0 ± 0.0	0.50 ± 0.03

[a] K_d values for NADH dissociation were determined using fluorescence titration data obtained at 25 °C. The values and standard errors were obtained by nonlinear least-squares fitting of the fluorescence titration data to the equation $\Delta F = \Delta F_{max}[NADH]/(K_d + [NADH])$, where ΔF is the corrected change in enzyme fluorescence upon addition of NADH and ΔF_{max} is the maximum change in fluorescence intensity. [b] K_d values for dissociation of NAD^+ from wild-type and mutant LDHs were determined indirectly by competition experiments.[41] [c] Not determined.

Thermal Stability. Generally, thermophilic enzymes are stable but poorly active at low temperatures. In contrast, most cold-adapted enzymes found in nature are less stable but substantially more active at low temperatures. Apparently, the increased flexibility caused by destabilization of the catalytic site or the tertiary structure contributes to the greater catalytic efficiency of a psychrophilic enzyme at low temperatures.[12,16] This activity−stability trade-off is also found for mutants that evolved in vitro that develop enhanced low-temperature activities.[25−27] However, certain cold-active enzymes are also very stable.[15,47,48] Therefore, an inverse relationship between thermal stability and activity at low temperatures is not always found. To determine if the mutant IPMDHs are as resistant to thermal denaturation as wild-type IPMDH, temperature-induced unfolding of all IPMDHs was monitored by circular dichroism spectroscopy because the ellipticity at 222 nm is a measure of protein secondary structure content. The denaturation curves of all IPMDHs are similar (Figure 3A). Therefore, the NAD^+-binding-residue mutations improved the catalytic activities without affecting the thermal stabilities of the mutants, which had been found previously[29,30] and has been found for low-temperature-adapted mutants of thermostable subtilase,[22] xylose isomerase,[24] and indoleglycerolphosphate synthase.[28]

Improvement of the Low-Temperature Activity of *T. thermophilus* LDH. Because a single example is not sufficient to demonstrate that, in general, the low-temperature activities of thermophilic dehydrogenases can be improved by mutating residues that interact with the adenine of NAD^+, we examined the low-temperature activity of thermophilic NADH-dependent dehydrogenase after slightly changing the side chain volume of a nonpolar residue that interacts with the adenine of NADH. We chose *T. thermophilus* lactate dehydrogenase (LDH) to test the generality of our hypothesis, as its NAD^+-complexed crystal structure has been determined.[49] In addition, LDH differs structurally and functionally from IPMDH. LDH is a homotetramer, and each subunit has the typical NAD^+-binding Rossmann fold; *T. thermophilus* IPMDH is a homodimer, and each polypeptide chain folds into two domains with parallel α/β motifs that are not Rossmann folds.[34] Furthermore, IPMDH decarboxylates and dehydrogenates its substrate simultaneously, whereas LDH catalyzes only a dehydrogenation.[34]

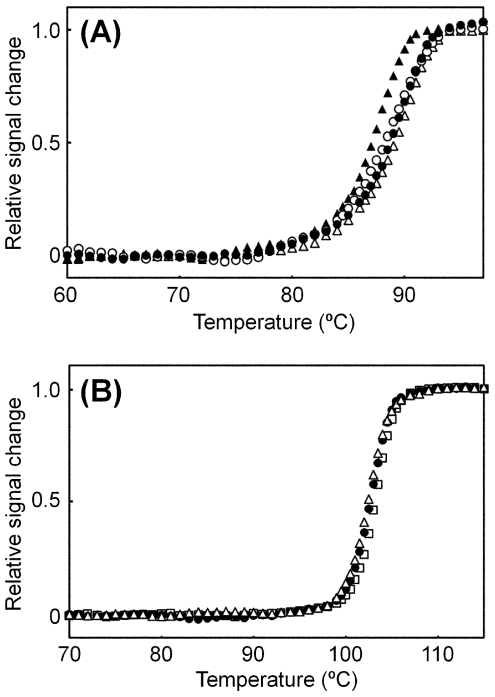

Figure 3. Thermal melting profiles for wild-type and mutant *T. thermophilus* IPMDHs and wild-type and mutant *T. thermophilus* LDHs. Ellipticities were monitored at 222 nm. The scan rate was 1.0 °C/min. The solutions consisted of 0.25 mg/mL protein, 20 mM potassium phosphate (pH 7.6), and 0.5 mM EDTA: (A) (●) *T. thermophilus* IPMDH, (○) L254V, (△) G255A, and (▲) I279V and (B) (●) *T. thermophilus* LDH, (△) A75G, and (□) V99I.

Two aliphatic residues, Ala75 and Val99, interact with the adenine of NAD^+ in LDH (Figure 4). We therefore produced

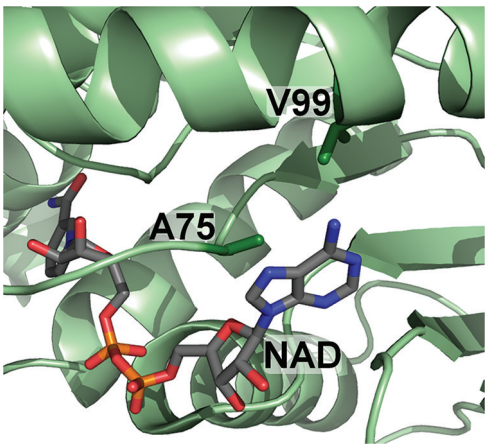

Figure 4. Active site of *T. thermophilus* LDH with bound NAD^+.[49] Two of the nonpolar residues that interact with the adenine of NAD^+ are identified. This figure was drawn using PyMOL (http://www.pymol.org).

the LDH mutants, A75G and V99I, by site-directed mutagenesis. These variants were expressed in *E. coli* and purified to homogeneity. Their kinetic parameters were determined from steady-state kinetic data obtained at 25 and 40 °C using NADH as the cofactor, as were those of wild-type LDH. The k_{cat} values for A75G are 1.9- and 2.7-fold larger at 25 and 40 °C, respectively, than that of wild-type LDH, and the K_m^{NADH}

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Table 5. Kinetic Constants for Wild-Type and Mutant *T. thermophilus* LDHs

	K_m^{NADH} (μM)[a]		k_{cat} (s^{-1})[a]		k_{cat}/K_m^{NADH} (s^{-1} μM^{-1})	
enzyme	25 °C	40 °C	25 °C	40 °C	25 °C	40 °C
wild type	31 ± 3	160 ± 20	0.20 ± 0.01	6.3 ± 0.4	0.0063	0.039
A75G	54 ± 8	240 ± 20	0.38 ± 0.02	17 ± 1	0.0071	0.072
V99I	33 ± 5	100 ± 10	0.20 ± 0.01	4.0 ± 0.2	0.0061	0.039

[a] K_m and k_{cat} were determined using steady-state kinetic data obtained at 25 and 40 °C with an assay solution of 50 mM HEPES (pH 7.5), 3.5 mM $MgCl_2$, 2 mM sodium pyruvate, and various concentrations of NADH. The values and standard errors of the kinetic constants were obtained by nonlinear least-squares fitting of the steady-state velocity data to the Michaelis–Menten equation.

values for A75G increased, but by a factor of <2, at the two temperatures (Table 5). A75G also has increased k_{cat}/K_m^{NADH} values at the two temperatures in comparison with those of wild-type LDH. Thus, the low-temperature activity of *T. thermophilus* LDH was also improved by removing a methyl from a nonpolar side chain that interacts with the adenine of NAD(H). Conversely, the kinetic parameters of V99I were similar to those of wild-type LDH. As given in Table 4, the $K_d^{NAD^+}$ value for A75G (0.50 ± 0.03 μM), determined by competitive fluorescence titration, is slightly better than that of the wild-type enzyme (0.70 ± 0.04 μM). Therefore, the improved k_{cat} value for A75G is probably not a consequence of the improved release of NAD^+.

We also assessed the thermal stabilities of the wild-type and mutant LDHs. The thermal melting curves of the mutants are very similar to that of wild-type LDH (Figure 3B). Therefore, as is true for the IPMDH mutants, small changes in side chain volumes of the residues that interact with NAD^+ improved the low-temperature activity of *T. thermophilus* LDH without compromising its thermal stability.

DISCUSSION

We describe here the selective mutagenesis of the thermostable 3-isopropylmalate dehydrogenase (IPMDH) and lactate dehydrogenase (LDH) from the extreme thermophile *T. thermophilus* that improved their low-temperature activities. We initially found that an amino acid substitution that affects coenzyme binding improves the low-temperature activity of *T. thermophilus* IPMDH. The saturation mutagenesis experiment supported the concept that a small change in the side chain volume of a nonpolar residue that interacts with the adenine of NAD^+ increases the turnover number. The low-temperature activity of *T. thermophilus* LDH was also enhanced by a similar amino acid substitution. In contrast to the substitution of a polar residue for a nonpolar residue, the substitution of a nonpolar residue for another nonpolar residue should maintain the electrostatic environment around the active site and not perturb the catalytic mechanism. The thermostabilities of the mutants were almost identical to those of the wild-type enzymes. Therefore, a small change in the side chain volume of a nonpolar residue that interacts with the adenine of NAD^+ may generally improve the low-temperature activities of thermophilic dehydrogenases without affecting their stabilities.

The kinetic mechanism of *E. coli* isocitrate dehydrogenase (ICDH) has been characterized by others and includes the release of product and NADPH as the rate-limiting catalytic step.[50] In addition to their similar tertiary structures, ICDH and *T. thermophilus* IPMDH catalyze similar oxidative decarboxylations. Therefore, similar kinetic mechanisms might be expected for ICDH and IPMDH. Our steady-state kinetic experiments showed that the $K_m^{NAD^+}$ values for the IPMDH mutants are greater than that of wild-type IPMDH (Table 2). Because the affinity of NAD^+ likely correlates with that of NADH, we hypothesized that mutations that destabilize the enzyme–substrate–NAD^+ complex also destabilize the enzyme–product–NADH complex and thus would facilitate the release of NADH, which should enhance catalysis. Indeed, the K_d^{NADH} value for I279V is significantly greater than that of wild-type IPMDH (Table 4). However, the K_d^{NADH} values for the other two mutants, L254V and G255A, are similar to that of wild-type IPMDH and therefore do not support the hypothesis that the increases in k_{cat} for the mutant enzymes are consequences of facilitated NADH release.

The rate-limiting step for the reduction of oxaloacetate catalyzed by *Thermus aquaticus* malate dehydrogenase also involves the release of NAD^+.[39] The catalytic rate for this thermophilic malate dehydrogenase was improved by replacement of an acidic residue that forms hydrogen bonds with the 2′- and 3′-hydroxyls of the adenosine ribose.[39] This mutation increases the rate of NAD^+ release and thereby improves its catalytic turnover number. Although LDH and malate dehydrogenase have similar protein folds and catalyze analogous reactions, the increase in the k_{cat} value for the reaction catalyzed by the LDH mutant A75G seemed not to be a consequence of weakened LDH–NAD^+ binding that would facilitate the release of NAD^+ because the $K_d^{NAD^+}$ value for A75G is slightly better than that for wild-type LDH (Table 4).

A free energy profile for a dehydrogenase-catalyzed reaction is illustrated in Figure 5. The energetic contributions of the amino acid substitutions that improve enzymatic activity can be evaluated using the experimentally determined steady-state kinetic parameters for the forward reaction;[16] the change in free

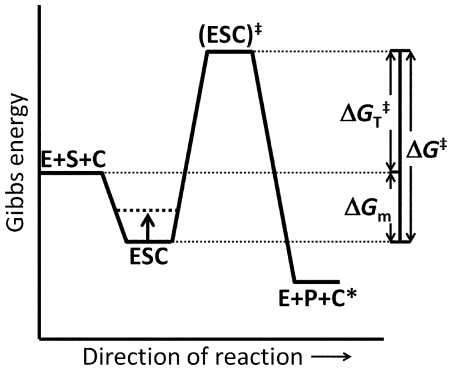

Figure 5. Gibbs free energy profile along the reaction coordinate. The dashed line shows the effect of a mutation that decreases the change in free energy upon NAD(H) binding. Abbreviations: E, enzyme; S, substrate; C, coenzyme; P, product; C*, product coenzyme; ESC, ground-state enzyme–substrate–coenzyme ternary complex; $(ESC)^‡$, transition-state ternary complex.

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Table 6. Thermodynamic Parameters of the Enzymatic Reactions of Wild-Type and Mutant *T. thermophilus* Dehydrogenases at 25 °C

enzyme	$\Delta G^{\ddagger a}$ (kJ/mol)	$\Delta\Delta G^{\ddagger b}$ (kJ/mol)	$\Delta G_m{}^c$ (kJ/mol)	$\Delta\Delta G_m{}^b$ (kJ/mol)	$\Delta G_T{}^{\ddagger d}$ (kJ/mol)	$\Delta\Delta G_T{}^{\ddagger b}$ (kJ/mol)
			IPMDH			
wild type	74.4	–	−29.0	–	45.3	–
L254V	70.2	−4.2	−22.0	7.0	48.2	2.9
G255A	69.9	−4.5	−20.5	8.5	49.3	4.0
I279V	72.1	−2.3	−25.0	4.0	47.0	1.7
			LDH			
wild type	77.0	–	−25.1	–	51.8	–
A75G	75.4	−1.6	−23.8	1.3	51.5	−0.3
V99I	77.0	0	−25.0	0.1	51.9	0.1

[a] $\Delta G^{\ddagger}$ is the activation free energy calculated from the experimentally determined k_{cat} value according to the equation $\Delta G^{\ddagger} = -RT \ln(k_{cat}h/k_BT)$, where R is the gas constant (8.31 J K^{-1} mol^{-1}), T is the temperature in kelvin, h is Planck's constant (6.63 × 10^{-34} J s^{-1}), and k_B is Boltzmann's constant (1.38 × 10^{-23} J K^{-1}). [b] $\Delta\Delta G^{\ddagger}$, $\Delta\Delta G_m$, and $\Delta\Delta G_T$ are the differences between the parameters for the mutant enzyme and the wild type. [c] ΔG_m is the change in free energy upon NAD(H) binding calculated from the $K_m{}^{NAD(H)}$ value. [d] $\Delta G_T{}^{\ddagger}$ is the difference in free energy between the initial state and transition state, calculated using the relationship $\Delta G_T{}^{\ddagger} = \Delta G^{\ddagger} + \Delta G_m$.

energy upon NAD(H) binding, ΔG_m, is calculated from the $K_m{}^{NAD(H)}$ value, and the activation free energy in the forward direction, $\Delta G^{\ddagger}$, which is the free energy difference between the ground-state and transition-state (enzyme−substrate−coenzyme) complexes, is calculated using k_{cat}. The calculated values for $\Delta G^{\ddagger}$, ΔG_m, and the difference in free energy between the initial and transition states, $\Delta G_T{}^{\ddagger}$, are listed in Table 6. The amino acid substitutions that improved the IPMDH and LDH k_{cat} values affected ΔG_m and $\Delta G_T{}^{\ddagger}$. An increased $\Delta G_T{}^{\ddagger}$ value was calculated for the mutated IPMDHs, whereas a marginally smaller $\Delta G_T{}^{\ddagger}$ value was calculated for LDH A75G. Moreover, the ΔG_m values of the mutants are substantially smaller than those of the corresponding wild-type enzymes. Therefore, the increased k_{cat} values (decreased $\Delta G^{\ddagger}$ values) found for the mutant enzymes are caused by the destabilization of the enzyme−substrate−coenzyme complexes rather than by the stabilization of the transition states; i.e., a reduction in the binding affinity of the coenzyme mostly affects k_{cat}.

It has been argued that the catalytic efficiency of a cold-adapted enzyme is reflected in the flexibility of segment(s) directly and indirectly involved in catalysis. Localized flexibility can be increased by reducing the strength of a hydrogen bond[15] or by removing a salt bridge.[51] Similarly, because glycine lacks a side chain, substitution with a glycine increases the flexibility of the peptide backbone, which would account for the catalytic behavior of LDH A75G. However, the IPMDH mutant, L254V, which has a β-branched chain residue substituted for a γ-branched residue, would seem to have a reduced flexibility because a β-branched side chain is conformationally more constrained than a γ-branched chain.[52] The other IPMDH mutant, G255A, which has an additional methyl group, also possibly reduces the flexibility of the surrounding regions. Therefore, given the less negative values of the free energy changes associated with NAD^+ binding (Table 6), these mutants likely have improved catalytic turnover numbers as a consequence of less than optimal hydrophobic packing of the adenine in the Michaelis complex.

The mutants with improved low-temperature activities obtained in this study have thermodynamic and kinetic properties similar to those of naturally occurring cold-adapted enzymes.[16,44−46] Several thermodynamic and kinetic features are common among naturally occurring cold-adapted enzymes, including smaller E_a and greater K_m values compared to those of their more thermostable homologues.[44−46] Fields et al. reported that substitutions of a residue located in the vicinity of the active site in the cold-adapted LDH-A_4 affected the values of E_a and K_m.[45] The smaller E_a value was ascribed to weakened binding of the substrate, which also increased the K_m value.[53] Psychrophilic enzymes also typically have larger k_{cat} values at a given temperature than their mesophilic and thermophilic homologues.[16] The IPMDH mutants that have improved k_{cat} values at the low temperatures also have smaller E_a values (Figure 2B) and increased $K_m{}^{NAD^+}$ values (Table 2). A smaller E_a value decreases the temperature dependency of thereaction rate, and thus, a similar reaction rate can be maintained as the temperature is lowered.[53] The LDH A75G also showed simultaneous increases in $K_m{}^{NADH}$ and k_{cat} (Table 5).

Nevertheless, the amino acid substitutions found in this study do not parallel those found for enzymes that have adapted to lower temperatures during evolution. Gly255 and Ile279 in IPMDH and Ala75 in LDH are strongly conserved in the homologous thermophilic, mesophilic, and psychrophilic enzymes. In addition, residue 254 of IPMDH is apparently always a Leu, Ile, or Met. Residues 255, 279, and 254 directly contact the adenine of NAD(H), and therefore when they were mutated, the K_m values increased (Table 2). Because NAD(H) is used in many different reactions, $k_{cat}/K_m{}^{NAD(H)}$, rather than k_{cat}, likely regulates enzymatic activity in vivo, which would explain why the mutations found in this study are not found in nature as adaptive mutations that increased the low-temperature activities of the mesophilic and psychrophilic enzymes. For in vitro applications involving enzymes, the kinetic parameter that controls the catalytic rate depends on the substrate (and coenzyme) concentrations.[54] In many industrial applications, substrate and coenzyme are present at nearly saturating concentrations. Therefore, in these situations, the catalytic power of an enzyme is determined by its k_{cat} value.

We also examined the physical relationships between adenine-binding residues and adenine for four other thermophilic dehydrogenases: ICDH from *T. thermophilus*, glyceraldehyde-3-phosphate dehydrogenase from *T. thermophilus*, alcohol dehydrogenase from *Sulfolobus tokodaii*, and glycerol-3-phosphate dehydrogenase from *Thermotoga maritima* (Figure S1 of the Supporting Information). A common feature among the four dehydrogenases is a glycine is present near the adenine of the bound coenzyme. Similar to residue 255 of IPMDH, these glycine residues are strongly conserved among the respective enzymes and, therefore, would be a mutagenesis

target for improving the low-temperature activity of these thermophilic dehydrogenases. The four coenzyme-binding sites also contain several nonpolar residues, most of which are not well conserved among their corresponding homologues, and no remarkable difference in amino acid type is found between the mesophilic and thermophilic homologues of these dehydrogenases. Only for the position corresponding to residue 337 of *S. tokodaii* alcohol dehydrogenase is a glycine found in most of the mesophilic homologues, whereas the thermophilic homologues contain residues other than glycine at this position (Figure S2 of the Supporting Information). Perhaps, therefore, the highly conserved glycine in the mesophilic alcohol dehydrogenases causes an increase in flexibility at the coenzyme-binding site, which would account for enhanced catalysis at low temperatures. The second shell of adenine-binding residues might also be important for the flexibility of the coenzyme-binding sites. However, no distinct trend in the amino acid type in the second shell that would be responsible for the difference in low-temperature activity between thermophilic and mesophilic homologues was apparent when we compared the mesophilic and thermophilic sequences.

Because we do not have detailed structural information for the mutants, the relationships between structure and activity cannot be explored at the molecular level. X-ray crystallography and nuclear magnetic resonance are powerful methods that are routinely used to characterize the structure of a protein at the atomic level. These structural biological approaches will allow us to identify the specific structural requirement(s) responsible for the enhanced low-temperature catalytic efficiencies. Efforts to do so will be made in the future.

For the work reported here, we clearly demonstrated that single amino acid substitutions each enhanced the catalytic turnover numbers of the two thermophilic dehydrogenases examined. However, even the mutant (IPMDH G255A) that has the most improved turnover number had an only 6.2-fold increase in its k_{cat} value. In contrast, the k_{cat} of the mesophilic *E. coli* IPMDH is 57-fold greater than that of *T. thermophilus* IPMDH at 30 °C.[30] It is usually assumed that a small change in an enzyme's structure should not have a large effect on its properties.[55] The presence of many beneficial substitutions is expected to produce a mutant enzyme with a dramatically increased catalytic efficiency when the effects of the substitutions are additive. Future efforts along this line will be directed at *T. thermophilus* IPMDH and LDH.

■ ASSOCIATED CONTENT

Supporting Information

Coenzyme-binding sites of four thermophilic dehydrogenases with NAD(P)$^+$ bound and a portion of the multiple-sequence alignment of the alcohol dehydrogenase sequences. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Department of Molecular Biology, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan. Telephone: +81-426-76-7139. Fax: +81-426-76-7145. E-mail: yamagish@toyaku.ac.jp.

■ ABBREVIATIONS

IPMDH, 3-isopropylmalate dehydrogenase; NAD$^+$, nicotinamide adenine dinucleotide; LDH, lactate dehydrogenase; D-3-IPM, D-3-isopropylmalate; ICDH, isocitrate dehydrogenase.

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