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Roles of Arg^{231} and Tyr^{284} of *Thermus thermophilus* isocitrate dehydrogenase in the coenzyme specificity

Takuro Yaoi*, Kentaro Miyazaki, Tairo Oshima

Department of Life Science, Tokyo Institute of Technology, Nagatsuta, Yokohama 227, Japan

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Abstract The coenzyme binding site of isocitrate dehydrogenase from *Thermus thermophilus* was analyzed by site-directed mutagenesis. The mutation analysis revealed that Arg^{231} and Tyr^{284} are involved in the discrimination between NAD and NADP, suggesting that these two residues interact with 2′-phosphate group of NADP.

Key words: Thermophile isocitrate dehydrogenase; Site-directed mutagenesis; Coenzyme specificity; *Thermus thermophilus*

1. Introduction

Isocitrate dehydrogenase (ICDH; (2*R*,3*S*)-isocitrate-$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42) and 3-isopropylmalate dehydrogenase (IPMDH, EC 1.1.1.85) belong to a new enzyme family, 'decarboxylating dehydrogenase' [1,2]. The primary sequences of these enzymes are homologous and their 3D structures resemble each other. It has been speculated that they evolved from a common ancestral enzyme. We have cloned and sequenced the genes of NADP-dependent ICDH [2] and NAD-dependent IPMDH [3,4] from an extreme thermophile, *Thermus thermophilus* HB8. It has been known that the nucleotide-binding domains of many dehydrogenases are remarkably similar to each other [5]. This domain consists of two $\beta\alpha\beta\alpha\beta$ motifs called the 'Rossmann fold' and was found initially in lactate dehydrogenase (LDH) [6]. However, no obvious Rossmann fold is present in decarboxylating dehydrogenases, suggesting that these enzymes have no evolutionary relationship to LDH-like dehydrogenases [7,8].

Despite the high sequence homology and similarity of 3D structure among the decarboxylating dehydrogenase family, the coenzyme specificity is distinct; the thermophile NADP-dependent ICDH prefers NADP to NAD [9], and the NAD-specific IPMDH cannot accept NADP instead of NAD [10]. Since the sequence homology is high between those two enzymes, the discrimination between NAD and NADP could be determined by a relatively limited number of amino acid residues. The crystallographic structure of the *E. coli* ICDH–NADP complex was determined at 2.5 Å resolution by Hurley et al. [7]. Although they reported that $Arg^{292\prime}$, Tyr^{345}, Tyr^{391} and Arg^{395} (a prime indicates a residue from the second subunit) interact with the 2′-phosphate group of NADP, two among the four residues, Tyr^{345} and $Arg^{292\prime}$, of the *E. coli* enzyme are conserved in the *T. thermophilus* enzyme (corresponding to Tyr^{284} and Arg 231′, respectively). This suggests that Tyr^{391} and Arg^{395} are not essential in the coenzyme discrimination. According to Hurley et al., possible hydrogen bonds are formed between Tyr^{345} and 2′-phosphate of NADP, and between $Arg^{292\prime}$ and the phosphate. These hydrogen bonds may also play important roles in binding of NADP with the thermophile enzyme.

In this study, implication of $Arg^{231\prime}$ and Tyr^{284} of the thermophile enzyme in the recognition of the 2′-phosphate of NADP was confirmed by site-directed mutagenesis.

2. Experimental

2.1. Construction of mutant enzymes

DNA manipulating enzymes used in this study were products of either Toyobo, Bethesda Research Laboratories or New England Biolabs. *E. coli* MV1190 (*Δ(lac-proAB), thi, supE, Δ(srl-recA)* 306::Tn*10 tet′), F′[traD* 36, *proAB, lacI*^{q} *lacZΔ* M15]) was used for DNA amplification and expression of mutated *icd* genes of *T. thermophilus* HB8 [2]. Site-directed mutagenesis was carried out according to the method of Kunkel [11]. Oligonucleotides used for generating mutations were 5′-CTCAAACTGCTCCGGAGCTTTCACCAG CTG-3′ for substituting Arg^{231} with Ala, and 5′-GATTAGGTTCTT-GCCGGCGAACTTGGGGGCG-3′ for substituting Tyr^{284} with Phe. Expression and purification of the wild-type and mutant enzymes were done as described previously [12]. All the enzymes used in this study were purified to homogeneity as judged by SDS-polyacrylamide gel electrophoresis. Mutant enzyme Arg^{231}Ala designates a mutant in which Arg^{231} is replaced with Ala, Arg^{231}Ala/Tyr^{284}Phe is a double mutant in which both Arg^{231} and Tyr^{284} are replaced with Ala and Phe, respectively.

2.2. Steady-state kinetic analyses

Michaelis constant, K_m, for isocitrate and catalytic constant, k_{cat}, were determined in steady-state kinetic experiments at 60 °C in 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonat e-NaOH buffer (pH 7.8) containing 5.0 mM $MgCl_2$, 5.0 mM NAD or NADP. Isocitrate concentration was varied in the range of 5–500 μM. The reaction was monitored at 340 nm. In order to determine K_m for NAD(P), the concentrations of coenzymes were varied in the range of 5–5000 μM employing a fixed isocitrate concentration (1.0 mM). It has been confirmed that no substrate inhibition occurred under these conditions. The k_{cat} values were the average of data obtained by modulating the concentrations of NAD(P) or isocitrate. Errors of individual measurements were within ±10% of the quoted mean.

3. Results and discussion

To confirm the interactions between 2′-phosphate group of NADP and $Arg^{231\prime}$ and Tyr^{284} of *T. thermophilus* ICDH, Arg^{231}Ala, Tyr^{284}Phe, and Arg^{231}Ala/Tyr^{284}Phe mutants were constructed. All mutant enzymes were 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

*Corresponding author. Fax: (81) (45) 924 5805.

Abbreviations: ICDH, isocitrate dehydrogenase; IPMDH, 3-isopropylmalate dehydrogenase; LDH, lactate dehydrogenase

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Table 1
Kinetic parameters of wild-type and mutant enzymes

Enzyme and coenzyme	K_m (μM)		k_{cat} (s^{-1})	k_{cat}/K_m	
	For coenzyme	For isocitrate		For coenzyme	For isocitrate
Wild-type					
NADP	7.2	8.9	71	9.9	8.0
NAD	3100	81	41	0.014	0.49
$Arg^{231}Ala$					
NADP	250	63	24	0.1	0.35
NAD	2200	78	35	0.016	0.42
$Tyr^{284}Phe$					
NADP	760	43	40	0.06	0.88
NAD	4300	52	29	0.007	0.56
$Arg^{231}Ala/Tyr^{284}Phe$					
NADP	1500	97	62	0.04	0.61
NAD	5100	110	36	0.007	0.32

that the overall structure of the mutant enzymes was not altered by the substitutions and that the influence of the mutations was limited to local regions.

The steady-state kinetic parameters of the wild-type and mutant enzymes are summarized in Table 1. The Michaelis constant (K_m) of the one point mutant enzymes increased 36–106 fold for NADP. In the NADP-dependent reaction, the K_m for isocitrate also increased 5–7 fold. In contrast, the K_m for isocitrate in the NAD-dependent reaction did not change significantly as a result of the substitutions. These mutations did not affect the K_m for NAD, nor the turnover number. The double mutation further increased the K_m for NADP without significant changes accompanying the K_m for NAD and k_{cat}. The K_m for isocitrate in the NAD-dependent activity was not significantly altered by the substitutions.

The present results suggest that $Arg^{231'}$ and Tyr^{284} of *T. thermophilus* ICDH interact with the 2′-phosphate group of NADP. These two residues are essential for the high affinity to NADP, but do not affect the affinity to NAD. Tyr^{284} of the thermophile ICDH is highly conserved among the NADP-dependent enzymes, while this residue is substituted with Ile (or Leu) in NAD-dependent IPMDH [10]. A mutant ICDH, $Tyr^{284}Ile$, was designed, but the mutant was expressed as insoluble material in *E. coli*, suggesting that the substitution affected the 3D structure and/or solubility of the enzyme protein. The position at 284 in the thermophile ICDH might be important for conformational stability of the enzyme, as well as the interaction with the coenzyme.

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