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Arg-94 is crucial to the catalysis of 3-isopropylmalate dehydrogenase from *Thermus thermophilus* HB8

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Abstract

The crucial role of Arg-94 in 3-isopropylmalate (IPM) dehydrogenase from *Thermus thermophilus* HB8 was elucidated by replacing the residue to lysine with site-directed mutagenesis. The k_{cat} value of the R94K mutant enzyme for IPM was significantly reduced to 1/170 compared with that of native enzyme, whereas the K_m for IPM was not much changed. It appeared that the major role of Arg-94 in exerting the enzymatic activity is not for the substrate recognition, but for the reaction catalysis, in such a way that Arg-94 facilitates stabilization of the transition-state in the decarboxylation step.
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Keywords: *Thermus thermophilus* HB-8; 3-Isopropylmalate dehydrogenase; Site-directed mutagenesis; Catalytic residue

1. Introduction

Prime interest of enzymology is to understand the detailed features of enzyme catalysis. Among those enzymes which have attracted attention is 3-isopropylmalate dehydrogenase (IPMDH, EC 1.1.1.85) that catalyzes the oxidative decarboxylation of (2*R*,3*S*)-3-isopropylmalate (IPM) to 2-oxoisocaproate in the rate-limiting penultimate step of the leucine biosynthetic pathway in microorganisms and plants. The IPMDH-encoding *leuB* gene from *Thermus thermophilus* HB8 was cloned, over-expressed 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 also described [4–6]. As to the chemical aspects, the stereospecificity of the hydride transfer reaction [7] and the stereochemical detail of the decarboxylation [8] in the IPMDH reaction were already elucidated by employing deuterated isopropylmalates as the substrates. Much studies in our laboratory have been devoted to the elucidation of catalytic mechanism of IPMDH through the bioorganic approach [8–12].

A similar enzymatic oxidative decarboxylation is found in the reaction of isocitrate dehydrogenase (ICDH, EC 1.1.1.42), functioning in the TCA cycle. A major difference between the enzyme reactions catalyzed by IPMDH and ICDH is found only in the mode of substrate

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recognition: IPMDH recognizes the hydrophobic isopropyl group of the substrate, whereas ICDH recognizes the ionized carboxymethyl group. Significant similarity of the overall reaction mechanisms as well as of the amino acid sequences between these two enzymes strongly suggests a common ancestral protein for the evolution of these enzymes [2,4,13,14].

The three-dimensional structure of the active site in an *E. coli* ICDH–substrate–Mg^{2+} complex was elucidated by X-ray crystallography [15]. Since the amino acid residues surrounding the isocitrate substrate in the ICDH active site were known to be well conserved in IPMDH of *T. thermophilus* HB8, the 3-D structural feature of an IPMDH–substrate–M^{2+} complex was proposed as depicted in Fig. 1. Further, the recent X-ray crystallographic result of a *T. thermophilus* IPMDH–IPM complex suggested that the interactions between the protein moiety and IPM involved Leu-91, Arg-94, Leu-134, Arg-132 and Arg-104, although the crucial hydrogen-bondings were not depicted (Fig. 2) [6]. However, since the elucidated structure appeared to have an intermediate conformation between an open- and closed-conformation, it has also been pointed out that the structural features of the IPMDH–IPM complex may not represent the realistic IPMDH–IPM–NAD^+ complex in the actual enzyme reaction [6].

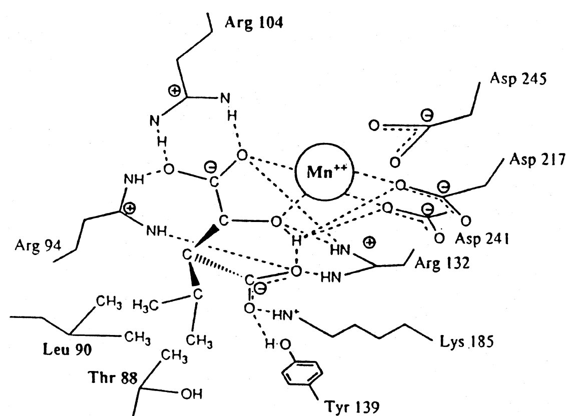


Fig. 1. Structure of the active site of IPMDH–substrate–M^{2+} complex proposed by adopting the ICDH–substrate–M^{2+} complex (see Ref. [10]).

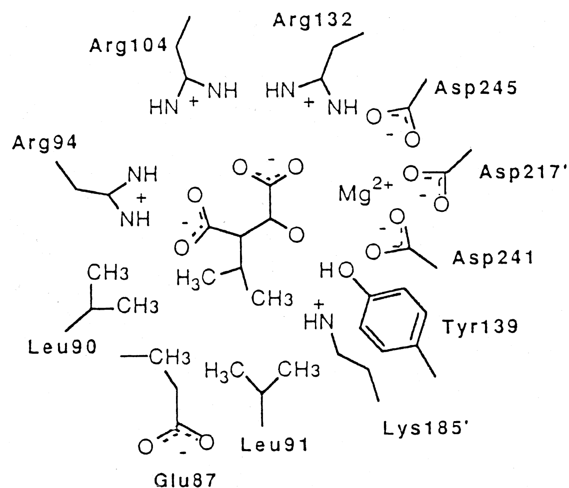


Fig. 2. The model of the substrate-binding region of IPMDH constructed by calculation of SAX methods (see Ref. [6]).

A possible mechanism for the enzyme reaction is shown in Fig. 3 [16]. However, while a prediction was made by computational calculation [17], neither a responsible residue involved in the initial proton abstraction from the hydroxyl group of the substrate prior to the hydride transfer nor a residue which is responsible for the later donation of a proton to the C-3 position after decarboxylation has been identified. The possible amino acid residues involved in the reaction catalysis are anticipated to be among Tyr-139, Lys-185, two aspartates, Asp-241, Asp-245 and three arginines, Arg-94, -104 and -132. Among these residues, Arg-94 apparently locates in the region of electrostatic contact with the carboxylate groups of the substrate [6]. Previously, Miyazaki et al. [18] showed that the mutation of Tyr-36 to Phe led to the loss of enzyme activity, and the X-ray crystallographic analysis of the Y36F mutant enzyme revealed that this mutation induced a significant conformational change of the residue Arg-94. Additionally described without any details was a preliminary experiment of site-directed mutagenesis, thus, mutation of Arg-94 to Gln caused complete loss of enzyme activity [18]. These observations allowed them to argue that Arg-94 plays an important role in the catalysis of the

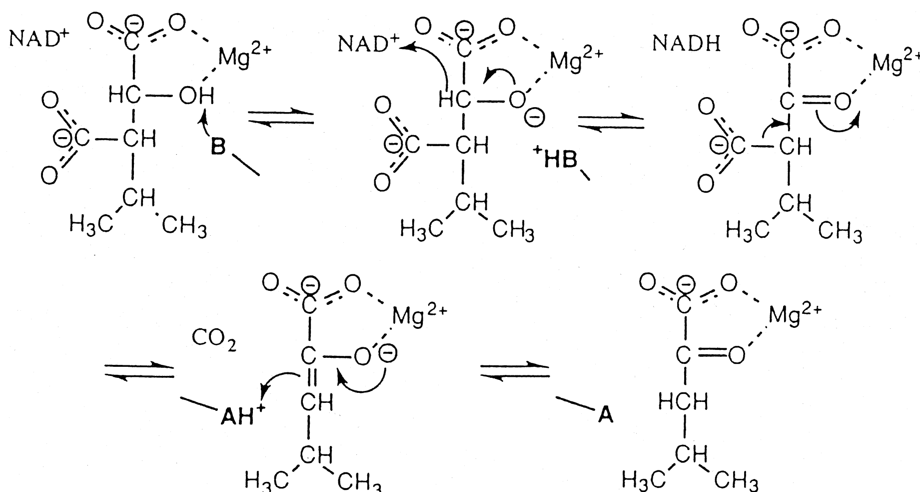


Fig. 3. A possible mechanism for the reaction of IPMDH.

enzyme. However, a crucial question arisen then was how Arg-94 is important and how it is involved in the catalysis of IPMDH, which has still been remained unsolved.

In the present study, we undertook a key site-directed mutagenesis experiment. Arg-94 of the thermophilic IPMDH from *T. thermophilus* HB8 was replaced to Lys so as to minimize the change in basicity and the length of the side-chain in order to clarify the role of Arg-94.

2. Materials and methods

2.1. Site-directed mutagenesis

Site-directed mutagenesis of the *T. thermophilus* IPMDH was carried out according to the method of Kunkel [19], using pUTL119 [20] as a template. The oligonucleotide used for generating the mutation was 5′-CCTGGCTTT-TCTTTAAGGAAAGAAG-3′ for R94K, which was synthesized by and purchased from Biologica (Japan). Generation of the mutation was confirmed by sequencing the resulting plasmid, pUTL119R94K, and by comparing with the sequence of the wild *T. thermophilus* IPMDH [2]. DNA sequence was analyzed by a LONG READIR 4200L-1 DNA sequencer (LI-COR, USA) according to the manufacturer's instructions.

2.2. Enzyme preparation and purification

E. coli HB101 was transformed with pUTL119R94K, and the gene containing the mutation of R94K was expressed in the transformant. The enzyme was purified by the method of Numata et al. [21] with slight modification. The transformed *E. coli* were grown overnight at 37°C in 3 l of 2 × YT medium containing 100 μg/ml ampicillin. Cells were harvested by centrifugation (5000 × *g*, 10 min), and were suspended in 20 mM potassium phosphate buffer (pH 7.6) containing 0.5 mM EDTA, and disrupted by Sonifier 250 sonic oscillator (Branson, USA) for a total period of 30 min. Unbroken cells were removed by centrifugation (20 000 × *g*, 90 min) and the supernatant was heat-treated (75°C, 20 min) to denature most of the mesophilic proteins in the extract. To the supernatant thus obtained, ammonium sulfate was added to 20% saturation. The solution was stirred at 4°C for 1 h, and was centrifuged at 20 000 × *g* for 30 min. The resulting supernatant was subsequently subjected to a column of Butyl-Toyopearl 650S (2.2 × 20 cm) equilibrated with 20 mM potassium phosphate buffer

Table 1
Kinetics parameters of wild and mutant IPMDH for substrates

	For IPM		For NAD^+
	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)
Wild type	3.81	44.3	734
R94K	2.62	0.255	271

(pH 7.6) containing 0.5 mM EDTA and ammonium sulfate of 20% saturation, using a FPLC system. The adsorbed protein was eluted with a gradient of ammonium sulfate of 20%–0% saturation. The eluted enzyme was dialyzed at 4°C for 3 h against 2 liters of 20 mM potassium phosphate buffer (pH 7.6) containing 0.5 mM EDTA, and the dialysate was then applied to Mono Q HR 10/10, using a FPLC system. The adsorbed protein was eluted with a gradient of NaCl of 0–0.4 M, and the fractions showing the IPMDH activity were used as the enzyme preparation. The enzyme purity was judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate by the method of Laemmli [22].

2.3. Enzymatic activity

The kinetic constants, K_m and k_{cat}, for IPM were determined in a standard manner, 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 500 μl. K_m for NAD^+ was determined in the presence of 1.0 mM 3-IPM. The initial velocities were determined by monitoring the formation of NADH as an increase of the absorbance at 340 nm using a Shimadzu UV-160A spectrophotometer.

2.4. Reagents

IPM was synthesized by the route established in our laboratory [11,12]. NAD^+ was purchased from Oriental Yeast (Tokyo, Japan). Restriction enzymes were the products from TaKaRa (Kyoto, Japan). Butyl-Toyopearl 650S was purchased from Tosoh (Japan). Mono Q HR 10/10 and FPLC system were purchased from Pharmacia Biotech (Sweden). All other reagents were of the purest grade available.

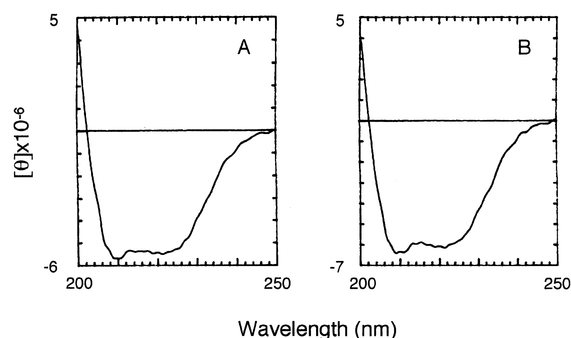


Fig. 4. CD spectra of wild type IPMDH (A) and R94K mutant (B).

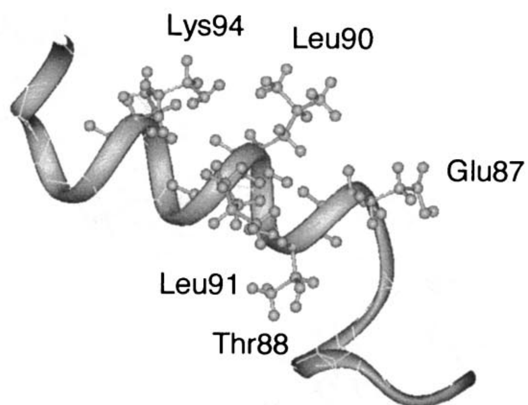
2.5. Molecular dynamics calculation

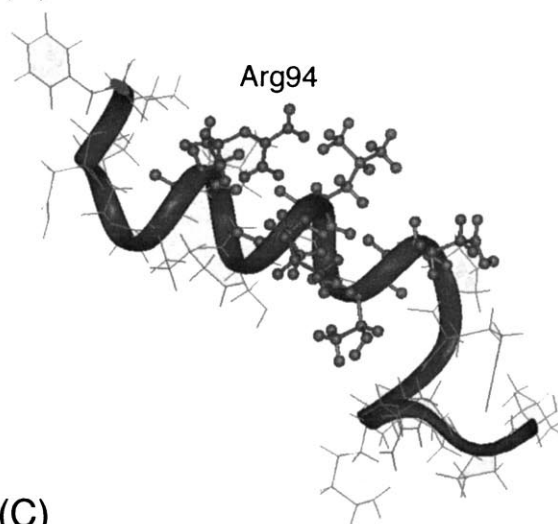
Computation was performed using a Silicon Graphics INDY. The package Insight II/Discover (Biosym Technologies, USA) with the consistent valence force field (CVFF) was used for energy minimization, RMD, and MD simulations. The crystallographic 3-D structure of IPMDH was used as the starting model [4], and Arg-94 was substituted with Lys with the Biopolymer module of the Insight II program. The starting structure was energy-minimized using the Steepest descents method. All the calculations were performed according to the instructions of the software.

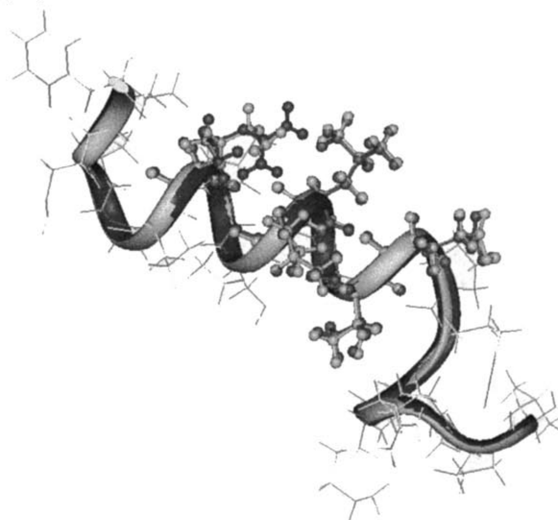
3. Results and discussion

The mutant enzyme (R94K) derived from IPMDH of *T. thermophilus* was constructed and purified to an electrophoretically homogeneous state as described in Section 2 (data not shown).

Fig. 5. Proposed structure of the region near Arg-94 calculated with molecular dynamics. (A) B94K mutant, (B) native enzyme, (C) overlap of the two enzyme.


(A)
Lys94
Leu90
Glu87
Leu91
Thr88


(B)
Arg94


(C)

The reaction kinetics of the resulting enzyme was analyzed, and the results are summarized in Table 1. The k_{cat} value of the enzyme for IPM was reduced to 1/170 compared with the native enzyme, which appears to suggest that Arg-94 is critically important for catalysis of native enzyme. On the other hand, the K_m value of the mutant enzyme for IPM was not so different from that of the native enzyme. These results clearly showed that the precise role of Arg-94 in enzymatic activity is not for the substrate recognition, but this residue is important for the reaction catalysis. The catalytic efficiency (k_{cat}/K_m) of the R94K mutant decreased accordingly for IPM. The kinetic constant for NAD^+ was not so affected in R94K mutant, indicating that Arg-94 is not concerned for the cofactor recognition either.

To confirm whether or not a major structural change has occurred, the CD spectrum of the mutant enzyme was compared with that of native enzyme (Fig. 4). Almost identical spectra were obtained. Although the CD spectrum is not so sensitive to a minor structural change, it appeared that R94K mutation did not induce major structural alteration. An additional support came from the molecular dynamics calculation, and a relevant part of the resulting stable conformation is shown in Fig. 5. Although this sort of calculation can only predict stable conformations of a molecule, these results at least suggested that no significant change in the whole structure of the enzyme may take place by R94K mutation.

As was referred above, R94Q mutation caused complete loss of enzyme activity [18]. However, R94K mutation in the present study allowed the mutant enzyme to retain the activity to some extent. This appeared to be due to the difference of the chemical nature between Gln and Lys. The latter mutation was not so drastic in terms of the basicity or ionic nature of the residue. Yet, the length of the Lys side chain in the mutant enzyme is a little shorter than that of the Arg in the native enzyme. Accordingly, the electrostatic interaction between the enzyme active site and an appropriate part of a substrate or an intermediate may well be weakened in the R94K mutant.

The results mentioned above led to propose the actual role of Arg-94 in enzymatic activity. It was already suggested that Arg-94 locates near the C-3 carboxylate group of the substrate IPM from the studies of 3-D structure of the enzyme [6]. The present study demonstrated that Arg-94 is important for the reaction catalysis, but not for the substrate recognition. It is well established that the IPMDH reaction involves two steps, i.e., the initial oxidation at C-2 and the subsequent decarboxylation at C-3. As we pointed out previously, a particular conformation of the oxodicarboxylate intermediate should be required for the decarboxylation step [11]. It seems therefore that Arg-94 is the residue responsible for the enzyme catalysis, in such a way that Arg-94 facilitates stabilization of the transition-state in the decarboxylation reaction. In other words, Arg-94 appears to be important in dictating the C3-carboxyl sigma bond being perpendicular to the plane of the C2 carbonyl group after the initial dehydrogenation step to fulfil the best overlap of the molecular-orbital for decarboxylation. In conclusion, it appears that the length, the zigzag conformation and the basicity of Arg-94 at this particular position is crucial to the reaction catalysis of IPMDH.

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