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Substrate Recognition of Isocitrate Dehydrogenase and 3-Isopropylmalate Dehydrogenase from *Thermus thermophilus* HB8[1]

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The substrate-binding sites of NADP-dependent isocitrate dehydrogenase and NAD-dependent 3-isopropylmalate dehydrogenase from *Thermus thermophilus* were analyzed by site-directed mutagenesis. Ser97 and Asn99 of isocitrate dehydrogenase were identified to be involved in the isocitrate recognition. In 3-isopropylmalate dehydrogenase, the corresponding residues, Leu90 and Leu91, appear to recognize the substrate by forming a hydrophobic pocket. Double mutation of Asp78 and Glu87 revealed that negative charge of these residues plays a crucial role in discriminating isopropylmalate from isocitrate.

Key words: active site, site-directed mutagenesis, substrate specificity, thermophilic enzymes.

Isocitrate dehydrogenase (ICDH: EC 1.1.1.42) and 3-isopropylmalate dehydrogenase (IPMDH: EC 1.1.1.85) belong to a unique enzyme family of bifunctional decarboxylating dehydrogenases, and the reactions catalyzed by these two enzymes are shown in Fig. 1. These enzymes act on structurally similar substrates containing a malate moiety, and catalyze chemically equivalent reactions: dehydrogenation at carbon-2 of the malate moiety to form an oxo group from a hydroxyl group, and decarboxylation at carbon-3. In fact, they catalyze the same stereospecific protonation of carbon-3 of the substrates (*1, 2*) and the same stereospecific hydride transfer. Moreover, a hydrogen atom is transferred to the proR position (A form) of the nicotinamide ring during the catalytic reaction of these enzymes (*3, 4*).

In addition to their chemically equivalent catalytic reactions (*3*), the two enzymes share a common structural framework (*5, 6*). The primary structures of ICDH resemble those of IPMDH (*5-7*). The three-dimensional structures of *Thermus thermophilus* IPMDH and *Escherichia coli* ICDH have been reported and are considerably similar to each other (*5, 6*). Although it is well known that the nucleotide-binding domains of many dehydrogenases consist of a similar structural motif called a "Rossmann fold" (*8*), no such motif is present in these two enzymes, supporting the idea that they belong to another dehydrogenase family. The active site structure of *E. coli* isocitrate dehydrogenase-substrate complex has been reported to a 2.5-Å resolution (*4*), and the residues surrounding the substrate, isocitrate, were well conserved in 3-isopropylmalate dehydrogenases from other organisms (*5, 7*). On the other hand, the substrate and coenzyme specificities of these enzymes are markedly different. Thus, ICDH and IPMDH would have evolved from a common ancestral enzyme and eventually obtained their own substrate and coenzyme specificities (*5, 7*).

We have previously cloned and sequenced the gene coding for NADP-dependent ICDH and that coding for NAD-dependent IPMDH from an extreme thermophile, *T. thermophilus* HB8 (*7, 9-11*). We found that Ser97 and Asn99 of *T. thermophilus* ICDH are involved in the substrate specificity (*12*). In *E. coli* ICDH, phosphorylation of Ser113, which corresponds to Ser97 of *T. thermophilus* ICDH, inhibits the enzyme activity by preventing substrate binding through the direct steric blocking and the electrostatic repulsion of the γ-carboxylate of isocitrate (*13*). The X-ray analysis of *E. coli* ICDH-isocitrate-NADP-Ca^{2+} complex indicated that there are the polar interactions between the γ-carboxyl of isocitrate and the nicotinamide ring nitrogen and amide group of NADP (*14*). A schematic drawing of the catalytic site of *T. thermophilus* ICDH is shown in Fig. 2B. Ser97 and Asn99 are expected to play important roles in substrate discrimination and in forming the interaction between the substrate and the coenzyme in the thermophile enzyme. In the case of *T. thermophilus* IPMDH, the two residues at the positions corresponding to Ser97 and Asn99 of ICDH are Leu90 and Leu91, respectively. These residues are highly conserved across the amino acid sequences of IPMDHs from different species (Table I). In addition to these two residues, two acidic residues, Asp78 and Glu87, of IPMDH may be also important, because they are expected to interact with the nicotinamide mononucleotide (NMN) (*15*). The proposed active site structure of the thermophile IPMDH is illustrated in Fig. 2A. In this study, we investigated the roles of Ser97 and Asn99 of ICDH, and those of Asp78, Glu87,

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

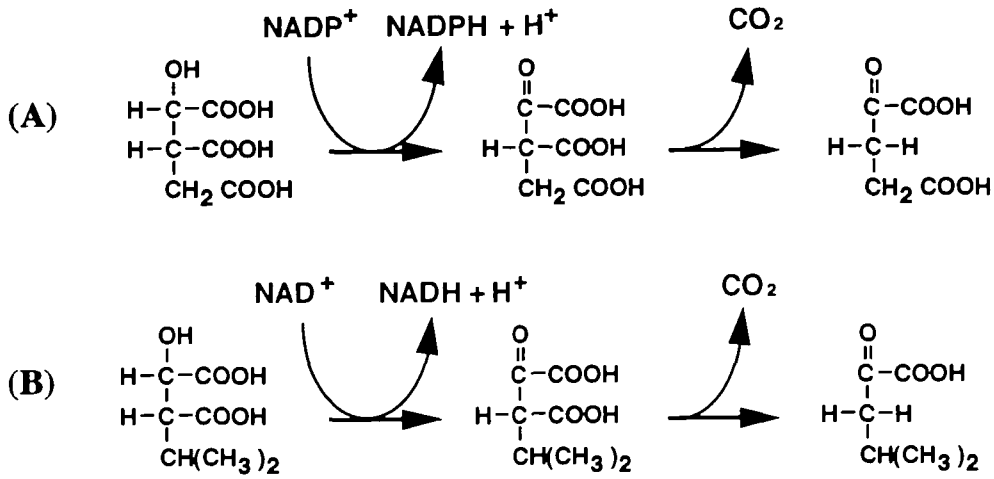

Fig. 1. **Reactions catalyzed by (A) NADP-dependent ICDH and (B) NAD-dependent IPMDH.**

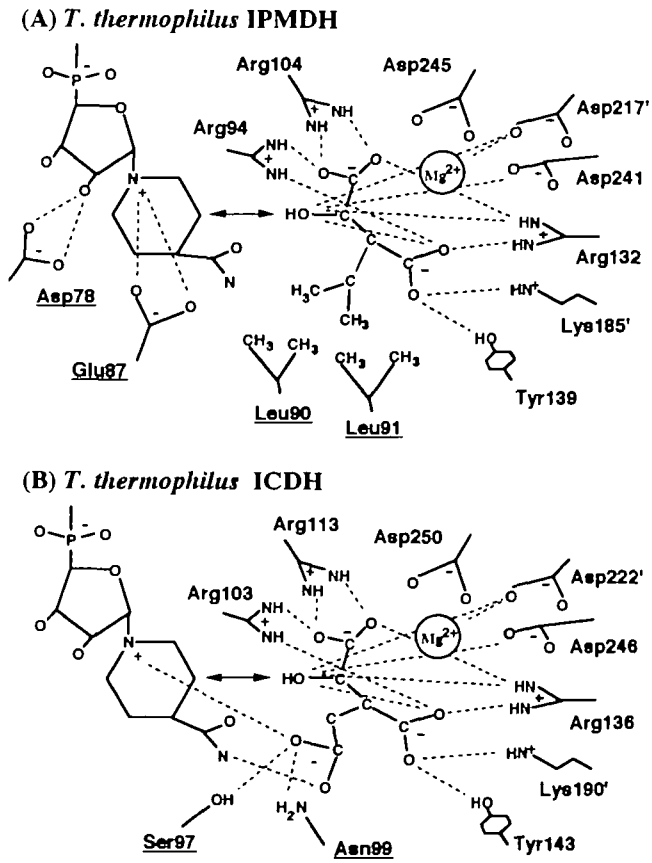

Fig. 2. **Schematic drawings of the catalytic site of (A) *T. thermophilus* IPMDH (*15, 18, 31, 32*) and (B) *T. thermophilus* ICDH (*12, 14*).** Only NMN phosphate moieties of coenzyme are shown. Arrows show C2 of the substrates and C4 of the nicotinamide (the atoms participating in hydride transfer). A prime indicates the residue from the second subunit (*e.g.*, Asp217'). Underlined residues are investigated in the present paper.

Leu90, and Leu91 of IPMDH from *T. thermophilus* in the substrate recognition by mutational analysis.

TABLE I. **Multiple sequence alignment of IPMDHs and ICDHs.** Residue numbers are based on *T. thermophilus* IPMDH and ICDH sequences.

source	sequence
NAD-dependent IPMDH	80 90
T. thermophilus [a]	KW-GLPRKIRP-TG-SL-K
T. aquaticus [b]	KW-ALPRKIRP-SG-AL-K
S. pombe [c]	-W--TNPNCRP-QG-KL-K
A. tumefaciens [d]	KW-GVPYEHRP-AG-RL-K
B. subtilis [e]	KW-QNLSELRP-KG-SI-K
B. coagulans [f]	KW-HNPASLRP-KG-GL-K
B. caldotenax [g]	KW-DNPPHLRP-KG-AI-K
B. napus [h]	KW-KNEKHLKP-TG-QL-A
L. lactis [i]	KW--GTGAVRP-QG-KI-K
S. platensis [j]	KW-NLPRPERP-TG-AL-A
NAD-dependent ICDH	
S. cerevisiae [k]	D----IGK-GHP-L-LTL-K
NADP-dependent ICDH	100
T. thermophilus [l]	LETPVGYGEK--A-VTL-K
E. coli [m]	LTTPVGGGIR--L-VAL-Q
Vibrio sp. [n]	LTTPVGGGMS--L-VAI-Q

Specific references: [a] Kirino et al., 1994 (*11*); [b] Kirino and Oshima, 1991 (*19*); [c] Kikuchi et al., 1988 (*20*); [d] Strizhov et al., 1990 (*21*); [e] Imai et al., 1987 (*22*); [f] Sekiguchi et al., 1986 (*23*); [g] Sekiguchi et al., 1987 (*24*); [h] Ellerstroem et al., 1992 (*25*); [i] Godon et al., 1992 (*26*); [j] Bini et al., 1992 (*27*); [k] Cupp et al., 1991 (*28*); [l] Miyazaki et al., 1992 (*7*); [m] Thorsness and Koshland, 1987 (*29*); [n] Ishii et al., 1993 (*30*).

MATERIALS AND METHODS

Preparation of Thermophile ICDH and IPMDH—All chemicals were of the highest commercial grade available.

Expression and purification of the wild-type and mutant enzymes were carried out as described previously (*12, 16*). All the enzyme preparations used in this study were purified to homogeneity as judged by SDS-polyacrylamide gel electrophoresis.

Construction of the 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, D(srl-recA)* 306::Tn*10 (tet r)*, F′[*traD* 36, *proAB, lacI q lacZΔ* M15]) was used for DNA amplification and expression of the wild-type and mutated *icd* and *leuB* genes of *T. thermophilus* HB8. Site-directed mutagenesis was carried out according to the method of Kunkel (*17*). Oligonucleotides used for generating mutations were follows:

5′-CCTTAGGGTGACGTTTGCGGCCTTCTCCCCGTA-3′ for Ser97Ala ICDH,
5′-CCTTAGGGTGACGTTTGCGACCTTCTCCCCGTA-3′ for Ser97Leu ICDH,
5′-CCTTAGGGTGACGTTTGCATCCTTCTCCCCGTA-3′ for Ser97Asp ICDH,
5′-CCTTAGGGTGACGGCGGCGCTCTTCTC-3′ for Asn99Ala ICDH,
5′-CCTTAGGGTGACGACGGCGCTCTTCTC-3′ for Asn99Val ICDH,
5′-TTAGGGTGACGTCGGCCGACTTCTCCCCGTA-3′ for Asn99Asp ICDH,
5′-CCTTAGGGTGACTTGAGCGCTCTTCTC-3′ for Asn99Gln ICDH,
5′-AGAGCTTCCTTAAGGTGAGGTTGGCGCTC-3′ for Val100Leu ICDH,
5′-CCTTAAGGAAAGTACTCCCGTCTCCG-3′ for Leu90Val IPMDH,
5′-CCTTAAGGAAAGGATCCCCGTCTCC-3′ for Leu90Ile IPMDH,
5′-GGCTTTTCCTTAACGAAAGATTCCCCGTCTCC-3′ for Leu90Asn IPMDH,
5′-GGCTTTTCCTTAACGAAACAAGCCCCGTCT-3′ for Leu91Val IPMDH,
5′-GGCTTTTCCTTAACGAAATAAGCCCCGTCT-3′ for Leu91Ile IPMDH,
5′-GGCTTTTCCTTAACGAAGCAAGCCCCGTCT-3′ for Leu91Asn IPMDH, and
5′-AAGCCCCGTCTCCGGGCTGATCTTCCGCGGAAGG-CCGTTCCACTTGG-3′ for Asp78Asn/Glu87Gln IPMDH.

Steady-State Kinetic Analyses—The Michaelis constant, K_m, for isocitrate and the catalytic constant, k_{cat}, were determined from steady-state kinetic experiments at 60°C in 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate-NaOH (HEPES) buffer (pH 7.8) containing 5.0 mM $MgCl_2$, 5.0 mM NADP. The K_m and k_{cat} of 3-isopropylmalate dehydrogenase were determined at 60°C in 50 mM HEPES buffer (pH 7.8) containing 100 mM KCl, 5.0 mM $MgCl_2$, 5.0 mM NAD. Initial velocity was determined by monitoring the formation of NADH at 340 nm. Individual measurements were within ±10% of the quoted mean.

TABLE II. **Kinetic parameters of the wild-type and mutant ICDHs for isocitrate.**

Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot\mu M^{-1}$)
Wild-type	8.9	71	8.0
Ser97Thr[a]	42	39	0.93
Ser97Ala	50	7.0	0.14
Ser97Leu	83	8.2	0.099
Ser97Asp	880	2.3	0.003
Asn99Leu[a]	126	7.3	0.058
Asn99Ala	212	5.9	0.028
Asn99Val	82	6.3	0.077
Asn99Asp	5,210	0.9	0.0002
Asn99Gln	21	6.2	0.3
Val100Leu	7.1	48	6.8

[a]Data from Miyazaki *et al.*, 1994 (*12*).

RESULTS AND DISCUSSION

Kinetics of Mutant ICDH—All mutant ICDHs and IPMDHs were catalytically active and resistant to heat treatment at 70°C for 20 min, like the wild-type enzyme (data not shown). These observations suggest that the overall structure of the mutant enzymes was not altered by the substitutions.

The steady-state kinetic parameters of the wild-type and mutant ICDHs for isocitrate are summarized in Table II. The replacement of Ser97 with hydrophobic residues (Ser97Ala and Ser97Leu) reduced k_{cat} for isocitrate by about 1/10 of the wild-type value and increased the K_m about 5-fold. Asn99Val and Asn99Leu mutation also increased K_m value about 10-20-fold. The k_{cat} of these mutants decreased by 1/10. Even homologous substitution reduced the catalytic efficiency k_{cat}/K_m: Ser97Thr and Asn99Gln mutations reduced k_{cat}/K_m, while Val100Leu did not alter K_m for the substrate compared with that of the wild-type. These results indicate that Ser97 and Asn99 of *T. thermophilus* ICDH are involved in the substrate recognition and catalytic function. Ser97Asp and Asn99Asp mutations largely increased K_m value (100-600-fold) and decreased k_{cat} (1/30-1/80), suggesting that negative charge of Asp opposes the γ-carboxylate of isocitrate. The reduction of k_{cat} may be caused by the weakening of substrate-coenzyme interaction. In support of this speculation, Ser97Thr and Asn99Leu mutations decreased K_m for NADP (*12*). For additional proof, recent X-ray analysis of *E. coli* ICDH-NADP-isocitrate complex revealed that the γ-carboxylate of isocitrate interacts with the nicotinamide ring of NADP (*14*).

Kinetics of Mutant IPMDH—The steady-state kinetic parameters of the wild-type and mutant IPMDHs are summarized in Table III. The replacements of Leu90 and Leu91 with other hydrophobic residues (Val and Ile) considerably affected the K_m (<13-fold), but hardly changed the k_{cat}, due probably to the streic-hindrance of the Cβ of Val and Ile. On the other hand, in spite of the shape similarity, Leu90Asn and Leu91Asn mutations largely

TABLE III. **Kinetic parameters of the wild-type and mutant IPMDHs for 3-isopropylmalate.**

Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot\mu M^{-1}$)
Wild-type	5.1	31	6.08
Leu90Val	41	13	0.32
Leu90Ile	20	15	0.75
Leu90Asn	770	2.2	0.0028
Leu91Val	65	14	0.22
Leu91Ile	65	13	0.20
Leu91Asn	1,011	79	0.078

TABLE IV. **Kinetic parameters of the wild-type and mutant IPMDHs.**

Enzyme	K_m (μM)		k_{cat}	k_{cat}/K_m ($s^{-1}\cdot\mu M^{-1}$)	
	For NAD	For IPM[a]	(s^{-1})	For NAD	For IPM[a]
Wild-type	40	5.1	31	0.77	6.1
Asp78Asn/Glu87Gln	591	17	43	0.073	2.5

[a]IPM, isopropylmalate.

increased K_m values (150-200-fold) due to the loss of the hydrophobicity. The results support the conclusions from our previous X-ray crystallographic study on the enzyme-substrate complex that these two residues form a hydrophobic pocket for the substrate binding, and size and shape of the side chains are important (*18*).

The k_{cat} of Leu90Asn decreased to 1/15, while that of Leu91Asn increased (about 1.5-fold). This suggests that Leu90 is located near the nicotinamide binding site and affects the substrate-NAD interaction. In fact, K_m for NAD of Leu90Asn (114 μM) increased about 3-fold while that of Leu91Asn (51 μM) did not change compared with that of the wild-type ICDH (40 μM).

In order to confirm the interaction between Asp78, Glu87 and the nicotinamide ring of NAD, Asp78Asn/Glu87Gln mutant was constructed. K_m for NAD of this mutant increased about 15-fold while that for the substrate and k_{cat} did not change considerably (Table IV). The results show that Asp78 and Glu87 are involved in the coenzyme recognition. In addition, the catalytic reaction of Asp78Asn/Glu87Gln was competitively inhibited by isocitrate ($K_i = 0.9$ mM), while that of the wild-type is not, suggesting that this mutant binds isocitrate at the active site. The negative charge of Asp78 and Glu87 of *T. thermophilus* IPMDH would obstruct the binding of isocitrate. These two residues are located in a loop between a β-strand and an α-helix containing Leu90 and Leu91. This loop is close to the active site (*18*), and the affinity for isocitrate might be altered by the drastic change of the electrostatic environment around the binding site for the γ-carboxyl moiety of the substrate.

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