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Tyr-139 in *Thermus thermophilus* 3-isopropylmalate dehydrogenase is involved in catalytic function

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The role of Tyr-139, which is thought to be located at the active site of *Thermus thermophilus* HB8 3-isopropylmalate dehydrogenase, has been investigated by site-specific replacement with phenylalanine. The replacement scarcely affected the Michaelis constant (K_m) for 3-isopropylmalate, but caused a 13-fold decrease of that for NAD. The catalytic constant (k_{cat}) showed a 14-fold decrease. Accordingly, the catalytic efficiency (k_{cat}/K_m) decreased for 3-isopropylmalate but not for NAD. The results suggest that Tyr-139 is involved in the catalytic function through interaction with 3-isopropylmalate.

Active site; Catalysis; 3-Isopropylmalate dehydrogenase

1. INTRODUCTION

3-Isopropylmalate dehydrogenase (IPMDH, EC 1.1.1.85) is an enzyme in the leucine biosynthetic pathway while isocitrate dehydrogenase (ICDH, EC 1.1.1.42) is a key enzyme of the tricarboxylic acid cycle and also plays a role in glutamate biosynthesis. These enzymes act on structurally similar substrates, HOOC(HO)CHCH(X)COOH, in which X represents the $CH(CH_3)_2$ of 3-isopropylmalate and CH_2COOH of isocitrate. Both enzymes catalyze a chemically analogous reaction: dehydrogenation at C2 to form a carbonyl group from a hydroxyl group; and decarboxylation at C3. In recent years, the three-dimensional structure of *Escherichia coli* ICDH [1] and that of *Thermus thermophilus* IPMDH [2] have been determined by X-ray crystallography and the structural similarity between them has been pointed out [2]. The structure of the active site was also determined for *E. coli* ICHD–substrate complex [3], and the residues surrounding the substrate, isocitrate, were found to be well conserved in IPMDH. Taking the high similarity of primary structures between these two dehydrogenases (~33%) [4] into account, they are assumed to have evolved from a common ancestral enzyme to obtain their own substrate specificities. The reaction mechanisms of the enzymes are, thus, considered to be common. In fact, both enzymes catalyze the same stereospecific protonation to carbon-3 of the substrates [5,6] and the same stereospecific hydride transfer; the hydride-accepting site of nicotinamide ring of coenzymes has been identified as A form (or proR configuration) [7,8].

In ICDH, the oxidation of isocitrate is believed to occur in two steps. In the first step, isocitrate is oxidized to oxalosuccinate by removal of a proton from the hydroxyl oxygen to a base and the transfer of a hydride to coenzyme; and, in the second step, the 3-carboxyl group of oxalosuccinate is lost as CO_2 with a large acceleration in rate by a factor of 10^8 [9,10]. Hurley et al. suggested that Tyr-160 and Lys-230′ (primed number indicates the residues which belong to the second subunit of the enzyme) in *E. coli* ICDH are linked to the 3-carboxyl of isocitrate by a hydrogen bond where they are favorably positioned to serve as an acid catalyst that protonates the C3 atom of the substrate after decarboxylation [8]. The corresponding residues are conserved in all IPMDHs so far reported, including *T. thermophilus* enzyme (Tyr-139 and Lys-185′, respectively).

In this paper, in order to analyze in detail the reaction mechanism of IPMDH based on its three-dimensional structure, Tyr-139, one of the candidates for the acid catalyst, was studied by site-directed mutagenesis. To minimize the conformational perturbation, the residue was changed to phenylalanine and the contribution of the hydroxyl group on the catalytic reaction was investigated.

2. EXPERIMENTAL

2.1. *Site-directed mutagenesis*

Site-directed mutagenesis was carried out according to the method of Kunkel [11]. The oligonucleotide used for generating the base substitutions was synthesized on an Applied Biosystems DNA synthesizer (381A); 5′ GGG CTC CCC GAA GAA TAT CCC CCC GGT G 3′.

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

Table I

Kinetic parameters of wild-type and mutant enzymes

Enzyme	K_m (μM)		k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)	
	3-Isopropylmalate	NAD		3-Isopropylmalate	NAD
Wild-type	1.26	40.9	13.6	10.8	0.333
Tyr139Phe	2.06	3.00	0.937	0.455	0.312

The base substitutions were confirmed by sequencing with the dideoxynucleotide chain-termination method [12].

2.2. *Enzyme preparation*

The gene containing the directed mutation was cloned into pTD-tacN [13] (derivative of pUC118) and expressed in *E. coli* strain MV1190 (*Δ(lac-proAB), thi, supE, Δ(srl-recA)*306::Tn*10* (*tet*^{r}), F'[*traD*36, *proAB*, *lacI*^{q}*lacZΔ*M15]). The purification procedure described previously [14] was used with slight modifications. Over-expressing *E. coli* cells were grown at 37°C for 4 h in 2 × YT medium (8 g Bacto tryptone, 5 g Bacto yeast extract, and 2.5 g NaCl) containing 100 μg/ml ampicillin, and after the addition of isopropyl-β-D-thiogalactoside at a final concentration of 0.1 mM the cells were further grown for 10 h. The cells collected (5,000 × *g*, 10 min) were resuspended in 10 mM Tris-HCl buffer (pH 7.6) containing 0.5 mM EDTA and subjected to sonication with a Branson sonifier B15P. The cell debris was removed by ultracentrifugation (100,000 × *g*, 20 min) and the supernatant was heat-treated (70°C, 20 min) to denature most of the proteins in the extract. The supernatant was subjected to high-performance liquid column chromatographies (Butyl-Toyopearl 650S and DEAE-Toyopearl 650S, Tosoh).

2.3. *Kinetic experiments*

The Michaelis constant (K_m) and catalytic constant (k_{cat}) for 3-isopropylmalate were determined in steady-state experiments at 60°C in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate-Na buffer (pH 8.0) containing 100 mM KCl, 5.0 mM $MgCl_2$, and 5.0 mM NAD. The K_m for NAD was determined in the presence of 1.0 mM 3-isopropylmalate. Initial velocities were determined by monitoring the formation of NADH at 340 nm on a Gilford Response spectrophotometer.

3. RESULTS AND DISCUSSION

The wild-type and mutant enzymes were purified to homogeneity as judged by polyacrylamide gel electrophoresis either in the presence or absence of SDS. The mutant enzyme was catalytically active and resistant to heat treatment at 70°C for 20 min just like the wild-type enzyme (data not shown). These observations suggest that the overall structure of the enzyme had not been disrupted and that the influence of the mutation was local.

The kinetic parameters of the wild-type and mutant enzymes are listed in Table I. The Michaelis constant, K_m, for 3-isopropylmalate was not changed singificantly by the replacement, while that for NAD decreased by 13-fold. The catalytic constant, k_{cat}, was greatly reduced to 7%, indicating that the hydroxyl group of the tyrosine residue is important for efficient catalysis. The catalytic efficiency represented by k_{cat}/K_m is, accordingly, altered only for 3-isopropylmalate and not for NAD. In conclusion, as in the case of *E. coli* ICDH, Tyr-139 in *T. thermophilus* IPMDH is indeed involved in the catalytic function through interaction with the substrate.

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