Ornithine cyclodeaminase/μ-crystallin homolog from the hyperthermophilic archaeon *Thermococcus litoralis* functions as a novel Δ¹-pyrroline-2-carboxyate reductase involved in putative trans-3-hydroxy-ι-proline metabolism

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**A B S T R A C T**

ι-Ornithine cyclodeaminase (OCD) is involved in ι-proline biosynthesis and catalyzes the unique deaminating cyclization of ι-ornithine to ι-proline via a Λ¹-pyrroline-2-carboxyate (Pyr2C) intermediate. Although this pathway functions in only a few bacteria, many archaea possess OCD-like genes (proteins), among which only AF1665 protein (gene) from *Archaeoglobus fulgidus* has been characterized as an NAD⁺-dependent ι-alanine dehydrogenase (AfAlaDH). However, the physiological role of OCD-like proteins from archaea has been unclear. Recently, we revealed that Pyr2C reductase, involved in trans-3-hydroxy-ι-proline (T3LHyp) metabolism of bacteria, belongs to the OCD protein superfamily and catalyzes only the reduction of Pyr2C to ι-proline (no OCD activity) [FEBS Open Bio (2014) 4, 240–250]. In this study, based on bioinformatics analysis, we assumed that the OCD-like gene from *Thermococcus litoralis* DSM 5473 is related to T3LHyp and/or proline metabolism (TlLhpI). Interestingly, TlLhpI showed three different enzymatic activities: AlaDH; N-methyl-ι-ala- nine dehydrogenase; Pyr2C reductase. Kinetic analysis suggested strongly that Pyr2C is the preferred substrate. In spite of their similar activity, TlLhpI had a poor phylogenetic relationship to the bacterial and mammalian reductases for Pyr2C and formed a close but distinct subfamily to AfAlaDH, indicating convergent evolution. Introduction of several specific amino acid residues for OCD and/or AfAlaDH by site-directed mutagenesis had marked effects on both AlaDH and Pyr2C reductase activities. The OCU_00387 gene, clustered with the TlLhpI gene on the genome, encoded T3LHyp dehydratase, homologous to the bacterial and mammalian enzymes. To our knowledge, this is the first report of T3LHyp metabolism from archaea.

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**A R T I C L E  H I S T O R Y**

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**1. Introduction**

ι-Proline plays many important roles in the protein structure and cell signaling and is synthesized within cells and organisms from an intermediate, glutamate γ-semialdehyde, commonly involved in biosynthesis pathways from ι-glutamate and ι-arginine: ι-glutamate → γ-glutamyl phosphate → glutamate γ-semialdehyde (Route 1); ι-arginine → ι-ornithine → glutamate γ-semialdehyde (Route 2) (Fig. 1A). The glutamate γ-semialdehyde then undergoes “non-enzymatic cyclization” to give the imine, Δ¹-pyrroline-5-carboxyrate (Pyr5C), which is subsequently converted to ι-proline by NAD(P)H-dependent Pyr5C reductase (EC 1.5.1.2) [1]. Route 1 is the main mechanism of ι-proline biosynthesis in bacteria, while eukaryotes use this route predominantly under stress and limited nitrogen conditions. Under high nitrogen input, Route 2 appears to be prominent and is mainly found in higher plants.

Alternatively, ι-proline is also directly synthesized from the amino acid ι-ornithine, itself an intermediate along the above Route 2, by ι-ornithine cyclodeaminase (OCD; EC 4.3.1.12) (Route 3) [2]. This unique enzyme initially catalyzes NAD⁺-dependent oxidative deamination from the α-amino group of ι-ornithine, and subsequently cyclization to form Δ¹-pyrroline-2-carboxyrate (Pyr2C) (Fig. 1B). The actual cyclization step in this pathway occurs with the participation of enzymes, in contrast to glutamate γ-semialdehyde → Pyr5C in Routes 1 and 2, described above. The final

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**Abbreviations:** OCD, ornithine cyclodeaminase; CRYM, μ-crystallin; AlaDH, ι-alanine dehydrogenase; Pyr2C, Λ¹-pyrroline-2-carboxyate; T3LHyp, trans-3-hydroxy-ι-proline; NMAAlaDH, N-methyl-ι-alanine dehydrogenase

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Fig. 1. (A) Pathways of L-proline biosynthesis. (B) Proposed hydrolytic (Scheme I) and non-hydrolytic schemes (Scheme II) for the conversion of L-ornithine to L-proline by OCD [9]. Step 6 corresponds to the reaction by Pyr2C reductase. Asterisks indicate putative intermediates. (C) T3LHyp pathway and the metabolic network with D-proline. (D) Schematic gene clusters related to T3LHyp and/or proline metabolism of archaea (A) and bacteria (B). Putative genes in the box were purified and characterized in this study (see (E)). TllHlp1 gene was fused with OCC_00362 and OCC_00367 genes (Supplementary Methods). C–C and C–T indicate a pair of catalytic amino acid residues of proline racemase superfamily enzymes (see Fig. S1B). Gray putative genes are sequentially similar to other (amino acid) transporters. (E) Purification of recombinant His6 tag proteins. Five micrograms each of purified protein were applied to 12% (w/v) gel (also (F)). (F) Purification of recombinant His6 tag TllHlp1 mutants.
step by OCD is NADH-dependent reduction of Pyr2C to l-proline. OCD belongs to the ornithine cyclodeaminase/μ-crystallin (OCD/CRYM) superfamily (see Fig. 4). It was recently reported that μ-crystallin (CRYM) from mammals, known as an NADP(H)-dependent T3 thyroid hormone (triiodothyronin)-binding protein without enzymatic catalysis, functions as an NAD(P)H-dependent ketimine reductase (EC 1.5.1.25) [3]. Pyr2C is one of the substrates of this enzyme.

Although Route 3 of l-proline biosynthesis is found in several bacteria, including Pseudomonas putida [2], Agrobacterium tumefaciens [4], Clostridium sporogenes [5], and Sinorhizobium melloti [6], many archaea possess OCD/CRYM family genes (proteins) (20–40% sequence identity with bacterial OCD). Among them, only AF1665 protein (gene) from the hyperthermophilic archaeon Archaeoglobus fulgidus has been characterized at the molecular level, and the protein functions as an L-alanine dehydrogenase like protein including AfAlaDH has been unclear in archaea. This gene was clustered with T3LHyp dehydratase and proline racemase genes involved in putative T3LHyp and/or proline metabolism. Molecular evolution of the OCD/CRYM family is also discussed.

2. Results

2.1. Candidates of target genes

Although it is unclear whether there are archaea capable of metabolizing T3LHyp as a sole carbon source, a homology search by the Protein-BLAST program was carried out against genome sequences of archaea using bacterial Pyr2C reductases (LhpH from Azospirillum brasilense (AbLhpI), GenBank: BA021622; LhpH from Colwellia psychrerythraea 34H (CpLhpI), GenBank: YP_268197) as the probe protein sequences. As described below, bacterial Pyr2C reductases are classified into two different subfamilies, in which CpLhpH and AbLhpI are contained, respectively (see Fig. 4). However, over 900 homologous genes (proteins) with sequence identities of 20–40% were found (data not shown). Similar results was also obtained when other OCD/CRYM family proteins including AfAlaDH was used instead of LhpH protein as the probe.

On the other hand, when bacterial T3LHyp dehydratases (LhpH from A. brasilense (AbLhpI), GenBank: BA021621; LhpH from C. psychrerythraea 34H (CpLhpI), GenBank: YP_268195) was used as the probe, 31 homologous genes were found. T3LHyp dehydratase belongs to the proline racemase superfamily, which also includes the archetypal proline racemase (EC 5.1.1.4) and T4LHyp epimerase (EC 5.1.1.8) [15,17]. In contrast to bacterial Pyr2C reductases, T3LHyp dehydratase form a single subclass in this superfamily. On the basis of two specific residues at the active sites, the members have been classified into three types: Cys-Cys type (proline racemase and T4LHyp epimerase); Cys-Thr type (T3LHyp dehydratase); Ser-Cys type (function unknown). Furthermore, two Cys-Cys type enzymes can be clearly distinguished in the phylogenetic tree. Based on this insight, we could select only three proline racemase-like genes as putative T3LHyp dehydratases, although it has been believed that the enzyme is found only in animals and fungi (and bacteria) [13,15]: T. litoralis DSM 5473 (OCC_00387, GenBank: KP_008428263; 45.6% identity with AbLhpI), Thermococcus sibiricus MM 739 (TSIB_0630, GenBank: KP_002994044; 47.1%), and Candidatus Korarchaeum cryptofilum OPFR (OPFR Kcr_0803, GenBank: KP_001737233; 44.1%) (Fig. 1). Interestingly, these genes were clustered with OCD-like genes: T. litoralis (OCC_00362→KP_008428257; 44.4% identity with AbLhpI), Thermococcus sibiricus MM 739 (TSIB_0630, GenBank: KP_002994044; 47.1%), and Candidatus Korarchaeum cryptofilum OPFR (OPFR Kcr_0803, GenBank: KP_001737233) (Fig. 1). In the cases of T. litoralis and T. sibiricus, another proline racemase-like gene (Cys-Cys type) and genes encoding two different types of putative heterooligomeric l-proline dehydrogenase (l-ProDH) [18,19] were also contained in this gene cluster. This analysis strongly indicated that the gene cluster is related to T3LHyp and/or proline metabolism. Therefore, in this study, we selected GenBank: OCC_00362, GenBank: OCC_00367, and GenBank: OCC_00387 genes from T. litoralis as target genes (referred to as TilHlpH genes, respectively).

2.2. Preparation of recombinant His₆-tag proteins

TilHlpH gene was fused with OCC_00362 and OCC_00367 genes by PCR (Supplementary Methods). After cloning all target genes into the vector pETDuet-1, the recombinant enzymes with attached His₆-tags at their N-termini were expressed in Escherichia coli and purified with an Ni²⁺-chelating affinity column (Fig. 1E). Apparent molecular masses of TilHlpH and TilHlpH, estimated by SDS–PAGE, were 40 (36.9) and 43 (38.1) kDa (values in parentheses indicate the calculated molecular mass of the enzyme with His₆-tag), and those estimated by analytic gel filtration were 72 and 75 kDa, respectively (data not shown). Therefore, the two enzymes appear to be dimeric.
2.3. Characterization of TILhpl as AlaDH

Although TILhpl shows only 44.3% sequence identity with AfAlaDH, we could detect both activities of the NAD+-dependent oxidative deamination of L-alanine and NADH-dependent reductive amination of pyruvate (in presence of 700 mM NH₄⁺): specific activities of 0.0265 and 4.43 (unit/mg protein), respectively. NAD⁺ was not used as a coenzyme. The optimum pH values of each reaction were 10–12 and 6–10.5, respectively (Fig. 2A). The kₑ/kₘ values for pyruvate for NH₄⁺/C₂ and NAD⁺/C₂ were 10–12 and 6–10.5, respectively (Fig. 2A). The kₑ/kₘ value for pyruvate for NH₄⁺/C₂ was 230-fold higher than that for L-alanine, caused by the ~120-fold lower kₘ value: namely, the reaction equilibrium favors the direction toward reductive amination (Tables 1 and 2). Activity was also observed with several L-amino acids and 2-oxo acids with a hydrophobic side chain (see below for L-proline). Among them, L-2-aminovalerolate and 2-oxovalerolate with a C₃ aliphatic side chain were the best substrates, and the kₑ/kₘ values were 146- and 4.7-fold higher than those for L-alanine and pyruvate, respectively. When pyruvate and NADH were alternatively incubated together with methylamine (50 mM) instead of NH₄⁺ as the nitrogen donor, reduction activity was detected. This result indicated that TILhpl functions not only as AlaDH but also N-methyl-L-alanine dehydrogenase (NMAlaDH; EC 1.4.1.17). Activities of AlaDH and NMAlaDH were also detected in HPLC analysis using the reaction products and zymogram staining analysis (Figs. 2B and 3A, B). kₑ/kₘ values for L-alanine and pyruvate of TILhpl were over 1000-fold lower than those of AfAlaDH [7]. Furthermore, the kₘ value for NH₄⁺/C₂ of TILhpl was clearly beyond the physiological level (Table 2). AfAlaDH shows similar optimum pH of the oxidative and reductive reactions (~7.0), and no significant activity for hydrophobic amino acids including L-2-aminovalerate.

![Graphs showing enzymatic properties of TILhpl Pyr2C and AbLhpI](image)

Fig. 2. Enzymatic properties of TILhpl Pyr2C. (A) Effect of pH on the activity as AlaDH (left panel) and Pyr2C reductase (right panel). 50 mM acetate-NaOH (pH 4.0–6.0) (square), 50 mM potassium phosphate (pH 6.0–8.5) (circle), and 50 mM Tris–HCl (pH 7.0–9.0) (triangle) for the reduction of pyruvate or Pyr2C (left axis and black symbol), and 50 mM Bis-tris propane (diamond) for the oxidation of L-alanine or L-proline (right axis and gray symbol), instead of 50 mM buffer under each standard assay condition. (B) Zymogram staining. Five micrograms each of purified protein was applied on 10% (w/v) gel. After electrophoresis, the gel was soaked at 50 °C (for TILhpl) or 30 °C (for AbLhpI) in staining solution in the presence of the indicated substrate and NAD⁺ (each of 10 mM). Protein stained with Coomassie Brilliant Blue (CBB) was used as a loading control. (C) Comparison of NADH-dependent AlaDH and Pyr2C reductase activities of wild-type and mutated TILhpl proteins. The assay was performed with standard assay solutions containing the indicated substrate (10 mM). AbLhpI and CpLhpI were assayed at 30 °C under the standard assay conditions for TILhpl. Data for AfAlaDH are from Ref. [7].
Pyruvate + NH₄Cl + NADH → 2-Oxobutyrate + NH₄Cl + NADH

**Table 1**

| Enzymes   | Substrates          | Specific activity (units/mg protein) | Kᵣ (mM) | kₑ (min⁻¹) | kₑ/Kᵣ (min⁻¹ mM⁻¹) |
|-----------|---------------------|-------------------------------------|---------|-------------|---------------------|
| Wild type | i-Alanine           | 0.0265 ± 0.0015                     | 18.2 ± 5.0 | 2.64 ± 0.55 | 0.147 ± 0.012       |
|           | i-2-Aminobutyrate   | 0.136 ± 0.010                       | 6.00 ± 0.29 | 6.71 ± 0.16 | 1.12 ± 0.03        |
|           | i-2-Aminovalerate   | 0.396 ± 0.030                       | 0.306 ± 0.009 | 6.56 ± 0.08 | 21.5 ± 0.4         |
|           | Leucine             | 0.248 ± 0.010                       | 1.32 ± 0.10 | 10.5 ± 0.4  | 8.00 ± 0.28        |
|           | N-Methyl-L-alanine  | 0.526 ± 0.025                       | 0.406 ± 0.044 | 11.6 ± 0.7  | 28.6 ± 2.1         |
|           | i-Proline           | 0.384 ± 0.015 (0.0376 ± 0.0005)ᵇ    | 1.12 ± 0.12 (13.1 ± 1.2)ᵇ | 13.8 ± 1.0 (2.27 ± 0.24)ᵇ | 12.3 ± 0.4 (0.250 ± 0.006)ᵇ |
|           | i-Pipicolate        | 0.219 ± 0.020                       | 0.608 ± 0.115 | 12.2 ± 1.3  | 20.3 ± 1.8         |
| V224D/A228K | i-Alanine         | 0.0133 ± 0.00010                   | 36.9 ± 0.9  | 2.12 ± 0.02  | 0.0575 ± 0.0008    |
|           | i-2-Aminovalerate   | 0.666 ± 0.050                       | 2.13 ± 0.30 | 7.72 ± 0.82 | 3.64 ± 0.12        |
|           | i-Proline           | 1.51 ± 0.10                         | 0.444 ± 0.008 | 36.5 ± 0.9  | 82.2 ± 1.1         |

ᵇ Under standard assay conditions in “Experimental procedures”. All substrate concentrations were 10 mM.

**Table 2**

| Enzymes   | Substrates          | Specific activity (units/mg protein) | Kᵣ (mM) | kₑ (min⁻¹) | kₑ/Kᵣ (min⁻¹ mM⁻¹) |
|-----------|---------------------|-------------------------------------|---------|-------------|---------------------|
| Wild type | Pyruvate            | 4.43 ± 0.50                         | 9.37 ± 0.23 | 315 ± 4     | 33.6 ± 0.4         |
|           | Oxalacetate         | 1.78 ± 0.18                         | 8.86 ± 0.91 | 192 ± 15 | 21.7 ± 0.5        |
|           | 2-Oxobutyrate       | 2.46 ± 0.25                         | 0.701 ± 0.020 | 92.0 ± 1.8 | 131 ± 1           |
|           | 2-Oxovalerate       | 2.59 ± 0.21                         | 0.969 ± 0.093 | 152 ± 11 | 157 ± 3           |
|           | 4-Methyl-2-oxovalerate | 1.02 ± 0.19                     | 2.52 ± 0.45 | 129 ± 18 | 51.4 ± 2.0        |
|           | NH₄⁺(+2-oxovalerate)ᵇ | 2.45 ± 0.22                     | 1450 ± 240 | 267 ± 33 | 0.183 ± 0.007    |
|           | Pyr2C               | 23.9 ± 2.5                         | 0.944 ± 0.090 | 995 ± 33 | 1058 ± 66        |
| V224D/A228K | Pyruvate            | 1.60 ± 0.15                         | 62.0 ± 12.2 | 413 ± 75 | 6.68 ± 0.11       |
|           | 2-Oxovalerate       | 7.00 ± 0.95                         | 1.82 ± 0.14 | 212 ± 16 | 116 ± 0          |
|           | Pyr2C               | 23.9 ± 35                          | 2.77 ± 0.44 | 33500 ± 4900 | 12900 ± 300     |

ᵇ Under standard assay conditions in “Experimental procedures”. All substrate concentrations were 10 mM.

**Fig. 3.** HPLC analysis of the reaction products. (A) Reaction product from pyruvate or 2-oxobutyrate by TlLhp1 (as AlaDH) in presence of NADH and NH₄Cl. Control indicates authentic i-alanine and i-2-aminobutyrate. (B) Reaction product from pyruvate by TlLhp1 (as NMAlaDH) in the presence of NADH and methylamine. Control indicates authentic N-methyl-L-alanine. (C) Reaction product from i-proline by TlLhp1 (as L-ProDH) in the presence of NAD⁺. After the enzymatic reaction, the product was treated by H₂O₂, and then analyzed. Control indicates authentic L-glutamate and γ-aminobutyrate (GABA), which are potentially yielded from Pyr5C and Pyr2C, respectively. (D) Reaction products from Pyr2C by TlLhp1 and from T3LHyp by TlLhpH and TlLhpI, in the presence of NADH. Control indicates authentic T3LHyp and L-proline. No peak corresponding to Pyr2C was observed, probably caused by no reaction with labeling reagent for amino acid analysis (data not shown).
(2-oxovalerate). This indicated strongly that TILHpl possesses largely different properties from AfAldh, and that the physiological function is not the metabolism of l-alanine and pyruvate.

2.4. Characterization of TILHpl as novel Pyr2C reductase in OCD/CRYM superfamily

In contrast to AfAldh [7], TILHpl showed NAD+-dependent oxidation activity for l-proline. The \( k_{cat}/K_{m} \) value in the presence of NADP\(^+\) was \(~50\)-fold lower than that in the presence of NAD\(^+\) (Table 1): the specificity for NAD\(^+\) is described above. In addition to l-proline, several l-proline analogs, including cis-4-hydroxy-l-proline (85%), trans-4-hydroxy-l-proline (70%), and l-pipecolate (49%), were active substrates (relative activity to l-proline in parentheses), whereas it is known that bacterial LhpL proteins are strictly specific to l-proline [13]. These activities were also detected in zymogram staining analysis (Fig. 2B). When the reaction product of l-proline was further oxidized by \(^2\)-O2, 4-aminobutyrate (GABA) was identified in HPLC analysis (Fig. 3C), indicating that TILHpl catalyzes the conversion of l-proline to Pyr2C. Generally, l-ProDH (EC 1.5.99.8) including archaeal enzymes [18,19] is a flavoprotein, and the reaction product is Pyr5C (if so, l-glutamate is yielded by the above \(^2\)-O2 treatment).

Therefore, we compared the dehydrogenation activity toward l-proline and other l-amino acids (Tables 1 and 2). The \( k_{cat}/K_{m} \) value for Pyr2C (1058 min\(^{-1}\) mM\(^{-1}\)) was 6.7-fold higher than that for 2-oxovalerate, caused by the high \( k_{cat} \) value. Furthermore, the ratio of Pyr2C to l-proline in \( k_{cat}/K_{m} \) was 86, suggesting the preference of the reaction equilibrium in the direction toward NADH-dependent reduction. Interestingly, the optimum pH of Pyr2C reductase was 4.0–6.0, clearly different from that of the reductive deamination of AlaDH (pH 6–10.5) (Fig. 2A). These results strongly indicated that Pyr2C is the preferred substrate for TILHpl. Although AbLhpL and CplHpl showed only 25.3% and 31.6% sequence identity to TILHpl, respectively, we unexpectedly detected AlaDH activity in both enzymes (Fig. 3C). Commonly, these bacterial enzymes showed significantly high ratios of Pyr2C to pyruvate in specific activity: 176 and 28, respectively (~5 for TILHpl). Namely, TILHpl possessed “vestigial” properties to AlaDH, probably due to the close phylogenetic relationship between them, as described in the main section.

Using the reductase activity toward Pyr2C, several properties of TILHpl as an enzyme from “hyperthermophiles” were identified: T. litoralis grows between 55 and 98 °C with an optimal growth temperature of 88 °C [20]. Although the optimum temperature was above 75 °C, and clearly higher than that of (mesophilic) AbLhpL, the assay could not be performed above 75 °C due to the thermolability of NADH (Fig. 5A). Thus, TILHpl appears to be a typical (hyper)thermophilic enzyme.

2.5. Phylogenetic analysis of TILHpl

As expected from the preliminary annotation, TILHpl belongs to the OCD/CRYM superfamily, including the archetype OCD (subfamilies 7 and 11) [2,21], CRYM/ketimine reductase (subfamily 5) [3], AlaDH (subfamily 2) [7,8], l-arginine dehydrogenase (subfamily 10) [22], l-lysine cyclodeaminase (EC 4.3.1.28; subfamily 8) [23], tauropine and strombine dehydrogenases (EC 1.5.1.23/22; subfamily 3) [24,25], l-2,3-diaminopropionate synthase (subfamily 4) [26], and Pyr2C reductase (from bacteria) (subfamilies 6 and 9) [13] (Fig. 4). TILHpl had a poor relationship to the bacterial LhpL and mammalian CRYM proteins, in spite of their similar activity, and formed a close but distinct subfamily to many archaeal OCD-like proteins, including AfAldh (novel subfamily 1). Surprisingly, two OCD-like proteins from “thermophilic bacteria”, Thermosdminibacter oceani (Toce_0647) and Thermovigina lieii (Tlie_1581), were closely related to TILHpl rather than (bacterial) AbLhpL and CplHpl, suggesting the possibility of horizontal gene transfer between bacteria and archaea.

Putative amino acid sequences of TILHpl contained essential amino acid residues for coenzyme binding (Rossmann-fold motif consisting of Gly-X-Gly-X-[Ala/Ser], where X indicates any amino acid) and the catalytic tetrad for interacting (and/or binding) with carboxyl and amino groups of substrate ([Arg/Lys]-Lys-Arg-Asp); Gly\(^{134}\)-Ala-Gly-Val-Gln-Ala\(^{139}\) and Arg\(^{35}\)-Lys\(^{67}\)-Arg\(^{110}\)-Asp\(^{103}\), respectively [2,8] (Fig. 5). Furthermore, TILHpl possessed a specific aspartate residue (Asp\(^{156}\)) for NAD\(^+(H)\)-dependent enzymes of the OCD/CRYM superfamily [2,7,23–26], conforming to the coenzyme specificity, described above.

2.6. Identification of catalytic amino acid residues

It is proposed that three amino acid residues of OCD, ES6-D228-K232 (numbering from P. putida), are related to the unique cyclization of l-ornithine [2] (Fig. 5). In TILHpl and AfAldh, these amino acid residues correspond to L52-V224-A228 and R52-D219-K223, respectively (conserved amino acid residues are underlined). To obtain insight into the catalytic mechanism of OCD/CRYM superfamily enzymes, we constructed three mutants of TILHpl, V224D/A228K (DK mutant), L52E/V224D/A228K (EDK mutant), and L52R/V224D/A228K (RDK mutant). They were overexpressed in E. coli cells as a His\(^6\)-tagged enzyme and purified with the same procedures as for the wild-type enzyme (Fig. 1F). Approximately 12-fold enhancement of Pyr2C reductase activity (6.7-fold for the oxidation activity for l-proline), but not AlaDH activity, was found in the DK mutant, caused by the increasing \( k_{cat} \) value (Tables 1 and 2). On the other hand, when the mutations were combined with L52E and L52R, AlaDH activities for the 2-oxo acids were completely eliminated, whereas Pyr2C reductase activity was the same as in the wild type (Fig. 2C).

2.7. Characterization of TILHplH

Potential T3LHyp dehydratase activity in TILHplH was initially assayed spectrophotometrically in the coupling system with TILHpl (in the presence of NADH). A significant decrease in absorbance at 340 nm was observed as a result of cooperative reactions by TILHplH (T3LHyp → Pyr2C) and TILHpl (Pyr2C → l-proline); 44.6 unit/mg protein of specific activity with T3LHyp. \( K_{m} \) and \( k_{cat} \) values for T3LHyp were 0.288 mM and 1710 min\(^{-1}\), respectively, comparable with those of AbLhpH (data not shown). On the other hand, optimum temperature for the activity was 80–100 °C, which was significantly higher than that of (mesophilic) AbLhpH (40–45 °C) (Fig. 5B). These results indicated that the TILHplH gene encodes T3LHyp dehydratase with enzymatic properties as a typical (hyper)thermophilic enzyme: this is the first example for archaea.

2.8. Physiological meaning of TILHpl and TILHplH

Although a minimal medium for the cultivation of T. litoralis is not available, we found that T. litoralis can grow in the complex medium [27] supplemented with l-proline, p-proline or T3LHyp as a sole carbon source, instead of maltose and peptone (data not shown). On the other hand, Pyr2C reductase and T3LHyp dehydratase activities in cell-free extract prepared from T. litoralis cells grown on such as carbon source were similar to those on maltose and peptone. These suggested one possibility that similar metabolic pathway of T3LHyp to bacteria and mammalian is operative in T. litoralis.

3. Discussion

The discovery of archaean reductase for Pyr2C revealed that OCD/CRYM superfamily enzymes with this activity are large:
subfamilies of 1, 5, 6, 7, 9, and 11 at least (Fig. 4). Furthermore, TlLhpI (and AbLhpI and CpLhpI) possesses not only Pyr2C reductase activity but also (slight but significantly detectable) AlaDH and NMAlaDH activities. Among OCD/CRYM members, enzymatic reactions by subfamilies 2 and 10 are homologous to AlaDH, and enzymes of subfamilies 3 and 4 catalyze similar reactions to NMAlaDH, in which taurine, glycine, and L-2,3-diaminopropionate are used as the nitrogen donor instead of NH₄⁺, respectively [24–26]. Furthermore, cyclodeaminases for L-ornithine and L-lysine possess the same initial step of the catalytic reaction as AlaDH and NMAlaDH (step 1 in Fig. 1B). This suggests that the origin of L-ProDH (Pyr2C reductase) activity (step 6) is the same as step 1. A common ancestor possessed NAD(P)⁺-dependent dehydrogenation activity toward broad primary and secondary amines, and (strict) substrate specificity convergently evolved in bacteria, mammalians, and archaea. If this hypothesis is true, TlLhpI acquired catalytic specificity as Pyr2C reductase later than AbLhpI and CpLhpI, because the relative high activity of AlaDH and broad substrate specificity of L-ProDH remain (Fig. 2B and C).

Although it is unclear whether OCD possesses (remaining) oxidative deamination activity for L-amino acid(s) except L-ornithine, the “potential” mechanism to produce Pyr2C (from L-ornithine) by TlLhpI corresponds to scheme I, because of the similarity to AfAlaDH, as described above. On the other hand, Glu⁶⁸ of OCD favorably interacts with the ε-amino group of L-ornithine. In CRYM, it is also proposed that Gly⁶⁰ at the equivalent position (numbering from mouse) is important for the binding of a large ligand, T3, because of the small side chain [28]. These insights explain why the substitution of Leu⁵² in hydrophilic glutamate or arginine in TlLhpI leads to a complete loss of reductive amination activity for hydrophobic 2-oxo acid substrates (Fig. 3C).

As in our previous proposal [13], dehydratase activity with T3LHyp in the proline racemase superfamily was acquired once at an early evolutional stage, because TlLhpH also forms a single subfamily together with enzymes from bacteria and mammalians (Fig. S1). Several (thermophilic) archaea and bacteria possess another proline racemase-like gene instead of the LhpH gene within the gene cluster containing the LhpI gene (Fig. 1D). The enzymatic function may be interconversion of L- and D-isomer of proline (and/or hydroxyproline), but not dehydration of T3LHyp: Pyr2C is potentially produced from D-proline by FAD-dependent D-ProDH (Fig. 1C). TlLhpI (this study; Fig. 2B) and L-ProDH (a4b4) [29] show activities not only for Pyr2C and L-proline but also Δ¹-piperidine-2-carboxylate and L-pipecolate, respectively, which are involved in the so-called “Δ¹-pipecolate pathway” of L-lysine metabolism [16]. Putative L-ProDH genes are also found within the gene cluster. Furthermore, (putative) pyruvate-formate lyase (PFL) and serine/threonine dehydratase genes, producing pyruvate and/or 2-oxobutyrate, are often located within the flanking region of the LhpI gene (Fig. 1D). These indicate the possibility that the TlLhpI gene (and the gene cluster) is also involved not only in T3LHyp but also in Δ¹-proline, L-proline and L-lysine metabolism, and several 2-oxo...
acids. Although *T. litoralis* can grow on T3LHyp as a sole carbon source, Pyr2C reductase (and T3LHyp dehydratase) activity is not significantly induced in cell-free extract, in contrast of bacteria [13], conforming to the putative functions in multiple metabolism described above. Development of gene disruption of *T. litoralis* would be useful for understanding more detailed physiological role(s) of *TlLhpI* gene.

4. Experimental procedures

4.1. General procedures

Basic recombinant DNA techniques were performed as described by Sambrook et al. [30]. PCR was carried out using a GeneAmp PCR System 2700 (Applied Biosystems) for 30 cycles in a 50 l reaction mixture containing 1 U of KOD FX DNA polymerase (TOYOBO), appropriate primers (15 pmol) and template DNA under the following conditions: denaturation at 98°C for 10 s, annealing at 50°C for 30 s and extension at 68°C for the time periods calculated at an extension rate of 1 kbp min⁻¹. DNA sequencing was carried out using the BigDye Cycle Sequencing Kit ver.3.1 (Applied Biosystems) and appropriate primers with the Genetic Analyzer 3130 (Applied Biosystems). Protein concentrations were determined by the method of Lowry et al. [31] with bovine serum albumin as the standard. SDS–PAGE was performed as described by Laemmli [32].

4.2. Strain and growth conditions

*T. litoralis* DSM5473 were grown under anaerobic conditions at 80°C in a complex medium, as described previously [27], in the absence of sulfur. If necessary, maltose, peptone and yeast extract were omitted, and L-proline, D-proline or T3LHyp (30 mM) was added alternatively.

4.3. Plasmid construction for expression of recombinant proteins

Primer sequences used in this study are shown in Table S1. In this report, the prefixes *Tl* (*T. litoralis*), *Af* (*A. fulgidus*), *Ab* (*A. brasilense*) and *Cp* (*C. psychrerythraea*) have been added to gene symbols or protein designations when required for clarity. *TlLhpI* (fusion of OCC_00362 and OCC_00367; see Supplementary Methods) and *TlLhpH* genes (OCC_00387) were amplified by PCR using primers containing appropriate restriction enzyme sites at the 5' and 3' ends and genome DNA of *T. litoralis* as a template. Each amplified DNA fragment was introduced into BamHI-PstI sites in pETDuet-1 (Novagen), a plasmid vector for conferring N-terminal His6-tag on expressed proteins, to obtain pET/TlLhpIWT and pET/TlLhpH.

4.4. Expression and purification of His6-tagged recombinant proteins

Basic recombinant DNA techniques were performed as described by Sambrook et al. [30]. PCR was carried out using a GeneAmp PCR System 2700 (Applied Biosystems) for 30 cycles in a 50 l reaction mixture containing 1 U of KOD FX DNA polymerase (TOYOBO), appropriate primers (15 pmol) and template DNA under the following conditions: denaturation at 98°C for 10 s, annealing at 50°C for 30 s and extension at 68°C for the time periods calculated at an extension rate of 1 kbp min⁻¹. DNA sequencing was carried out using the BigDye Cycle Sequencing Kit ver.3.1 (Applied Biosystems) and appropriate primers with the Genetic Analyzer 3130 (Applied Biosystems). Protein concentrations were determined by the method of Lowry et al. [31] with bovine serum albumin as the standard. SDS–PAGE was performed as described by Laemmli [32].

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4.5. Enzyme assay

All enzyme assays were performed at 50 °C unless otherwise indicated.

Pyr2C reductase was assayed routinely in the direction of Pyr2C reduction by measuring the oxidation of NADH at 340 nm, using a Shimadzu UV-1800 spectrophotometer (Shimadzu GLC Ltd., Tokyo, Japan). The standard assay mixture contained 10 mM Pyr2C in 50 mM potassium phosphate (pH 6.0) buffer. The reactions were started by the addition of 100 µl of a 1.5 mM NADH solution to a final volume of 1 ml. To assay the reverse reaction, the reaction mixture consisted of 50 mM Bis–tris propane (pH 10.5) and 10 mM l-proline. The reaction was started by the addition of 15 mM NAD+ (100 µl) with a final reaction volume of 1 ml. One unit of enzyme activity refers to 1 µmol NADH produced/min. Pyr2C was enzymatically synthesized from T3LHyp, described previously [13].

AlaDH activity was measured as oxidative deamination of l-alanine and as reductive amination of pyruvate. For the oxidative deamination assay, the standard assay mixture contained 50 mM Bis–tris propane (pH 10.5), 10 mM l-alanine, and 1.5 mM NAD+. The reductive amination assay mixture consisted of 50 mM Tris–HCl (pH 8.0), 700 mM NH4Cl, 10 mM pyruvate, and 0.15 mM NADH. For the assay of NMAAlaDH activity, 50 mM methylamine was used instead of 700 mM NH4Cl. T3LHyp dehydratase was assayed spectrophotometrically in the coupling system with TILhpI. The reaction mixture consisted of 50 mM Tris–HCl (pH 8.0), 0.15 mM NADH, and 10 µg purified TILhpI. The reaction was started by the addition of 100 mM T3LHyp (100 µl) with a final reaction volume of 1 ml. The enzyme was alternatively assayed by the colorimetric method based on the reaction of 2-aminobenzaldehyde with Pyr2C, which yields a yellow reaction product [15]. This method was used to determine the optimum temperature for activity.

4.6. Reaction product analysis

Purified TILhpI (10 µg) was added to the following four mixtures (1 ml): for AlaDH activity, 50 mM Tris–HCl (pH 8.0) buffer containing 10 mM pyruvate (or 2-oxobutyrate), 500 mM NH4Cl, and 10 mM NADH; for NMAAlaDH activity, 50 mM Tris–HCl (pH 8.0) buffer containing 10 mM pyruvate, 100 mM methylamine, and 10 mM NADH; for i-ProDH activity; 50 mM Bis–tris propane (pH 10.5) buffer containing 10 mM l-proline and 10 mM NAD+; for T3LHyp dehydratase activity of TILhpI; 50 mM Tris–HCl (pH 8.0) buffer containing 10 mM T3LHyp, 10 mM NADH, and TILhpI (10 µg). After incubation at 50 °C overnight, each enzyme product was then analyzed by a Hitachi L-8900 amino acid analyzer (Tokyo, Japan), using ion exchange chromatography followed by post-column derivatization with ninhydrin. In the case of i-ProDH activity, the product was further oxidized by the addition of 30% H2O2 (20 µl) to the mixture before HPLC analysis [33].

4.7. Zymogram staining analysis

Purified TILhpI was separated at 4 °C on non-denaturing PAGE with 10% (w/v) gel, which was performed by omitting SDS and 2-mercaptoethanol from the solution used in SDS–PAGE. The gels were then soaked in 10 ml staining solution consisting of 50 mM Bis–tris propane (pH 10), 0.25 mM nitroblue tetrazolium (NBT), 0.06 mM phenazine methosulfate (PMS), 10 mM substrate, and 10 mM NAD+ at 50 °C for 5 min in the dark. Dehydrogenase activity appeared as a dark blue band.

4.8. Site-directed mutagenesis

The mutation in TILhpI was introduced by sequential steps of PCR [34] using sense and antisense primers (Table S1) and pET/TILhpI (for V224D/A228K (DK) mutant) or pET/TILhpI(dk) (for L52E/V224D/A228K (EDK) and L52R/V224D/A228K (RDK) mutants) as a template. Briefly, the codons used for single mutants were as follows: L52E, CTC → AGG; L52R, CTC → CAG; V224D, CTC → GAC; A228K, GCC → AAG. The region of the mutated genes was confirmed by subsequent sequencing in both directions. Mutant proteins were expressed and purified by the same procedures as for the wild-type enzyme.

4.9. Amino acid sequence alignment and phylogenetic analysis

Protein sequences were analyzed using the Protein-BLAST and Clustal W program distributed by DDBJ (DNA Data Bank of Japan (www.ddbj.nig.ac.jp)). Multiple sequence alignment was performed with the following default parameters: Protein Weight Matrix, Gonnet; GAP OPEN, 10; GAP EXTENSION, 0.20; GAP DISTANCES, 5; NO END GAPS, no; ITERATION, none; NUMITER, 1 and CLUSTERING, NJ. The phylogenetic tree was constructed by the neighbor-joining method [35]. Bootstrap resampling was performed 1000 times.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febio.2014.07.005.

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