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Bifunctional isocitrate–homoisocitrate dehydrogenase: A missing link in the evolution of β-decarboxylating dehydrogenase

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Abstract

β-Decarboxylating dehydrogenases comprise 3-isopropylmalate dehydrogenase, isocitrate dehydrogenase, and homoisocitrate dehydrogenase. They share a high degree of amino acid sequence identity and occupy equivalent positions in the amino acid biosynthetic pathways for leucine, glutamate, and lysine, respectively. Therefore, not only the enzymes but also the whole pathways should have evolved from a common ancestral pathway. In *Pyrococcus horikoshii*, only one pathway of the three has been identified in the genomic sequence, and PH1722 is the sole β-decarboxylating dehydrogenase gene. The organism does not require leucine, glutamate, or lysine for growth; the single pathway might play multiple (i.e., ancestral) roles in amino acid biosynthesis. The PH1722 gene was cloned and expressed in *Escherichia coli* and the substrate specificity of the recombinant enzyme was investigated. It exhibited activities on isocitrate and homoisocitrate at near equal efficiency, but not on 3-isopropylmalate. PH1722 is thus a novel, bifunctional β-decarboxylating dehydrogenase, which likely plays a dual role in glutamate and lysine biosynthesis in vivo.

Keywords: 3-Isopropylmalate dehydrogenase; Isocitrate dehydrogenase; Homoisocitrate dehydrogenase; Dual specificity; Ancestral enzyme; β-Decarboxylating dehydrogenase

It is commonly assumed that homologous proteins have developed new functions, including new enzymatic specificities, by evolving from a common ancestral protein. Such ancestral protein is usually a "missing link," probably because its more specialized descendants confer evolutionary advantages to the organism, allowing them to replace the ancestor. The β-decarboxylating dehydrogenases comprise 3-isopropylmalate dehydrogenase (IPMDH; EC 1.1.1.85), isocitrate dehydrogenase (ICDH; EC 1.1.1.42), and homoisocitrate dehydrogenase (HICDH; EC 1.1.1.115). They share a structurally similar substrate, HOOC(HO)CHCH(R)COOH, in which "R" represents the γ-moiety: $CH(CH_3)_2$ of 3-isopropylmalate, the CH_2COOH of isocitrate, and the CH_2CH_2COOH of homoisocitrate (Fig. 1, the third step of the biosynthetic pathway). The reactions catalyzed by these enzymes are essentially equivalent: dehydrogenation at carbon-2 of the malate moiety to form an oxo group from a hydroxyl group, and decarboxylation at carbon-3 using $NAD(P)^+$ as a coenzyme. Besides the similarities in the chemical structure of substrates and catalytic reactions, they also share a high degree (~30–40%) of sequence identity [1–3]. Moreover, the three-dimensional structures resemble each other [4,5]. Therefore, they are clearly homologous. In addition to these similarities at the molecular level, the metabolic roles of these enzymes are also equivalent: IPMDH catalyzes the third step in the biosynthesis of leucine, ICDH of glutamate, and HICDH of lysine via α-aminoadipate

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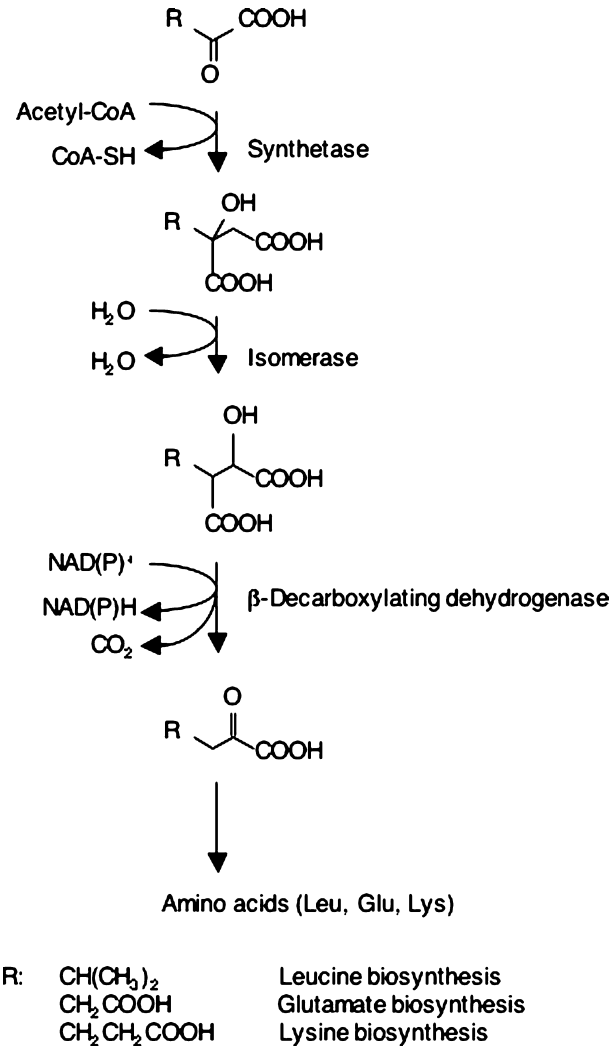

Fig. 1. Common pathway for leucine, glutamate, and lysine biosynthesis.

(Fig. 1). It is very likely that not only the enzymes but also the biosynthetic pathways have diverged from a common ancestral pathway to obtain their own specificities. Nevertheless, no organism has been discovered to possess such an ancestral pathway or enzyme thus far.

In search of β-decarboxylating dehydrogenase genes in published genome databases, I found that the hyperthermophilic archaeon *Pyrococcus horikoshii* has only a single β-decarboxylating dehydrogenase gene, PH1722 [6]. The organism does not require leucine, glutamate, or lysine for growth [7]. This raises the interesting possibility that PH1722 has retained ancestral, broad substrate specificities which allow it to play multiple roles in the pathway. In order to address to this question, the gene was cloned, sequenced, and expressed in *Escherichia coli*. The recombinant protein was solubilized and refolded into active enzyme from inclusion bodies. Kinetic analysis was used to determine its substrate specificity using 3-isopropylmalate, isocitrate, and homoisocitrate as substrates.

Materials and methods

Gene cloning. PCR primers were designed based on the reported nucleotide sequence of PH1722: 5′GGGGAACATATGTATAAGG TAGCGGTTATAAAAGGA-3′ (forward) and 5′-GGGGGGGGAT CCTTAACTTATCTCGAGCAGTATCC-3′ (reverse). The PCR mixture contained 1× KOD-Plus-buffer (Toyobo), 1 mM $MgCl_2$, 0.2 mM each of dNTP, 25 pmol each of primers, 10 ng of genomic DNA, and 1 U KOD-Plus-DNA polymerase (Toyobo) in a total volume of 50 μl. Reaction mixtures were heated at 94 °C for 1 min, followed by 25 cycles of incubation at 94 °C for 30 s, 55 °C for 15 s, and 68 °C for 1 min. The product (~1050 bp) was isolated by agarose gel electrophoresis, purified in a Qiagen QIAquick spin column, and eluted in 50 μl water. The sample was then digested with *Nde*I and *Bam*HI, and inserted into the corresponding sites of the pET-28a(+) vector (Novagen), to yield the expression plasmid pET28PH1722. This construct fuses a 6× His-tag to the N-terminus of the protein to facilitate purification by immobilized-metal affinity column chromatography. The sequence of the cloned DNA insert was determined on an Applied Biosystems automatic DNA sequencer (ABI PRISM 310 Genetic Analyzer) using an Applied Biosystems BigDye (version 3) kit. Multiple sequence alignment was performed by using the program CLUSTAL W [8].

Gene expression and protein purification. Competent cells of *E. coli* BL21-CodonPlus (DE3)-RIL (Stratagene) were transformed with pET28PH1722 and grown overnight at 37 °C on an LB (1%(w/v) tryptone, 0.5%(w/v) yeast extract, and 1%(w/v) NaCl)-agar plate containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol. A single colony was picked and grown in 10 ml LB broth containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol. The culture was then transferred to 1 L LB broth containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol, and agitated at 37 °C. When the OD_{600} reached 0.9, isopropyl-β-D-thiogalactopyranoside was added to give a final concentration of 1 mM and the cells were grown for an additional 5 h. Cells were harvested by centrifugation (5000*g*, 10 min, 4 °C) and disrupted by using the BugBuster kit (Novagen). Inclusion bodies were then prepared following the BugBuster kit protocol. Partially purified inclusion bodies were dissolved in 20 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl and 8 M urea (buffer A). Aggregates were removed by centrifugation (20,000*g*, 10 min, 4 °C) and the supernatant was loaded onto an Amersham Biosciences Hi-Trap Chelating HP column (1 ml) pre-equilibrated with buffer A. The column was washed with buffer A containing 20 mM imidazole, and bound proteins were eluted with a linear gradient of imidazole from 20 mM to 0.4 M in buffer A. Fractions containing a ~42 kDa protein as the sole band on an SDS–polyacrylamide gel were pooled.

Protein refolding. N-Lauroylsarcosine was added to ~5 ml of the purified inclusion bodies to 0.3%(v/v). The sample was then dialyzed against 1 L of 20 mM Tris–HCl (pH 8.5) containing a lower concentration of the detergent, at room temperature. At first, the concentration of the detergent in the dialysis buffer was adjusted to 0.15%. It was then lowered to 0.075% and finally to 0.003% by stepwise adjustment. Aggregated material observed after the final dialysis was removed by centrifugation (20,000*g*, 10 min, 4 °C). The supernatant, which contained the soluble enzyme, was stored at 4 °C and used for kinetic assays.

Determination of protein. Protein was measured by using a Bradford protein assay reagent (Pierce) and bovine γ-globulin as a standard (Bio-Rad).

Enzyme assays. Kinetic assays were carried out at 70 °C in 50 mM *N*-2-hydroxyethylpiperadine-*N*′-2-ethanesulfonate · NaOH (pH 7.8), 0.2 M KCl, 0.2 mM $MnCl_2$, substrate (3-isopropylmalate, isocitrate, or homoisocitrate), NAD^+, and an appropriate amount of enzyme. To determine kinetic constants (i.e., Michaelis constant, K_m and turnover number, k_{cat}) for isocitrate and homoisocitrate, 1 mM NAD^+ was used as a coenzyme. For constants for NAD^+, 0.5 mM isocitrate was used

as a substrate. Initial velocities were determined by monitoring the formation of NADH at 340 nm on a spectrophotometer using the molecular coefficient $\varepsilon = 6300\ M^{-1}\ cm^{-1}$.

Results

Molecular cloning of the P. horikoshii PH1722 gene

I conducted a BLAST search for a β-decarboxylating dehydrogenase gene in the genomic database of *P. horikoshii* (available via Internet at http://www.bio.nite.go.jp/dogan/HomologySearch?GENOME_ID=) using the amino acid sequence of *Thermus thermophilus* HICDH (GenBank Accession No. AB075751) as a query and identified the open reading frame PH1722, which was annotated as IPMDH by the genome project [6]. The gene comprised 969 bases, encoding a slightly shorter amino acid sequence (323) than the other known β-decarboxylating dehydrogenases. From multiple amino acid sequence alignment with other β-decarboxylating dehydrogenases, the open reading frame appeared to lack the conserved N-terminal region (Fig. 2, boxed region). I then surveyed upstream sequence in the genome, and identified a UUG codon located 66 bases upstream of the initially identified initiation codon, GUG. Because the UUG codon is often used as an initiation codon in this organism, and a purine-rich putative ribosome-binding site was identified several nucleotides upstream of the UUG codon (data not shown), I predicted that the UUG codon is the correct translation initiation site and cloned the newly identified gene, comprising 1035 bases, by PCR. The product was cloned into a vector and sequenced (Fig. 3). The nucleotide sequence was identical to that reported by the genome project [6].

Amino acid sequence similarities to those of other β-decarboxylating dehydrogenases

When the deduced amino acid sequence (Fig. 3) was compared with those of other β-decarboxylating dehy-

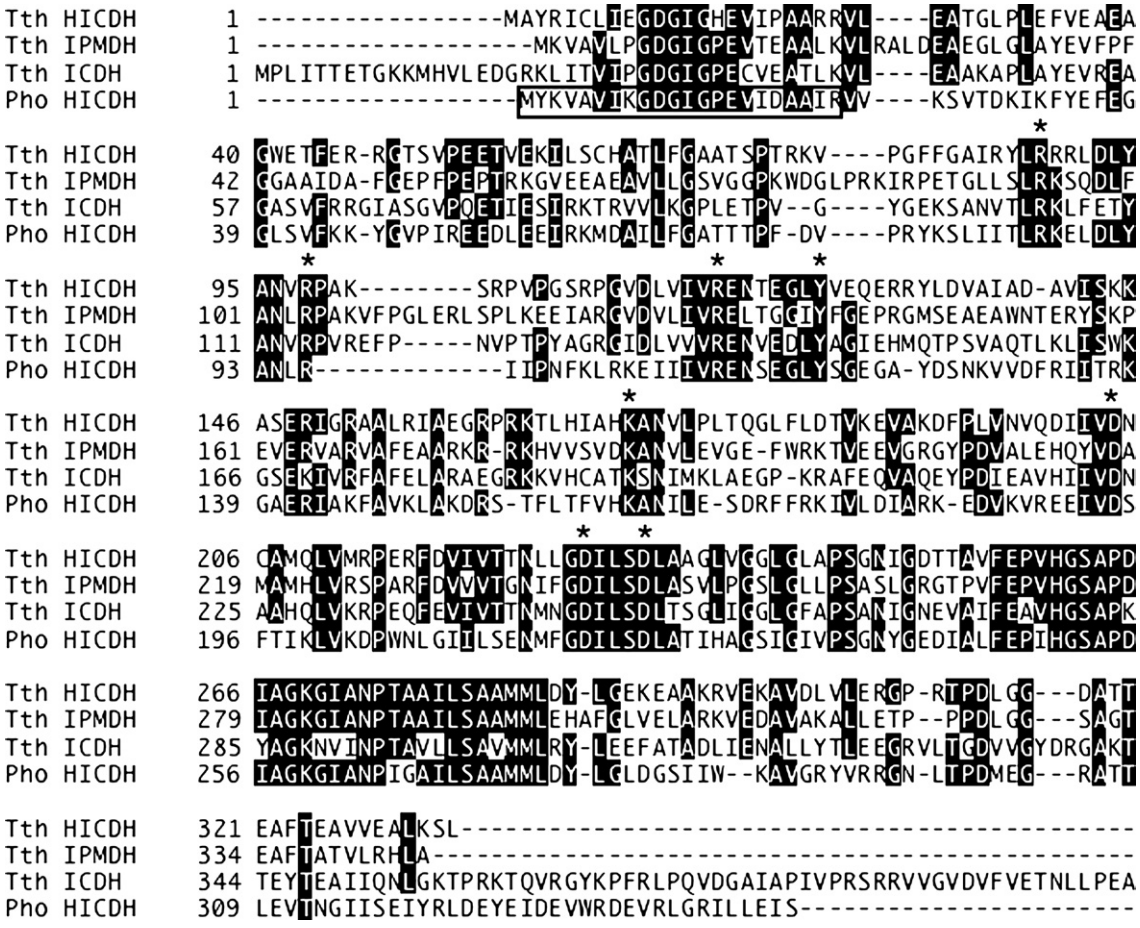

Fig. 2. Multiple alignment of the amino acid sequences (enzyme code and GenBank accession numbers in parentheses) of the PH1722 protein from *P. horikoshii* (Pho HICDH, AB197920), HICDH from *T. thermophilus* (Tth HICDH, AB075751), IPMDH from *T. thermophilus* (Tth IPMDH, K01444), and ICDH from *T. thermophilus* (Tth ICDH, M85277). Extended C-terminal residues of Tth ICDH (494 amino acids) were omitted. Letters on black background indicate residues that are conserved in ⩾75% of the sequences. Asterisks indicate eight key amino acid residues in the active site [4,5]. The sequence which was lacking in the reported PH1722 protein in the whole genome analysis [6] is boxed.

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ATG TAT AAG GTA GCG GTT ATA AAA GGA GAT GGG ATA GGA CCA GAG GTA ATA GAT GCT GCT  60
 M   Y   K   V   A   V   I   K   G   D   G   I   G   P   E   V   I   D   A   A

ATT CGG GTG GTA AAA TCT GTG ACA GAT AAA ATT AAA TTT TAT GAA TTC GAA GGG GGT TTA 120
 I   R   V   V   K   S   V   T   D   K   I   K   F   Y   E   F   E   G   G   L

AGT GTT TTT AAA AAA TAT GGG GTG CCA ATC AGG GAG GAG GAT CTC GAG GAA ATT AGG AAG 180
 S   V   F   K   K   Y   G   V   P   I   R   E   E   D   L   E   E   I   R   K

ATG GAT GCT ATA TTA TTT GGA GCA ACG ACG ACA CCC TTT GAT GTT CCT AGA TAT AAG AGC 240
 M   D   A   I   L   F   G   A   T   T   T   P   F   D   V   P   R   Y   K   S

TTA ATT ATA ACG TTG AGG AAG GAA CTT GAC TTG TAC GCA AAT CTA AGG ATA ATT CCA AAC 300
 L   I   I   T   L   R   K   E   L   D   L   Y   A   N   L   R   I   I   P   N

TTT AAG TTG AGA AAA GAG ATA ATT ATA GTT AGA GAA AAT AGT GAA GGG CTA TAC AGT GGA 360
 F   K   L   R   K   E   I   I   I   V   R   E   N   S   E   G   L   Y   S   G

GAA GGA GCA TAC GAT AGT AAC AAG GTA GTT GAC TTT AGG ATA ATA ACA AGG AAA GGT GCG 420
 E   G   A   Y   D   S   N   K   V   V   D   F   R   I   I   T   R   K   G   A

GAG AGG ATA GCT AAA TTT GCA GTA AAA CTT GCA AAA GAT AGG AGC ACC TTC TTA ACC TTT 480
 E   R   I   A   K   F   A   V   K   L   A   K   D   R   S   T   F   L   T   F

GTT CAT AAG GCA AAC ATA CTT GAG AGT GAT AGA TTC TTC AGA AAA ATT GTC CTA GAC ATT 540
 V   H   K   A   N   I   L   E   S   D   R   F   F   R   K   I   V   L   D   I

GCT AGG AAG GAA GAT GTT AAA GTT AGA GAA GAA ATA GTT GAC TCT TTT ACA ATA AAG TTA 600
 A   R   K   E   D   V   K   V   R   E   E   I   V   D   S   F   T   I   K   L

GTG AAA GAC CCT TGG AAC TTG GGA ATA ATC CTT TCT GAA AAT ATG TTT GGT GAC ATC CTT 660
 V   K   D   P   W   N   L   G   I   I   L   S   E   N   M   F   G   D   I   L

TCA GAT CTG GCA ACG ATA CAT GCT GGT AGC ATC GGG ATC GTT CCC AGC GGA AAC TAT GGG 720
 S   D   L   A   T   I   H   A   G   S   I   G   I   V   P   S   G   N   Y   G

GAG GAT ATA GCA TTG TTT GAG CCA ATA CAT GGC TCT GCT CCG GAT ATA GCT GGC AAG GGA 780
 E   D   I   A   L   F   E   P   I   H   G   S   A   P   D   I   A   G   K   G

ATA GCA AAT CCA ATA GGG GCA ATA CTG AGT GCT GCA ATG ATG CTG GAT TAC CTT GGT TTA 840
 I   A   N   P   I   G   A   I   L   S   A   A   M   M   L   D   Y   L   G   L

GAT GGT TCT ATC ATC TGG AAA GCT GTT GGG AGG TAT GTG AGG AGA GGA AAC CTA ACA CCG 900
 D   G   S   I   I   W   K   A   V   G   R   Y   V   R   R   G   N   L   T   P

GAT ATG GAG GGG AGA GCA ACA ACT CTG GAG GTT ACT AAT GGG ATA ATC TCG GAG ATA TAT 960
 D   M   E   G   R   A   T   T   L   E   V   T   N   G   I   I   S   E   I   Y

CGC TTG GAT GAG TAC GAA ATA GAT GAG GTT TGG AGG GAT GAA GTT AGA CTT GGG AGG ATA 1020
 R   L   D   E   Y   E   I   D   E   V   W   R   D   E   V   R   L   G   R   I

CTG CTC GAG ATA AGT                                                          1035
 L   L   E   I   S
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Fig. 3. Nucleotide and deduced amino acid sequences of PH1722 from *P. horikoshii*.

drogenases (i.e., HICDH, IPMDH, and ICDH from *T. thermophilus*), conserved residues that are involved in the active site [4,5] were also identified in the PH1722 enzyme: R86, R96, R111, Y118, K163, D194, D218, and D222 (marked by asterisks in Fig. 2). In contrast, residues that would recognize the γ-moiety of the substrates (amino acid residues from F73 to T84) were not aligned.

Gene expression, protein purification, and protein refolding

The cloned gene was expressed in *E. coli* using a conventional pET system [9]. No activity was detected in the soluble fraction, which suggested the formation of inclusion bodies. This was verified by SDS–polyacrylamide gel electrophoresis of the supernatant and precipitate (data not shown). The precipitate was collected and

dissolved in urea-containing buffer. The solubilized protein was then purified on an immobilized-metal affinity column. In order to refold the enzyme, the purified protein was dialyzed against urea-free buffer. However, this procedure resulted in rapid accumulation of aggregates in the dialysis tube, so *N*-lauroylsarcosine was added to the sample to a concentration of 0.3%(v/v). The concentration of the detergent was lowered by stepwise dialysis to 0.003%(v/v), at which point most of the protein was collected in soluble, active form.

Catalytic properties

The substrate specificity of the purified 6× His-tagged PH1722 was determined using 3-isopropylmalate, isocitrate, and homoisocitrate as substrates. The enzyme was active in the presence of K^+, Mn^{2+}, and NAD^+, and used homoisocitrate and isocitrate as a substrate but not 3-isopropylmalate. For isocitrate, the kinetic constants were $K_m = 16.4\ \mu M$, $k_{cat} = 14.8\ s^{-1}$, and $k_{cat}/K_m = 9.02 \times 10^5\ M^{-1}\ s^{-1}$. For homoisocitrate, the kinetic constants were $K_m = 18.3\ \mu M$, $k_{cat} = 13.7\ s^{-1}$, and $k_{cat}/K_m = 7.49 \times 10^5\ M^{-1}\ s^{-1}$. Thus, the enzyme used the two substrates at near equal efficiency. The enzyme preferentially used the coenzyme NAD^+ over $NADP^+$ as predicted from the sequence [3,10,11]. The kinetic constants were determined to be $K_m = 77.1\ \mu M$, $k_{cat} = 21.8\ s^{-1}$, and $k_{cat}/K_m = 2.83 \times 10^5\ M^{-1}\ s^{-1}$.

Discussion

Unlike many organisms that have two or three β-decarboxylating dehydrogenases, *P. horikoshii* has only one, PH1722, encoded in its genome [12]. Because the organism is a tryptophan auxotroph but not a leucine-, glutamate- or lysine-auxotroph [7], an interesting possibility is that PH1722 plays multiple roles in a single pathway of amino acid biosynthesis. I cloned and sequenced the gene (adding 66 bases to the known 5′-region of PH1722), and expressed the protein in *E. coli*. Using a conventional pET system, high-level expression was achieved. However, virtually all of the PH1722 protein formed inclusion bodies. Its solubility was not improved by lowering the growth temperature and/or the concentration of isopropyl-β-D-thiogalactopyranoside, by altering the induction timing and/or host strains, or by adding chaperone plasmid.

I found that after addition of detergent to the sample, followed by dialysis, the protein efficiently refolded into an active form, which was used for kinetic analysis of the substrate specificity. The enzyme was found to be bifunctional for homoisocitrate and isocitrate. HICDH from *T. thermophilus* is known to be another bifunctional β-decarboxylating dehydrogenase [13]. However, that enzyme uses isocitrate ($k_{cat}/K_m = 4.2 \times 10^5\ M^{-1}\ s^{-1}$) far more efficiently than homoisocitrate ($k_{cat}/K_m = 2.3 \times 10^4\ M^{-1}\ s^{-1}$). Although the assay conditions are different, PH1722 had catalytic efficiencies, k_{cat}/K_m, comparable to the higher of those of HICDH from *T. thermophilus*. Therefore, although enzymes with broad specificities are usually less efficient than more specialized proteins and thus tend to be missing links, bifunctional isocitrate–homoisocitrate dehydrogenase is efficient enough to allow *P. horikoshii* to use a single enzyme instead of two. As for the other HICDHs, enzymes from the yeasts *Schizosaccharomyces pombe* [3] and *Saccharomyces cerevisiae* [13], and an archaeon, *Methanococcus jannaschii* [14], are all homoisocitrate-specific. Thus, substrate specificities of tentatively named HICDHs vary greatly depending on the source organism. Non-biased bifunctional isocitrate–homoisocitrate dehydrogenase is clearly a novel member of the β-decarboxylating dehydrogenase family. I here propose that *P. horikoshii* PH1722 be referred to as an isocitrate–homoisocitrate dehydrogenase and that it be classified under a new EC number. Although the substrate specificity of PH1722 is broad, the enzyme does not possess IPMDH activity. Therefore, *P. horikoshii* would likely not use the common leucine biosynthetic pathway (i.e., 2-isopropylmalate synthetase—2-isopropylmalate isomerase—3-isopropylmalate dehydrogenase as in Fig. 1): some other pathway may exist which remains unclear. In *T. thermophilus*, because there are three specific biosynthetic pathways for leucine, glutamate, and lysine, it is not necessary for the HICDH to play a dual role. I suggest that its dual specificity may simply be an intrinsic enzymatic property of no physiological consequence.

Recently, Yasutake et al. [15] reported the crystal structure of PH1724 from *P. horikoshii* and found that, unlike other homocitrate-specific enzymes, it has a unique structure in the region that recognizes the γ-moiety of the substrate. The authors considered it possible that the difference is related to substrate specificity; they speculated the enzyme to be dually specific for 2-isopropylmalate and homocitrate. In an experiment that helped to clarify its functions, Lombo et al. [16] carried out an in vivo complementation test of a LysU-deficient mutant of *T. thermophilus*. They were successful in growing the strain in the absence of lysine in minimal medium by supplying it with the PH1724 gene, demonstrating that PH1724 has homoaconitase activity and is involved in lysine biosynthesis. As yet we have no experimental evidence that the enzyme recognizes 2-isopropylmalate as a second substrate. However, because PH1722, which belongs to the same gene cluster as PH1724, lacks IPMDH activity and can be considered not to function in leucine biosynthesis, it is reasonable to predict that PH1724 is active toward citrate as well as homocitrate, but not to 2-isopro-

pylmalate. In any case, it will be worthwhile to experimentally determine the substrate specificity of PH1724.

Some enzymes possessing broad substrate specificities have been reported, many of which are from hyperthermophilic archaea: β-glucosidase/β-mannosidase from *Pyrococcus furiosus* [17], carbamoyl phosphate synthetase from *P. furiosus* [18], aromatic aminotransferase from *P. horikoshii* [19], and carboxypeptidase/aminoacylase from *P. horikoshii* [20]. In these organisms, the genome sizes are smaller (e.g., 1.74 Mbp for *P. horikoshii* and 1.9 Mbp for *P. furiosus*) than in common bacteria (e.g., 4.6 Mbp for *E. coli*). It is reasonable that one protein has multiple tasks, limiting the number of genes yet catalyzing the same reactions as the multiple enzymes of organisms with larger genomes. In any case, these proteins can be regarded as ancestral enzymes and PH1722 is the first example discovered in central metabolism.

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