論 文 の 内 容 の 要 旨

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論文題目

Studies on function and evolution of β-decarboxylating dehydrogenase

（β 脱炭酸型脱水素酵素の機能および進化に関する研究）

Introduction

Homoisocitrate dehydrogenase (HICDH) catalyzes the fourth step reaction in the novel α-aminoadipate (AAA) lysine biosynthetic pathway; together with the paralogous enzymes, isocitrate dehydrogenase (ICDH) and 3-isopropylmalate dehydrogenase (IPMDH), are called three brothers forming the divergent β-decarboxylating dehydrogenase family. HICDH is involved in lysine biosynthesis, while ICDH and IPMDH are involved in the TCA cycle and leucine biosynthesis, respectively. Each β-decarboxylating dehydrogenase should have evolved from a common ancestor, which possesses broad substrate specificity to catalyze the various metabolic reactions in the early stage of life. The broad substrate specificity and evolutionary relationship of β-decarboxylating dehydrogenase provide several interesting implications regarding the overall process of gene family evolution by gene duplication and functional divergence from ancestral genes.

1. Characterization of β-decarboxylating dehydrogenases from various organisms

Phylogenetic analyses from various organisms have shown the enzymes in β-decarboxylating dehydrogenase family can be separated into three groups: IPMDH group; ICDH group; and ICDH/IPMDH/HICDH-mixed group. Characterization of ICDH and IPMDH from *Escherichia coli* and *Thermus thermophilus* has shown that ICDH and IPMDH strictly discriminate their substrates from each other; however, HICDH activity is detectable for most enzymes. This suggests that HICDH activity may be retained as an evolutionary leftover in substrate-specific ICDH or IPMDH of bacteria and archaea. The existence of HICDH activity in ICDH or IPMDH from various organisms suggests that the common ancestor of β-decarboxylating dehydrogenase would have HICDH activity.

2. Evolutionary analysis of chemically synthesized common ancestor of β-decarboxylating dehydrogenase

Phylogenetic and molecular evolutionary analyses were conducted for ICDH, IPMDH and HICDH from a large set of microorganisms. To examine the catalytic feature of ancestral-type β-decarboxylating dehydrogenase, we aligned amino acid sequence of ICDHs, IPMDHs and all the β-decarboxylating dehydrogenases, and designed ancestral-type ICDH, IPMDH, and β-decarboxylating dehydrogenases, each with the ancestral-type sequence inferred by maximum likelihood method. The genes for the ancestral enzymes of ICDH group, IPMDH group and the common ancestor of all three groups were chemically synthesized and expressed in *E.coli* cells. Although the ancient-type IPMDH was accumulated as inclusion bodies in *E.coli* cells, other two proteins were produced in soluble fractions. ICDH ancestor did not show activity for isocitrate, 3-isopropylmalate, nor homoisocitrate; however, the chemically synthesized common ancestor of all three groups exhibited distinct enzymatic activity for 3-isopropylmalate using NAD^+ as a coenzyme. Furthermore, trace amount of 2-oxoglutarate was detected by overnight reaction with the ancestral-type enzyme using $NADP^+$ as a coenzyme, although activity detection by monitoring an increase in NADH was impossible due to extremely low activity. These results suggest that ancient β-decarboxylating dehydrogenase possessed dual functions at least. Complete loss of activity for homoisocitrate might suggest that a large loop to accommodate larger γ-moiety of homoisocitrate in the substrate-binding pocket was removed in the common ancestral-type enzyme during amino acid sequence alignment.

3. Characterization of β-decarboxylating dehydrogenase homolog (TK0280) from *T. kodakarensis*

The hyperthermophilic archaeon *Thermococcus kodakarensis* is suggested to synthesize lysine through the AAA pathway starting from 2-oxoglutarate as the initial compound. Interestingly, *T. kodakarensis* has only a single set of the lysine biosynthetic gene cluster carrying *TK0280* gene, but has no other enzymes (gene clusters) specific to the leucine, glutamate and arginine biosynthesis. Therefore, I proposed that *T. kodakarensis* has an ancient-type multi-functional metabolic pathway that can produce not only lysine but also leucine, glutamate, and arginine. The characterization of the chemically synthesized ancient-type enzymes could not provide fully convincing evidence for presenting the ancestral feature of broad substrate specificity (Chapter 2); however, phylogenetic analyses suggest that TK0280 is in the ICDH/IPMDH/HICDH-mixed group and present close to the root of phylogenetic tree for β-decarboxylating dehydrogenase from various microorganisms, which suggests that TK0280 would exhibit all the activities for the substrates: homoisocitrate, isocitrate and 3-isopropylmalate.

The *TK0280* gene was cloned and overexpressed in *E. coli* and catalytic properties and subunits organization were investigated. TK0280 is a tetramer composed of four identical subunits, each with a molecular weight of about 42 kDa. TK0280 exhibits distinct activity for homoisocitrate, isocitrate, and 3-isopropylmalate (Fig. 1). This result indicates that TK0280 is a promiscuous enzyme that can recognize several related compounds as substrates, and also suggests that TK0280 is potentially involved in the synthesis of lysine, leucine and glutamate in *T. kodakarensis*. The patchwork hypothesis in the evolution of metabolic pathways implicates that the ancestral enzymes should possess broad substrate specificity and be involved in multi-metabolic pathways. Thus, the results above indicate that TK0280 is an ancestral-type enzyme and may be very close to the common ancestor of β-decarboxylating dehydrogenase.

4. Site-directed mutagenesis of TK0280

In HICDH from *T. thermophilus*, it is known that Arg85 is a key determinant for the substrate recognition, due to the fact that the single substitution of Arg85 altered its substrate specificity. According to the crystal structure of HICDH from *T. thermophilus*, Arg85 is located in the substrate-binding pocket and suggested to interact with the γ-carboxylate of homoisocitrate. The pairwise amino acid sequence alignment with *T. thermophilus* HICDH shows that, Leu83 is at the equivalent position of Arg85 of *T. thermophilus* HICDH. To further elucidate the key residues related to the substrate

specificity of TK0280, site-directed mutagenesis was conducted for Leu83.

Enzymatic analysis using 3-isopropylmalate, isocitrate, and homoisocitrate revealed that the mutant L83S shows narrow substrate specificity (Fig. 1) with increased turnover number for the reaction using homoisocitrate as the substrate. Although the catalytic efficiency, k_{cat}/K_m, was decreased due to the increased K_m value for homoisocitrate, the substrate preference of homoisocitrate to isocitrate was enhanced from ~30 fold to ~100 fold. The results above further suggest that the specificity is determined by a limited number of amino acid residues in TK0280 and that TK0280 is designed to exhibit broad substrate specificity.

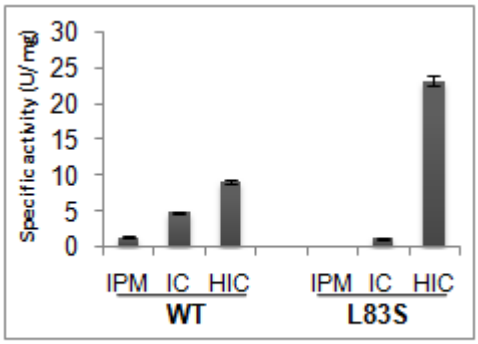

Figure 1. Substrate specificity of WT and L83S

Conclusion

TK0280 from hyperthermophilic archaeon *T. kodakarensis*, the fourth enzyme involved in AAA lysine biosynthetic pathway, possesses features of ancestral-type enzyme with broad substrate specificity and may serve for multi-metabolic pathways, which is in line with the patchwork hypothesis on the evolution of metabolic pathways. The mutation at Leu83 of TK0280 indicates that Leu83 is one of key determinants for the substrate specificity in conferring promiscuous activity. This study could provide valuable insights on the evolution of β-decarboxylating dehydrogenase family and also give a clue to the evolution of lysine, leucine and other amino acid biosynthetic pathways.