Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
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Published online: 22 May 2014.

To cite this article: Masaaki FUJITA, Hideyuki TAMEGAI, Tadashi EGUCHI & Katsumi KAKINUMA (2001) Novel Substrate Specificity of Designer 3-Isopropylmalate Dehydrogenase Derived from Thermus thermophilus HB8, Bioscience, Biotechnology, and Biochemistry, 65:12, 2695-2700, DOI: 10.1271/bbb.65.2695

To link to this article: http://dx.doi.org/10.1271/bbb.65.2695

Biosci. Biotechnol. Biochem., **65** (12), 2695-2700, 2001

Novel Substrate Specificity of Designer 3-Isopropylmalate Dehydrogenase Derived from *Thermus thermophilus* HB8

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Received June 22, 2001; Accepted August 23, 2001

Redesigning of an enzyme for a new catalytic reaction and modified substrate specificity was exploited with 3-isopropylmalate dehydrogenase (IPMDH). Point-mutation on Gly-89, which is not in the catalytic site but near it, was done by changing it to Ala, Ser, Val, and Pro, and all the mutations changed the substrate specificity. The mutant enzymes showed higher catalytic efficiency (k_{cat}/K_m) than the native IPMDH when malate was used as a substrate instead of 3-isopropylmalate. More interestingly, an additional insertion of Gly between Gly-89 and Leu-90 significantly altered the substrate-specificity, although the overall catalytic activity was decreased. Particularly, this mutant turned out to efficiently accept D-lactic acid, which was not accepted as a substrate by wild-type IPMDH at all. These results demonstrate the opportunity for creating novel enzymes by modification of amino acid residues that do not directly participate in catalysis, or by insertion of additional residues.

Key words: 3-isopropylmalate dehydrogenase; designed catalytic reaction; point mutation

Major advancement of various genome projects provides great opportunity in using protein resources for the production of useful materials. Protein engineering is a modern approach, based on gene manipulation, to prepare modified enzymes having higher or lower catalytic activity and broader or stricter substrate specificity from wild-type enzymes. Particularly interesting is to create a novel enzyme that can catalyze a different chemical reaction from the "parent" enzyme. Random mutation and screening of the desired reactivity is one way to this end, but a more rational way is to modify the well-established "parent" enzyme in terms of reaction mechanism and kinetics as well as precise three-dimensional structure. With this information in hand, we may proceed to surgery on the enzyme by substitution or addition of amino acid residues in the desired region of the polypeptide backbone. Herein, we describe our recent studies along this line.

3-Isopropylmalate dehydrogenase (IPMDH, EC 1.1.1.85) is among the well-studied enzymes that catalyzes the oxidative decarboxylation of (2*R*, 3*S*)-3-isopropylmalate (IPM) to 2-oxoisocaproate in the penultimate rate-limiting step of the leucine biosynthetic pathway in microorganisms and plants (Fig. 1). The IPMDH-encoding *leuB* gene from *Thermus thermophilus* HB8 was cloned, overexpressed in *Escherichia coli*,[1] and sequenced.[2] The purification and general characterization of the enzyme was already reported[3] and the crystal structures of several enzyme states were identified.[4-6] As to the chemical aspects, the stereospecificity of the hydride transfer reaction[7] and the stereochemical details of the decarboxylation[8] in the IPMDH reaction had already been discovered using deuterated isopropylmalate as the substrate. Much study has also been devoted in our laboratory to clarifying the catalytic mechanism of IPMDH by a bioorganic approach.[8-12]

Isocitrate dehydrogenase (ICDH, EC 1.1.1.42), functioning in the TCA cycle, catalyzes a similar oxidative decarboxylation to that of IPMDH. The major difference between the enzyme reactions catalyzed by IPMDH and ICDH is found in the mode of substrate recognition. Thus, similarities of the overall reaction mechanisms as well as the amino acid sequences between the two enzymes strongly suggest a common ancestral polypeptide for their evolu-

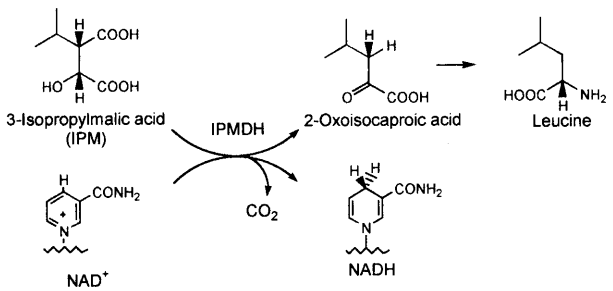

Fig. 1. The Mechanism of the IPMDH Reaction.

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tion.[2,4,13,14]

Based on the crystallographic structure of the non-productive *E. coli* ICDH-substrate-Ca^{2+} complex,[15] three-dimensional features of *T. thermophilus* HB8 IPMDH-substrate-M^{2+} complex were initially proposed as depicted in Fig. 2.[10] Later, X-ray crystallographic analyses of the IPMDH-IPM complexes of *T. thermophilus*[6] and *Thiobacillus ferrooxidans*[16] have been reported recently, which imply a closer interactive structure of the substrate and active site of enzyme as illustrated in Fig. 3. All this accumulated information prompted us to exploit the redesign of IPMDH for a new chemical catalyst with modified substrate specificity.

Redesigning or engineering of enzymes is usually done by modifying the residues that directly participate in the enzymatic catalysis.[17,18] However, this approach includes the intrinsic drawback of possible loss of all the enzymatic activity due to the failure of important interactions. One way for avoiding this risk should be to modify a residue that does not participate directly in the enzymatic activity, but is located in the proximity of the active site. Along this line, in order to redesign IPMDH for new substrate specificity by site-directed mutagenesis on the IPMDH gene, amino-acid substitutions were concentrated mainly on Gly-89 (Fig. 4) in this study. Since the isopropyl group on the C-3 position of IPM is recognized by Glu-87, Leu-90, and Leu-91 of IPMDH as shown in Fig. 3,[6,16] we anticipated that the Gly-89 mutation might cause modification of the recognition profile of the C-3 substituent, and thereby induce the change of substrate specificity. Furthermore, additional insertion of Gly between Gly-89 and Leu-90 (G89GG mutant here after) was also done to look for any change of chemical catalysis. We describe here some interesting results we obtained, and propose a new way for creating novel enzymes.

Materials and Methods

Site-directed mutagenesis. Site-directed mutagenesis was done by the method of Kunkel,[19] using pUTL119[20] as a template. The oligonucleotides used for generating the mutation were, 5′-TAAGGAAAGAAGCGCCGTCTCCGG-3′ for G89A, 5′-CTTAAGGAAAGAAGACTAGTCTCCGGGC-3′ for G89S, 5′-TTAAGGAAAGAAGTACTGTCTCCGGGC-3′ for G89V 5′-TTAAGGAAAGAAGCGGAGTCTCCGGGCGG-3′ for G89P, 5′-CTTAAGGAAAGAAGACCCCCCGTCTCC-3′ for G89GG, which were synthesized by and purchased from Biologica (Japan). The resulting plasmids were named pUTL119G89A, pUTL119G89S, pUTL119G89V, pUTL119G89P, and pUTL119-G89GG, respectively.

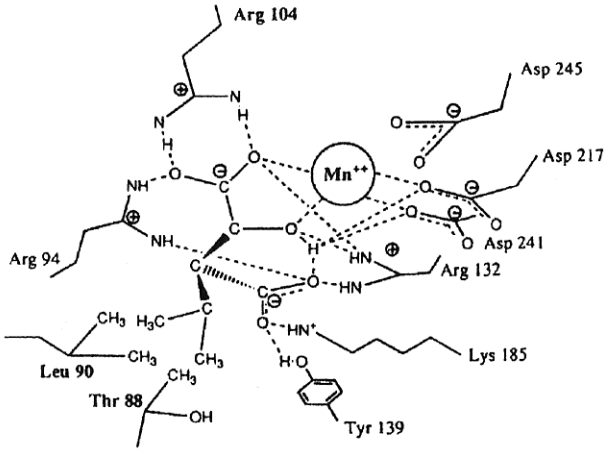

Fig. 2. A Postulated Feature of the Active Site of IPMDH-Substrate-M^{2+} Complex by Adopting the ICDH-Substrate-M^{2+} Complex, See Ref. 10.

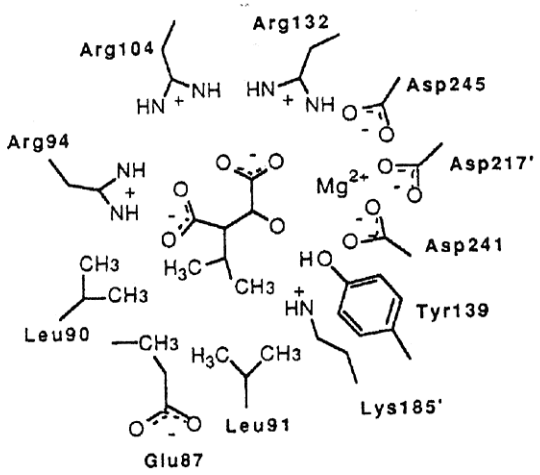

Fig. 3. The Mode of Substrate-Binding In IPMDH-Substrate-NAD-M^{2+} Complex Shown by X-Ray Crystallography, See Ref. 6.

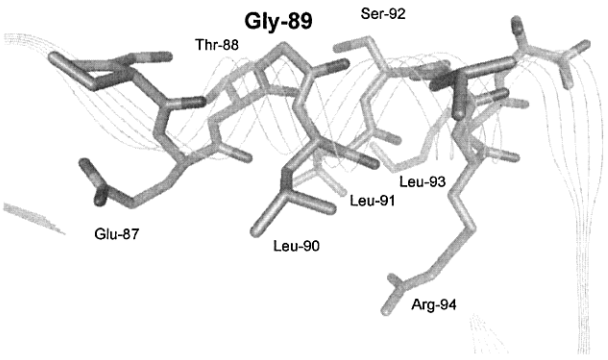

Fig. 4. The Structure Around Gly-89.

Enzyme Preparation and Purification. E. coli HB101 was transformed with each of the recombinant plasmids, and the gene containing each mutation was expressed in each transformant. The resulting mutant enzymes were separately purified by the

method described previously.[21] Enzyme purity was judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate by the method of Laemmli,[22] and stained with Coomassie brilliant blue R-250.

Enzymatic activity. All the kinetic constants, K_m and k_{cat}, of each mutant enzyme for malate, 3-alkylmalates and lactic acid were measured in the steady-state experiments at 60°C in 50 mM HEPES-NaOH buffer (pH 7.8) containing 100 mM KCl, 5.0 mM $MgCl_2$, and 5.0 mM NAD^+ in a total volume of 600 μl. The initial velocity was measured by monitoring the formation of NADH by an increase of absorbance at 340 nm using a Shimadzu UV-160A spectrophotometer.

Reagents. (2*R*, 3*S*)-3-Isopropylmalic acid and other 3-alkylmalates were synthesized by the route established in our laboratory.[9] NAD^+ was purchased from Oriental Yeast (Tokyo, Japan). Restriction enzymes were the products of TaKaRa (Kyoto, Japan). All other reagents were of the highest grade available.

Molecular dynamics calculation. Computation for the G89GG mutant was done using a Silicon Graphics INDY. The package Insight II/Discover (Biosym Technologies, USA) and Insight II/Homology with the consistent valence force field (CVFF) were used for energy minimization, RMD, and MD simulations. The crystallographic 3-D structure of IPMDH was used as the starting model,[4] and a Gly residue was inserted between Gly89 and Leu90 with the Homology module of the Insight II program. The starting structure was energy-minimized using the Steepest descents method. All the calculations were done following the instructions in the software.

Product analysis. For the charactarization of the reaction product with G89GG, an enzyme reaction was done at 60°C for 2 hours in 50 mM HEPES-NaOH buffer (pH 7.8) containing 100 mM KCl, 5.0 mM $MgCl_2$, 5.0 mM NAD^+, and 5 mM D-lactic acid in a total volume of 1 ml. To the resulting mixture, 1 ml of 2,4-dinitrophenylhydrazine in CH_3OH (saturated solution) and 2 drops of HCl was added, to form a spectrophotometrically visible hydrazone derivative. The whole mixture was then heated to 60°C for 30 min. After the solvent was concentrated to 50 μl using a centrifugal concentrator, the reaction product was put through high pressure liquid chromatography (HPLC). HPLC was done on a HITACHI L-6250 Intelligent pump apparatus equipped with a ODS-1251-N column (4.6 mm × 250 mm, Senshu Kagaku, Japan) at a flow rate of 1 ml/min with CH_3CN and H_2O (4:6) and with a L-4000H UV Detector and a D-2500 Chromato-Integrator. The elution was monitored by UV absorbance at 380 nm. Further, appropriate HPLC fractions were analyzed by liquid chromatography-mass spectrometry (LC-MS). Mass spectra were recorded on a Finnigan LCQ (San Jose, CA, USA) equipped with an electrospray ionization (ESI) source.

A synthetic standard, the 2,4-dinitrophenylhydrazone derivative of pyruvic acid, was prepared by the method as described above, and preparative TLC was done on a Merck Kieselgel 60 F_{254} plate Art with $CHCl_3$ and CH_3OH (3:1) to purify the product. The synthetic standard was confirmed by its ^{1}H-NMR (CD_3OD) spectrum, which was recorded on a Bruker DRX 500 (500 MHz) spectrometer.

Results and Discussion

Four Gly-89 mutant enzymes derived from *T. thermophilus* IPMDH were constructed and purified to homogeneity as described in the Materials and Methods section (data not shown).

The reaction kinetics of each mutant enzyme was analyzed using various malate derivatives, and the results are summarized in Table 1. It appears from these data that the mutation of Gly-89 caused no

Table 1. Kinetic Parameters for the Catalysis of Various Substrates by Wild-type and Mutated IPMDH

Substrate	Wild		G89A		G89S		G89V		G89P		G89GG	
	K_m (μM)	k_{cat} (sec^{-1})	K_m	k_{cat}	K_m	k_{cat}	K_m	k_{cat}	K_m	k_{cat}	K_m	k_{cat}
(2*R*, 3*S*)-alkylmalate												
3-H-	1600	33	400	29	320	99	380	17	950	18	54	0.033
3-methyl-	2.7	9.7	180	14	89	31	54	9.9	240	18	480	0.047
3-ethyl-	2.9	20	160	13	180	15	55	6.4	150	12	330	0.049
3-iso-propyl-	1.9	14	100	9.2	78	18	120	9.9	110	8.7	1200	0.071
3-iso-butyl-	6.4	6.4	91	8.7	59	5.4	64	5.6	76	4.4	1600	0.011
3-tert-butyl-	12	17	27	2.5	34	2.4	61	4.3	17	1.0	2000	0.094
3-iso-amyl-	7.4	11	82	7.7	48	13	63	8.7	64	5.5	1600	0.074
D-lactate	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.50	0.088

N.D.; not detected.

lethal change in enzymatic activity, because the k_{cat} of these enzymes for various alkylmalates were not much changed from the native enzyme. In contrast, the K_m of the mutant enzymes for alkylmalates were significantly affected by these mutations. Interestingly, these mutants (especially G89S) apparently showed higher catalytic efficiency for malate than that of wild IPMDH (Fig. 5). Clearly, these mutations caused a change in the substrate specificity of IPMDH rather than in catalytic activity. These results well exemplify that modification of an amino-acid residue that is not directly related to the enzyme activity and is positioned near to the catalytic residues may cause a change of substrate specificity of the enzyme without loss of activity. This is probably due to the induction of a conformational change of the proximal array of the mutated site into a productive form, while the catalytic sites remain unchanged. These findings appear to be important for the design of useful modified enzymes.

We turned our attention from the single substitution of Gly-89 to the additional insertion of a small residue (Gly in this case) between Gly-89 and Leu-90. The G89GG mutant enzyme was prepared and purified similarly as above. The k_{cat} of G89GG for alkylmalates turned out to be significantly lower than those of wild IPMDH and other mutant enzymes obtained in this study. Surprisingly however, the K_m for malate was much lower and somewhat comparable to that of active enzymes, including the wild type (Table 1). This result clearly suggested a possible positive affinity to sterically smaller substrates than alkylmalates. The oxidation reaction was attempted using D-lactate as a substrate in the presence of NAD^+. Although the wild-type IPMDH and the substitution mutants constructed in this study were unable to oxidize this small molecule, but surprisingly, the G89GG mutant clearly oxidized D-lactate into pyruvate (Table 1). Apparently, a novel substrate-specificity was acquired by this mutation. In order to confirm the reaction product, the reaction mixture was derivatized and analyzed by HPLC and LC-MS. As a synthetic standard, a 2,4-dinitrophenylhydrazone derivative of pyruvic acid was prepared; (^{1}H NMR (CD_3OD): δ 2.20 (s 3H), δ 8.11 (d, 1H J= 9.5 Hz), δ 8.31 (dd, 1H J=3 Hz, 9.5 Hz), δ 9.03 (d, 1H J=3 Hz): δ 2.26 (s 3H), δ 8.33 (d 1H J=9.5 Hz), δ 8.39 (dd 1H J=2.5 Hz, 10 Hz), δ 9.06 (d 1H J= 2.5 Hz)). The retention time of the reaction product and the standard on HPLC was identical (Fig. 6), and the corresponding peaks from LC-MS analyses clearly showed similar spectra including the molecular ion peak at m/z 267 (Fig. 7). Thus, it appeared that the enzymatic reaction product was pyruvic acid. All these data imply that the G89GG mutant acquired an ability for novel chemical catalysis for oxidation of 2-hydroxycarboxylic acid.

To get some insight into what occurred in the enzyme, a molecular dynamics simulation was done upon G89GG, which suggested that an α-helix containing the Gly-89 residue was shifted by one turn backward and a significant change occurred in the proximal loop region but not in the α-helix. Significantly repositioned residues in the substrate-bind-

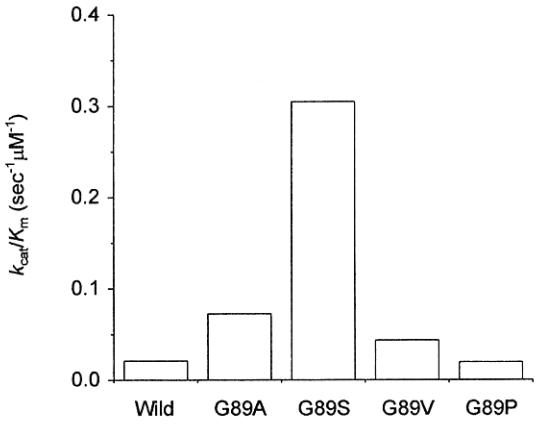

Fig. 5. Enzyme Efficiency of Wild and Mutant Enzymes for Malate.

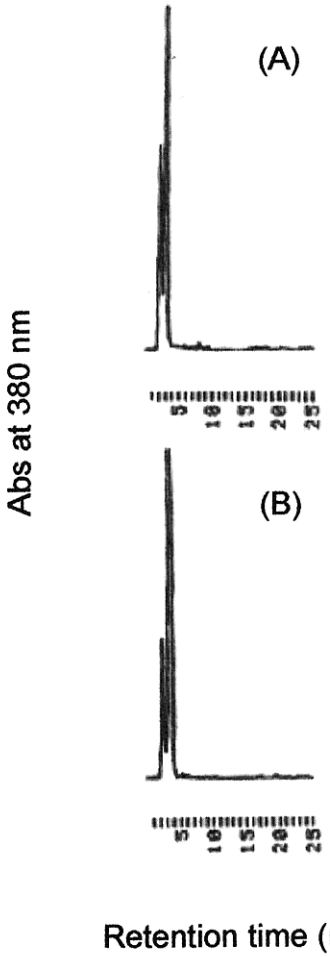

Fig. 6. HPLC Charts of Pyruvic Acid 2,4-Dinitrophenyl Hydrazone Derived from the Enzyme Reaction Product (A) and That from the Synthetic Standard (B).

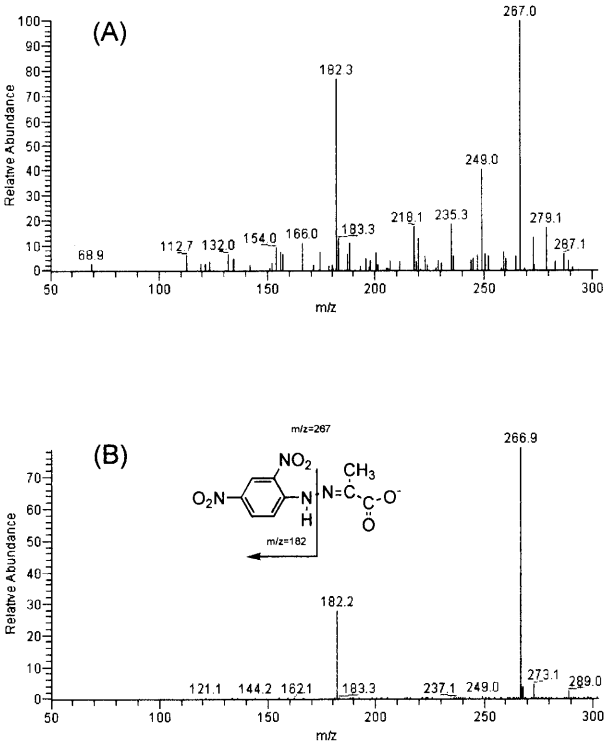

Fig. 7. Mass Spectra of Pyruvic Acid 2,4-Dinitrophenyl Hydrazone Derived from the Enzyme Reaction Product (A) and That from the Synthetic Standard (B).

ing pocket include Leu-90, Leu-91, and Arg-104. The roles of these residues in IPMDH have been described from various aspects.[6,16,23] Leu-90 and Leu-91 participate in the recognition of the isopropyl group of IPM, and Arg-104 is regarded as the residue that interacts with the 1-carboxyl group of IPM (Fig. 3). Particular concern is the location and structure of Arg-104. The flip-over of this residue from the binding pocket to the outward direction may be a reason for implementing the novel substrate specificity for D-lactate. A more detailed description of the mechanism of this intriguing change of substrate-specificity is the subject of future study.

In this study, we succeeded in changing the substrate specificity of IPMDH by modifying Gly-89, which is located in proximity to the active site but does not participate directly in the enzyme reaction. The Gly-89 mutation allowed broadening of the substrate specificity. Particularly interesting was the insertional mutation to make G89GG, which acquired a novel catalytic activity for the oxidation of lactate. In conclusion, it appears that substitution of an appropriate amino acid as well as insertion of an additional amino acid opens a new opportunity for creating novel unnatural enzymes.

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan. We wish to thank Drs. Yoshitaka Matsushima, Akira Chiba (Tokyo Institute of Technology), and Yoko Hayashi-Iwasaki (Tokyo College of Pharmacy and Life Science) for helpful technical assistance.

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