Characterization of Homoisocitrate Dehydrogenase Involved in Lysine Biosynthesis of an Extremely Thermophilic Bacterium, *Thermus thermophilus* HB27, and Evolutionary Implication of β-Decarboxylating Dehydrogenase*

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Although the presence of an enzyme that catalyzes β-decarboxylating dehydrogenation of homoisocitrate to synthesize 2-oxoadipate has been postulated in the lysine biosynthesis pathway through α-amino adipate (AAA), the enzyme has not yet been analyzed at all, because no gene encoding the enzyme has been identified until recently. A gene encoding a protein with a significant amino acid sequence identity to both isocitrate dehydrogenase and 3-isopropylmalate dehydrogenase was cloned from *Thermus thermophilus* HB27. The gene product produced in recombinant *Escherichia coli* cells demonstrated homoisocitrate dehydrogenase (HICDH) activity. A knock-out mutant of the gene showed an AAA-auxotrophic phenotype, indicating that the gene product is involved in lysine biosynthesis through AAA. We therefore named this gene *hicdh*. HICDH, the gene product, did not catalyze the conversion of 3-isopropylmalate to 2-oxoisocaproate, a leucine biosynthetic reaction, but it did recognize isocitrate, a related compound in the tricarboxylic acid cycle, as well as homoisocitrate as a substrate. It is of interest that HICDH catalyzes the reaction with isocitrate about 20 times more efficiently than the reaction with the putative native substrate, homoisocitrate. The broad specificity and possible dual function suggest that this enzyme represents a key link in the evolution of the pathways utilizing citrate derivatives. Site-directed mutagenesis study reveals that replacement of Arg85 with Val in HICDH causes complete loss of activity with isocitrate but significant activity with 3-isopropylmalate and retains activity with homoisocitrate. These results indicate that Arg85 is a key residue for both substrate specificity and evolution of β-decarboxylating dehydrogenases.

Among β-decarboxylating dehydrogenases, 3-isopropylmalate dehydrogenase (IPMDH; EC 1.1.1.85), an enzyme catalyzing the fourth reaction in leucine biosynthesis, and isocitrate dehydrogenase (ICDH; EC 1.1.1.42), one of the enzymes in the tricarboxylic acid cycle, catalyze similar β-decarboxylating dehydrogenation of structurally similar compounds, 3-isopropylmalate and isocitrate, respectively (Fig. 1). These enzymes share structural and functional similarities and are therefore thought to have diverged from a common ancestral enzyme (1–4). Many studies have been done to elucidate the structural and functional basis for substrate specificity, heat stability, and evolution of this protein family (5–9).

Conversion of homoisocitrate to 2-oxoadipate is supposed to occur in lysine biosynthesis through α-amino adipate (AAA), a pathway that has been believed to be present only in fungi and yeast (Fig. 1) (10–13). The enzyme containing β-decarboxylating dehydrogenation activity involved in this process is homoisocitrate dehydrogenase (HICDH; EC 1.1.1.115). The reactions catalyzed by HICDH proceed in a manner similar to those by IPMDH and ICDH. In addition, IPMDH and ICDH show distinct identity in amino acid sequence to each other. Until recently, no genes have been identified and characterized as *hicdh* even in *Saccharomyces cerevisiae*, although its whole genome sequence has been determined (14). Recently, Chen and Jeong (3) reported that the gene named *YIL094C* of *S. cerevisiae* and the gene with the accession number T38612 of *Schizosaccharomyces pombe* could be likely candidates encoding HICDHs based on the amino acid sequence alignment for this protein family and the three-dimensional structures of IPMDH and ICDH. They further showed that the T38612 product actually had HICDH activity but did not characterize it further. This new member of β-decarboxylating dehydrogenases is expected to lead us not only to elucidation of the substrate recognition but also to understanding evolution of the protein family as well as cellular metabolism including amino acid biosynthesis. However, unlike with ICDH and IPMDH, there is little information on properties of HICDH.

*Thermus thermophilus* HB27, an extremely thermophilic bacterium, optimally grows at 70 °C. We recently found that this bacterium synthesizes lysine through AAA as an intermediate (15) like fungi and yeast, although all bacteria and plants are believed to synthesize lysine through diaminopimelate. Sequence analysis of the cloned genes that were shown to be involved in the lysine biosynthesis suggested that the first half of the biosynthesis (from 2-oxoglutarate to AAA) proceeded in the same manner as that of fungi and yeast. The reactions were also suggested to be similar to those of leucine biosynthesis and a part of the tricarboxylic acid cycle (16). The second half of the biosynthesis from AAA to lysine is, on the other hand, similar to the conversion of glutamate to ornithine in arginine biosynthesis (17). We have succeeded in cloning of most of the genes involved in lysine AAA biosynthesis in *T. thermophilus* HB27...
Knockout of hicdh in T. thermophilus HB27 and Auxotrophic Complementation Test—The plasmid for knockout of hicdh gene of T. thermophilus HB27, respectively, by PCR using synthetic oligonucleotides, 19HICN and HICDH-Ct. PCR conditions were as follows: step 1, 94 °C for 2 min; step 2, 94 °C for 1 min; step 3, 55 °C for 1 min; step 4, 72 °C for 2 min; step 5, 72 °C for 7 min. Steps 2–4 were repeated 30 times using KOD-plus. An amplified fragment was digested with XhoI and cloned into p18DFHCII. The resulting plasmid named p18DFHCKmR was used for knockout of the hicdh gene of T. thermophilus HB27 by the method described previously (17). Colonies growing on TM plate (20) supplemented with 50 μg/ml of kanamycin were picked, and disruption of the genes was confirmed by Southern hybridization. For the auxotrophic complementation test, the knockout mutant, named MJ101, was inoculated on the minimal medium (MM plate) (21) and the MM plate supplemented with 0.1 mM AAA or 0.1 mM lysine. 

**Phylogenetic Analysis**—A phylogenetic tree was constructed using the neighbor joining method. Sequence data for the analysis were obtained from the GenBankTM and PIR data bases. The amino acid sequences were aligned by ClustalW (25) on the DDBJ. Using this aligned data, a phylogenetic tree was constructed by a computer program, Mega 2 (26).

**Expression of hicdh, icd, and leuB from T. thermophilus HB27 in E. coli and Preparation of the Crude Extract—NdeI and EcoRI recognition sites were introduced around the start codon and the termination codon of hicdh from T. thermophilus HB27, respectively, by PCR using synthetic oligonucleotides, 19HICN and HICDH-Ct. PCR conditions were as follows: step 1, 94 °C for 2 min; step 2, 94 °C for 1 min; step 3, 55 °C for 1 min; step 4, 72 °C for 2 min; step 5, 72 °C for 7 min. Steps 2–4 were repeated 30 times using Ex-taq (Takara Shuzo). The amplified fragment was digested with HindIII and EcoRI and cloned into pUC18. After the nucleotide sequence was verified, an NdeI- and EcoRI-digested fragment was cloned into pET22b (+) (Novagen, Darmstadt, Germany). The resulting plasmid, pET-HICDH101, was used for expression of hicdh. To construct the plasmids for overexpression of leuB and icd genes from T. thermophilus HB27, PCRs with the chromosomal DNA were performed by using primers 19IebN1/19IebC2 and ICN/ICC, respectively. PCRs were performed under the same conditions used for amplifying the hicdh gene. Amplified fragments were digested with HindIII and EcoRI, subcloned into pUC119 for leuB and pUC18 for icd, to yield pUC119-sleuB and pUC18-ICD, respectively. For overproduction of HICDH, E. coli BL21-Codon-Plus (DE3)-RIL harboring pET-HICDH101 was cultured in 2× YT medium containing 50 μg/ml kanamycin and 30 μg/ml chloramphenicol. When the E. coli cells were grown to give an A600 of 0.5, isopropyl-β-D-thiogalactopyranoside (final concentration 1 mM) was added. The culture was continued for an additional 12 h after the induction. For overexpression of leuB and icd, E. coli JM109 harboring pUC119-sleuB or pUC18-ICD was cultured in the same way for the hicdh overexpression.

E. coli cells that overexpressed hicdh, icd, or leuB from T. thermophilus HB27 were suspended in 5 ml of buffer IV (20 mM potassium phosphate, pH 7.5, 0.5 mM EDTA) and disrupted by sonication. The supernatant prepared by centrifugation at 40,000 × g for 20 min was heated at 70 °C for 20 min, and denatured proteins from E. coli cells were removed by centrifugation.

**Purification of Recombinant Enzymes of HICDH—**E. coli cells of 10 g (wet weight) harboring pET-HICDH101 were suspended in 20 ml of buffer IV and disrupted by sonication. The supernatant prepared by centrifugation at 40,000 × g for 20 min was heated at 85 °C for 20 min, and denatured proteins from E. coli cells were removed by centrifugation. 

**Purification of Recombinant HICDH—**E. coli cells of 10 g (wet weight) harboring pET-HICDH101 were suspended in 20 ml of buffer IV and disrupted by sonication. The supernatant prepared by centrifugation at 40,000 × g for 20 min was heated at 85 °C for 20 min, and denatured proteins from E. coli cells were removed by centrifugation. Supernatant fractions were applied onto an anion exchange column, DEAE (Hi-Light, Japan), pre-equilibrated with buffer IV, washed with buffer IV containing 0.1 M NaCl, and eluted with buffer IV containing 0.2 M NaCl. Ammonium sulfate was added to the eluted fractions at a final concentration of 45%, and the resultant precipitate was collected by centrifugation at 40,000 × g for 30 min, dissolved in buffer IV, and applied onto a Hi-load 26/60 Superdex200 prep grade column (Amersham Biosciences, Uppsala, Sweden) containing 0.2 M NaCl. ICDH from T. thermophilus HB27 was also purified by the method described previously (27).

Purity of the recombinant enzyme was verified by SDS-12% PAGE. Proteins were determined by the method of Bradford (28) by using a
enzymes was defined as the amount of enzyme that produced 1/H9262 the half-life (remaining activity in the supernatant was measured. For determining proteins were removed by centrifugation (23,500 for 20 min and immediately chilled in ice water. Then aggregated solved in buffer IV (0.5 mg/ml) was incubated at various temperatures for 5 min. For determination of the kinetic constants for isocitrate, 

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\text{Kinetic parameters were calculated by using an initial velocity pro-}
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\text{Site-directed mutagenesis was}
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\text{identified. Construction of the expression plasmid for the altered}
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\text{HICDH was carried out under the same strategy used for wild-type_hicd. Construction of other altered HICDHs (HICDH/4Sc, HICDH/5Sc, HICDH/3Sc, HICDH/2Sc, and HICDH/R85V) was carried out in the same way by using a different pair of primers.}
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Bio-Rad protein assay kit (Nippon Bio-Rad, Tokyo, Japan). Sedimentation equilibrium analysis of the purified HICDH was carried out with a Beckman Optima XL-A analytical ultracentrifuge fitted with a Beckman An-60Ti analytical rotor. The subunit organization of the enzyme was analyzed according to the procedure of Van Holde and Baldwin (29).

**Heat Stability of HICDH and ICDH of _T. thermophilus_ HB27**—Heat stability of HICDH and ICDH was analyzed as follows. For calculating the melting temperature (Tm) of the enzymes, purified enzyme dissolved in buffer IV (0.5 mg/ml) was incubated at various temperatures for 20 min and immediately chilled in ice water. Then aggregated proteins were removed by centrifugation (23,500 × g for 5 min), and the remaining activity in the supernatant was measured. For determining the half-life (t1/2) of the enzymes, the enzyme dissolved in buffer IV (0.5 mg/ml) was incubated at 90 °C, and an aliquot was taken at an appropriate interval followed by immediate cooling in ice water. After centrifugation to remove denatured proteins, the remaining activity of the supernatant was measured.

**Enzyme Assays**—Ten microliters of the enzyme solution (0.1 mg/ml for isocitrate and 0.2 mg/ml for homoisocitrate) was added to the reaction mixture (50 mM HEPES, pH 8.0, 200 mM KC1, 5 mM MgCl2, 1 mM NAD+, and 25–5000 μM homoisocitrate) that was preincubated at 60 °C for 5 min. For determination of the kinetic constants for isocitrate, 4–400 μM isocitrate was added to the reaction mixture in place of homoisocitrate. The reaction was monitored at 60 °C by following the increase in absorbance at 340 nm. Specific activity was determined by using a 400 μM concentration of each substrate, and 1 unit of the enzymes was defined as the amount of enzyme that produced 1 μmol of NADH or NADPH/min at 60 °C in the enzymatic reaction.

Kinetic parameters were calculated by using an initial velocity program, HYPER, of Cleland (20). Results

**Cloning and Sequence Analysis of the _hicdh_ Gene**—Putative candidates for HICDHs have been proposed in two yeasts (3). In addition, the whole genome sequence has been determined (31) for a radiation-resistant bacterium, *Deinococcus radiodurans*, that is closely related to *T. thermophilus* in taxonomy and suggested to possess the lysine biosynthetic system through AA found in *T. thermophilus* (32). *D. radiodurans* possesses a homolog of yeast HICDH genes in addition to putative _leu B_ and _icd_. Based on the amino acid sequences of the putative HICDHs from yeast and the *Deinococcus* strain and Blast analysis against the incomplete *T. thermophilus* HB27 genome sequence (available on the World Wide Web at www.g2l.bio.uni-goettingen.de/blast/blast_search.html), we designed a pair of primers (HICD-N1 and HICD-C1) to amplify a portion of _hicd_ of *T. thermophilus* HB27 by PCR. The resulting amplified DNA fragment of about 640 bp carried a sequence that was similar to that of the _hicdhleuB_ family but obviously different from those of _leu B_ and _icd_ from *T. thermophilus* (33–35). We then tried to clone the DNA fragment of *T. thermophilus* HB27 that hybridized against the amplified DNA fragment.

When the chromosomal DNA was digested with several restriction enzymes and subjected to Southern hybridization using the amplified DNA fragment as the probe, _SacI_ digestion gave a positive signal of ~2.6 kb. The DNA fragment was recovered and inserted into pUC18. A clone positive in the colony hybridization assay was picked, and the plasmid contained in the transformant was named _pHICD-Sac2600_. In this _SacI_ fragment, three open reading frames were found. One of the open reading frames encoded a protein showing 44 and 45% identity in amino acid sequence to _IPMDH_ and _ICDH_ from *T. thermophilus_ HB8, respectively (33–35). The other two open reading frames did not show amino acid sequence similarity to other proteins whose functions were identified.

Based on the crystal structures of _IPMDH_ and _ICDH_, amino acid residues responsible for the recognition of a common port, the malate moiety, of the substrates and amino acid residues determining the substrate specificity can be easily predicted (36–38). As expected, amino acid residues involved in

### RESULTS

| Name  | Sequence |
|-------|----------|
| HICD-N1 | 5'-CACGCGAGACAAGGTTAGGGCTGCCGGCGC-3' |
| HICD-C1 | 5'-CGGAGTCATAGAGGGCGGCGCAGGCTGGTACGTC-3' |
| DFHICN1 | 5'-GGGAGGTTAGGGCTGCCGGCGCAGGCTGGTACGTC-3' |
| DFHICC1 | 5'-CGTGTACTAGAGGGCGGCGCAGGCTGGTACGTC-3' |
| DFHICC2 | 5'-CGGAGTCTAGAGGGCGGCGCAGGCTGGTACGTC-3' |

**TABLE I**

Synthetic oligonucleotides using in this study
Amino acid sequence alignment of HICDHs, ICDHs, and IPMDHs from various sources. Closed circles, amino acid residues that interact with the malate moiety of isocitrate and 3-isopropylmalate; open circles, amino acid residues that interact with the adenine moiety of homoisocitrate. Amino acid residues conserved in all the enzymes are marked with asterisks. The amino acid residues responsible for the recognition of the malate moiety of the substrate are all conserved even in the putative HICDH from Thermus thermophilus and HICDH (Fig. 2). For the recognition of the malate moiety of isocitrate and 3-isopropylmalate, the amino acid residues that interact with the adenine moiety of homoisocitrate are boxed. Amido residues conserved in all the enzymes are marked with asterisks. TicICDH, ICDH from T. thermophilus HB27 (this study); EcICDH, ICDH from E. coli (P08200); ScHICDH, putative ICDH from S. cerevisiae (P40485); SpHICDH, ICDH from S. pombe (T38621); TtHICDH, HICDH from T. thermophilus HB27 (this study); TtIPMDH, IPMDH from T. thermophilus HB27 (this study); TtIPMDH, IPMDH from Thiobacillus ferrooxidans (JX0286).

Dual Roles of Homoisocitrate Dehydrogenase of T. thermophilus

HICDH and ICDH from T. thermophilus HB27—We next constructed plasmids for expression in E. coli of hicdh, icd, and leuB from T. thermophilus HB27 and determined the specific activities of the gene products after removal of most of the proteins in the E. coli crude extract by heat treatment and successive centrifugation. Thermus HICDH gave a specific activity of 564 units/mg with homoisocitrate as the substrate in an NAD-dependent manner. Surprisingly, the enzyme was able to catalyze the reaction using isocitrate as a substrate with much higher specific activity (8,579 units/mg). On the other hand, HICDH did not recognize 3-isopropylmalate as a substrate. A similar analysis was also performed with crude extract containing ICDH or IPMDH. ICDH catalyzed the reaction with both isocitrate (14,835 units/mg) and homoisocitrate (764 units/mg), whereas, as expected, IPMDH recognized 3-isopropylmalate (2,418 units/mg) and homoisocitrate (8,579 units/mg). On the other hand, HICDH did not recognize 3-isopropylmalate as a substrate. A similar analysis was also performed with crude extract containing ICDH or IPMDH. ICDH catalyzed the reaction with both isocitrate (14,835 units/mg) and homoisocitrate (764 units/mg), whereas, as expected, IPMDH recognized 3-isopropylmalate (2,418 units/mg) as a substrate but did not utilize homoisocitrate and isocitrate as substrates at all. Therefore, we analyzed only HICDH and ICDH in detail in the following experiments.

Expression and Purification of HICDH—16 mg of HICDH was prepared through three purification steps from 1 liter of culture with over 95% purity on SDS-PAGE (data not shown). We carried out sedimentation equilibrium analysis to evaluate the quaternary structure of HICDH. By using a partial specific volume of 0.754 that was calculated from the amino acid composition, data were fitted best with the dimer-tetramer equilibrium: equilibrium constants for monomer-dimer and monomer-tetramer association of 1.19 M⁻¹ and 3.80 × 10⁶ M⁻³, respectively. According to the equilibrium constants, we estimate that HICDH is present as a mixture of homodimer and homotetramer with a ratio of about 25 and 75%, respectively. When the enzyme concentration is in the range of 0.25–1.0 mg/ml. We therefore treated HICDH as a homotetramer in the kinetic analysis described below. The heat stability of HICDH and ICDH was analyzed by two different assay methods. When melting temperatures (Tm) were examined by incubating the purified enzyme at various temperatures for 20 min,

Knockout of hicdh in T. thermophilus HB27—We performed a knockout experiment of the homolog to examine whether or not this gene is related to lysine biosynthesis in T. thermophilus HB27. A knockout mutant, MJ101, did not grow on minimal medium (Fig. 4). However, growth of the mutant was restored by the addition of AAA or lysine, indicating that the homolog is related to lysine biosynthesis of the microorganism and has a role in a reaction step before AAA synthesis as expected. We hereafter call this homolog hicdh.

Specific Activities of HICDH, ICDH, and IPMDH from T. thermophilus HB27—In an NAD-dependent manner. Surprisingly, the enzyme was able to catalyze the reaction using isocitrate as a substrate with much higher specific activity (8,579 units/mg). On the other hand, HICDH did not recognize 3-isopropylmalate as a substrate. A similar analysis was also performed with crude extract containing ICDH or IPMDH. ICDH catalyzed the reaction with both isocitrate (14,835 units/mg) and homoisocitrate (764 units/mg), whereas, as expected, IPMDH recognized 3-isopropylmalate (2,418 units/mg) as a substrate but did not utilize homoisocitrate and isocitrate as substrates at all. Therefore, we analyzed only HICDH and ICDH in detail in the following experiments.

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homoisocitrate and isocitrate as the substrates, we used both HICDH. As shown above, because HICDH recognized both enzyme at 90° and specificity even in HICDH. In order to elucidate the residues deter-
mining the difference in the substrate specificity between yeast and Thermus HICDHs, we constructed six altered HICDHs from T. thermophilus HB27 in each of which 1–7 amino acid replacements were introduced in the loop region. An altered enzyme, HICDH/7Sc, containing the same sequence as that of S. cerevisiae HICDH in the loop displayed activity for homoisocitrate but not for isocitrate (Fig. 5). On the other hand, two other altered enzymes, HICDH/4Sc and HICDH/5Sc, containing YSSP sequence like HICDH/7Sc and the yeast en-
yzymes, recognized isocitrate as well as homoisocitrate as sub-
strates, although the activity was significantly decreased in the altered enzymes. These results suggested that Arg^2 and/or Tyr^36 were required for recognizing isocitrate. Enzyme assays for HICDH/8R5V, HICDH/2Sc, and HICDH/3Sc, all of which possessed Val at position 85, revealed that the altered enzymes completely lost activity for isocitrate but acquired activity with 3-isopropylmalate as a substrate.

**Discussion**

A gene coding for a new member of β-decarboxylating dehydrogenase, HICDH, has been cloned from an extreme thermophile, Thermus thermophilus HB27, and its involvement in lysine biosynthesis has been demonstrated. Although HICDH is indispensable for lysine AAA biosynthesis and its primary function in vivo must be the conversion of homoisocitrate to 2-oxoadipate, kinetic analysis showed that the enzyme can also utilize isocitrate, an intermediate of the tricarboxylic acid cycle, 20 times more efficiently compared with using homoisocitrate as a substrate (Table II, Fig. 3). This may indicate that the kinetic constant of an enzyme determined in vitro does not always reflect its physiological role, or it may be possible that HICDH will substitute for ICDH in some cases. On the other hand, the catalytic efficiency in terms of k_cat/K_m of ICDH for the ICDH reaction is in a range similar to that of HICDH for the same reaction. Therefore, this may suggest that ICDH can function in lysine AAA biosynthesis as well.

HICDH and ICDH share similarities in primary sequence and reaction mechanism and are therefore thought to have diverged from a common ancestral enzyme (3). A common an-
cestor is thought to have broad substrate specificity (1), so an HICDH from T. thermophilus HB27 that can catalyze reactions contained in both lysine AAA biosynthesis and the tricarboxylic acid cycle may still have features of the ancestral-type enzyme. It should be noted that we recently found that homocitrate synthase, which catalyzes synthesis of homocitrate from 2-oxoglutarate and acetyl-CoA, the first reaction in lysine biosynthesis has been demonstrated. Although HICDH contain in both lysine AAA biosynthesis and the tricarboxylic acid cycle may still have features of the ancestral-type enzyme. It should be noted that we recently found that homocitrate synthase, which catalyzes synthesis of homocitrate from 2-oxoglutarate and acetyl-CoA, the first reaction in lysine biosynthesis has been demonstrated. Although HICDH...
from E. coli, two residues (Ser113 and Asn115 in the numbering of E. coli ICDH) are indicated by x-ray structural analysis to be responsible for isocitrate recognition (36). Ser113 forms a hydrogen bond to the $\gamma$-H9253-carboxylate group of isocitrate, and Asn115 interacts both with the $\gamma$-H9253-carboxylate group of isocitrate and with NADP$^+$. Due to the polar environment of the $\gamma$-moiety-binding site of the ICDH, the hydrophobic isopropyl moiety of 3-isopropylmalate is excluded from the site. In HICDH, regions probably recognizing the $\gamma$-moiety are also occupied by amino acid residues different from those in ICDH and IPMDH (Figs. 1 and 2). Chen and Jeong (3) aligned amino acid sequences of several enzymes in this protein family and suggested Tyr106 and Ile110 (numbering according to HICDH from S. cerevisiae) as the residues that were necessary for recognition of the $\gamma$-moiety of homoisocitrate. They speculated that the phenolic hydroxyl group of Tyr 106 formed a hydrogen bond to $\gamma$-carboxylate of homoisocitrate analogous to Ser113 in E. coli ICDH. In HICDH from T. thermophilus HB27, however, the corresponding position is occupied by phenylalanine. This indicates that the mechanism for recognition of homoisocitrate has to be reconsidered in HICDH.

Thermus HICDH recognizes both homoisocitrate and isocitrate as substrates (Table II) in contrast to HICDHs from S. pombe and S. cerevisiae, where the yeast enzymes catalyze the reaction with only homoisocitrate and neither isocitrate nor 3-isopropylmalate (3). To elucidate the difference in the mechanism for substrate recognition between Thermus and yeast HICDHs, we introduced amino acid replacements into the $\beta$-strand 3-$\alpha$-helix 4 loop region of Thermus HICDH. An altered enzyme, HICDH/7Sc, containing the same sequence as that of S. cerevisiae HICDH in the loop displayed activity for homoisocitrate but not for isocitrate, indicating that the loop actually does contain the determinants for substrate specificity in HICDH. Among the altered HICDHs, all of the enzymes with Val at position 85 completely lost the ability to utilize isocitrate as a substrate. These results indicate that Arg85 is required for recognition of isocitrate as a substrate in Thermus HICDH.

**TABLE II**

| Enzyme | Homocitrate | Isocitrate |
|--------|-------------|------------|
|        | $K_m$ (µM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$·µM$^{-1}$) | $K_m$ (µM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$·µM$^{-1}$) |
| HICDH  | 7,486 ± 500 | 171 ± 8 | 2.3 × 10$^4$ | 405 ± 15 | 171 ± 2 | 4.2 × 10$^3$ |
| ICDH   | 114 ± 2 | 5 ± 0 | 4.4 × 10$^4$ | 67 ± 6 | 68 ± 3 | 1.0 × 10$^6$ |

**Fig. 5.** Specific activity of HICDH constructed by site-directed mutagenesis. Names of enzymes and their amino acid sequences in the loop between $\beta$-strand 3 and $\alpha$-helix 4 are shown. Black, white, and hatched bars indicate specific activities as HICDH, ICDH, and IPMDH, respectively. Each specific activity was determined by triplicate experiments. S.E. is below 5% of the indicated value for every enzyme.

**Fig. 6.** Superimposition of three-dimensional structures around the active sites of IPMDH of T. ferrooxidans (green; PDB 1A05) and ICDH of E. coli (magenta; PDB 1AI2) with bound substrates. Isocitrate (IC) is colored in orange and red, and 3-isopropylmalate (IPM) is in blue and red. Mg$^{2+}$ is in yellow. NADP$^+$ bound in ICDH is also shown. The figure was drawn using WebLab™ Viewer Lite software, version 3.2 (Accelrys Inc., San Diego, CA)
However, this result looks confusing, because the corresponding position (116 in E. coli ICDH) is also occupied by Val in ICDH. Val116 in E. coli ICDH does not directly interact with substrate nor NADP⁺, but replacement of the residue by Leu or Ser converted the ICDH to preference toward 3-isopropylmalate (9). This suggests that substrate recognition of the loop residues differs between these enzymes.

The phylogenetic tree of HICDH, ICDH, and IPMDH from various sources indicates that HICDH from T. thermophilus HICDHs, knockout experiment, the gene was shown to be involved in residues differs between these enzymes. recation to bind a malate moiety seem similar between the

is 45%, and the basic mechanisms for reactions and substrate specificity of the putative ICDH has to be ana-

catalyzed by a putative ICDH. In order to prove this hypothe-

reaction of lysine AAA biosynthesis in

Sulfolobus/H9252

divergent point between IPMDH and ICDH groups. This sug-

grows at extremely elevated temperatures, we may assume wild-type enzyme, which may ensure that the microorganism extremely high thermostability comparable with that of the

archaeaon, Pyrococcus horikoshii, However, PH1722, an HICDH homolog, in the gene cluster of T. thermophilus through AAA as in the case of Sulfolobus tokodaii strain 7 and Sulfolobus favicon, whose genome sequences have recently been determined (40, 41). Recently, expression of the gene cluster was shown to be regulated by LysM, an Lrp-like transcriptional regulator, depending on the presence or absence of lysine (42), although the functions of the cluster have not yet been analyzed. Based on these observations, it is speculated that the two Sulfolobus species also synthesize lysine through AAA. However, it is of interest that these microorganisms carry no hcdh-homologous genes other than icd and leaB in their genomes. IPMDH (LeuB) was isolated from S. tokodaii strain 7 and analyzed in detail (2). The Sulfolobus IPMDH cannot utilize homoisocitrate or isocitrate as substrates (2). This may suggest that the HICDH reaction of lysine AAA biosynthesis in Sulfolobus could be catalyzed by a putative ICDH. In order to prove this hypothe-
sis, substrate specificity of the putative ICDH has to be ana-
yzed. In any case, PH1722 in Pyrococcus and the putative ICDHs in the Sulfolobus species could be keys to elucidate evolution of β-decarboxylating dehydrogenase. It should be also noted that the HICDH group is monophyletic, with a deep divergent point between IPMDH and ICDH groups. This sug-
gests that HICDH has not evolved from IPMDH or ICDH but rather directly from an ancestral enzyme of β-decarboxylating dehydrogenase.

Amino acid sequence identity between HICDH and ICDH is 45%, and the basic mechanisms for reactions and substrate recognition to bind a malate moiety seem similar between the two enzymes. However, although these enzymes also showed similar catalytic efficiency (kcat/Km) for the reaction with homoisocitrate or isocitrate, profiles in the kinetic parameters were different; HICDH has larger kcat and Km values, and ICDH has smaller kcat and Km values. In addition, HICDH is a much more heat-stable enzyme than ICDH. Determination of structures of both enzymes may lead us to understand not only the detailed catalytic mechanism but also molecular evolution along with the heat stability of these enzymes.

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