論文の内容の要旨

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論文題目

Nitrogen assimilation of *Hydrogenobacter thermophilus* TK-6
（*Hydrogenobacter thermophilus* TK-6 の窒素同化代謝）

Nitrogen is a primary element that is indispensable for life to constitute its body, as well as carbon, hydrogen, and oxygen, and its assimilation is an important central metabolism in all organisms.

Hydrogenobacter thermophilus TK-6 is a thermophilic, hydrogen-oxidizing, obligately autotrophic bacterium. Analysis of the 16S rRNA sequence suggested that *Hydrogenobacter* species are located on the deepest branch in *Bacteria* along with other *Aquificales* species. Biochemical studies on *H. thermophilus* have brought a number of novel findings. One of them is the reductive TCA cycle, a unique central metabolism in which carbon dioxide is assimilated. It was demonstrated that reduced ferredoxin is used as an electron donor in this cycle.

In contrast to the detailed studies on the carbon anabolism, the nitrogen anabolism has not been investigated in *H. thermophilus*. The objective of this study was to elucidate the nitrogen assimilatory metabolism in this bacterium. The metabolic pathways in which ammonium or nitrate is assimilated as the nitrogen source were estimated, and individual enzymes were purified and characterized in this study (Fig. 1).

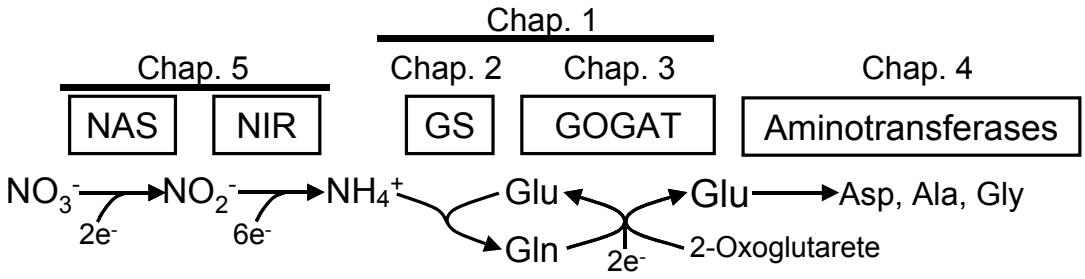

Fig. 1. Nitrogen assimilatory pathway in *H. thermophilus*. Chapter numbers indicate in which chapter each pathway or enzyme was examined.

Chapter 1. Screening for ammonium-assimilating enzymes

In Chapter 1, I screened for enzyme activities that can serve to assimilate ammonium, using the crude extract of *H. thermophilus*. In most bacteria, it is known that ammonium is assimilated by glutamate dehydrogenase (GDH) or a coupling reaction of glutamine synthetase (GS) and glutamate

synthase (GOGAT) as shown in Fig. 2. GDH activity was not detected in *H. thermophilus*. While GS activity was detected in the crude extract, NADPH-dependent GOGAT activity, which is a common type of GOGAT in non-photosynthetic bacteria, was not detected. Several possible activities that are alternative to GDH or GOGAT were tested, such as amino acid dehydrogenases and glutamine amidotransferase. However, none of them was detected. Unexpectedly, ferredoxin-dependent GOGAT (Fd-GOGAT) activity was detected. Fd-GOGAT has been found only in cyanobacteria and plants, but not in non-photosynthetic bacteria. This aroused interests in the GS-GOGAT pathway of *H. thermophilus*, and therefore each enzyme was purified and characterized in Chapter 2 and Chapter 3.

GDH: $NH_3 + 2\text{-OG} + 2e^- \leftrightarrow Glu$

GS: $NH_3 + Glu + ATP \rightarrow Gln + ADP + Pi$

GOGAT: $Gln + 2\text{-OG} + 2e^- \rightarrow 2\times Glu$

GS-GOGAT: $NH_3 + 2\text{-OG} + 2e^- + ATP \rightarrow Glu + ADP + Pi$

Fig. 2. GDH, GS, and GOGAT reactions. 2-OG, 2-oxoglutarate.

Chapter 2. Glutamine synthetase

In Chapter 2, GS was purified from *H. thermophilus* and enzymatically characterized. Purified GS was a homomultimer (probably a homododecamer) of a 55 kDa polypeptide. The GS gene was identified and its primary sequence was homologous to those of GSI-β, one of the GS families. Kinetic parameters were determined, and they were comparable to those of known GS, except for the high K_m value for Glu. It was demonstrated that *H. thermophilus* GS is subjected to a posttranscriptional modification, an adenylyl/deadenylyl mechanism to regulate its activity. Some GSI-βs were known to be regulated by this modification, but it was not clear when this mechanism evolved. The existence of this regulation in *H. thermophilus* suggests that the adenylylating regulation originated before the divergence of the *Aquificales* from other bacteria.

Chapter 3. Glutamate synthase

In Chapter 3, Fd-GOGAT was purified from *H. thermophilus* and enzymatically characterized. GOGAT is classified according to its specificity for the electron donor. Fd-GOGAT had been found only in plants and cyanobacteria, whereas the other bacteria have NADPH-dependent GOGAT.

The purified enzyme from *H. thermophilus* was shown to be a monomer of a 168 kDa polypeptide homologous to Fd-GOGATs from phototrophs. In contrast to known Fd-GOGATs, the *H. thermophilus* GOGAT exhibited glutaminase activity. Furthermore, ferredoxin specificities of this enzyme were examined by using Fd1, Fd2, and Fd3, ferredoxins from *H. thermophilus*. Consequently, *H. thermophilus* GOGAT did not react with Fd3, a plant-type ferredoxin containing a [2Fe-2S] cluster, but with Fd1 and Fd2, bacterial-type ferredoxins containing [4Fe-4S] clusters. Interestingly, the *H. thermophilus* GOGAT was activated by some of the organic acids in the reductive TCA cycle, the central carbon metabolic pathway of this organism. This type of activation has not been reported for any other GOGATs, and this property may enable the control of nitrogen assimilation by carbon metabolism. In the study of this chapter, it was clearly demonstrated that *Hydrogenobacter thermophilus*, a hydrogen-oxidizing chemoautotrophic bacterium, possess Fd-GOGAT like phototrophs. This was the first observation of an Fd-GOGAT in a non-photosynthetic organism to my knowledge.

Chapter 4. Aminotransferases

In many organisms, aminotransferase is know to serve in the synthesis and the catabolism of most amino acids, transferring the amino group of the amino acid into the 2-oxo acid. Studies in Chapter 2 and Chapter 3 indicated that the GS-GOGAT pathway assimilates ammonium into Glu in *H. thermophilus*. To verify Glu can be used for a nitrogen donor for other metabolites synthesis, aminotransferases from *H. thermophilus* were examined in Chapter 4.

Aminotransferase activities in the crude extract were assayed using Glu, Asp, Ala, Gly, and their corresponding 2-oxo acids as substrates. Consequently, the following four activities were detected (Fig. 3): glutamate:oxaloacetate aminotransferase (GOT), glutamate:pyruvate aminotransferase (GPT), glutamate:glyoxylate aminotransferase (GGT), and alanine:glyoxylate aminotransferase (AGT). In attempt to purify the enzymes with these activities, three aminotransferases, AT1, AT2, and AT3, were purified from *H. thermophilus*. It was shown that GOT, GPT, and AGT activities were derived from AT1, AT2, and AT3, respectively. AT1 and AT2 also had GGT activity. Kinetic parameters suggested that these three enzymes were enough efficient to serve as an aminotransferase. Interestingly, phylogenetic analysis showed that AT2 and AT3 were phylogenetically located at unusual positions when compared with known aminotransferases.

Study in this chapter demonstrated that several amino acids can be synthesized in *H. thermophilus* using Glu as an nitrogen donor.

GOT: Glu + oxaloacetate ↔ 2-OG + Asp (**AT1**)
GPT: Glu + pyruvate → 2-OG + Ala (**AT1** & **AT2**)
GGT: Glu + glyoxylate → 2-OG + Gly (**AT2**)
AGT: Ala + glyoxylate → pyruvate + Gly (**AT3**)

Fig. 3. Aminotransferase reactions catalyzed by AT1, AT2, and AT3.

Chapter 5. Nitrate and nitrite reductases

While studies in Chapter 2, 3, and 4 elucidated the metabolism where ammonium is assimilated into several amino acids, it remained unknown how nitrate was assimilated as the nitrogen source. To solve this question, nitrate- or nitrite-reducing enzymes were investigated in Chapter 5. Like GOGAT, assimilatory nitrate reductase (NAS) and nitrite reductase (NIR) are classified on the basis of their electron donor. Bacterial ferredoxin-dependent NAS and NIR were reported only in cyanobacteria with a few exceptions of the other bacteria.

Ferredoxin-dependent NAS activity was detected in the crude extract of *H. thermophilus*, while no NAD(P)H-dependent NAS activity was detected. An enzyme possessing this activity was purified, and it exhibited NAS activity using Fd1 as the electron donor. This clearly demonstrated that *H. thermophilus* has a ferredoxin-dependent NAS, which is homologous to known enzymes. In the upstream region of this NAS gene (*nasB*), a NIR-like gene (*nirB*) was found (Fig. 4). NirB harbored the "ferredoxin-binding site", which is conserved in genes encoding ferredoxin-dependent NIR. *nirB* was cloned into a pET-21c or pUC19 vector, and heterologously expressed in *E. coli*. Soluble fractions of the recombinants contained the ferredoxin-dependent NIR activity, and the recombinant protein was purified. The purified protein showed a ferredoxin-dependent NIR activity using Fd1 as the electron donor. However, this protein lacked the N-terminal region of NirB because its translation started at the

GTG codon that is located downstream of the expected initial codon, and it can not be excluded at present that NirB in its native form has different enzymatic properties.

Studies in this chapter indicated that a ferredoxin-dependent enzyme is involved in the nitrate reduction of *H. thermophilus*, and suggested that ferredoxin might also be used in the nitrite reduction.

Fig. 4. Physical map of the *nasB* and *nirB* gene cluster in the *H. thermophilus* genome.

Conclusions

This study elucidated nitrogen assimilatory pathways in *H. thermophilus*. Further, detailed analyses of each enzyme provided several novel findings, such as distinctive enzymatic properties and unexpected distributions of some metabolic features in *H. thermophilus*. Reaction pathways revealed in this study are conserved among other bacteria. This supports the speculation that the fundamental framework of the nitrogen anabolism was established before the divergence of organisms. In contrast to reaction pathways, some of enzymatic properties or electron donors are shown to be different from those of many bacteria. It could be argued that individual enzymes continued to evolve after the divergence, adapting to the physiological environment of each organism.

One of the characteristics of nitrogen anabolism in *H. thermophilus* is the deep involvement of ferredoxin (Fig. 5), which is involved also in the carbon anabolism of this bacterium, as is the case with cyanobacteria. This stimulates further interests in redox metabolisms of *H. thermophilus*.

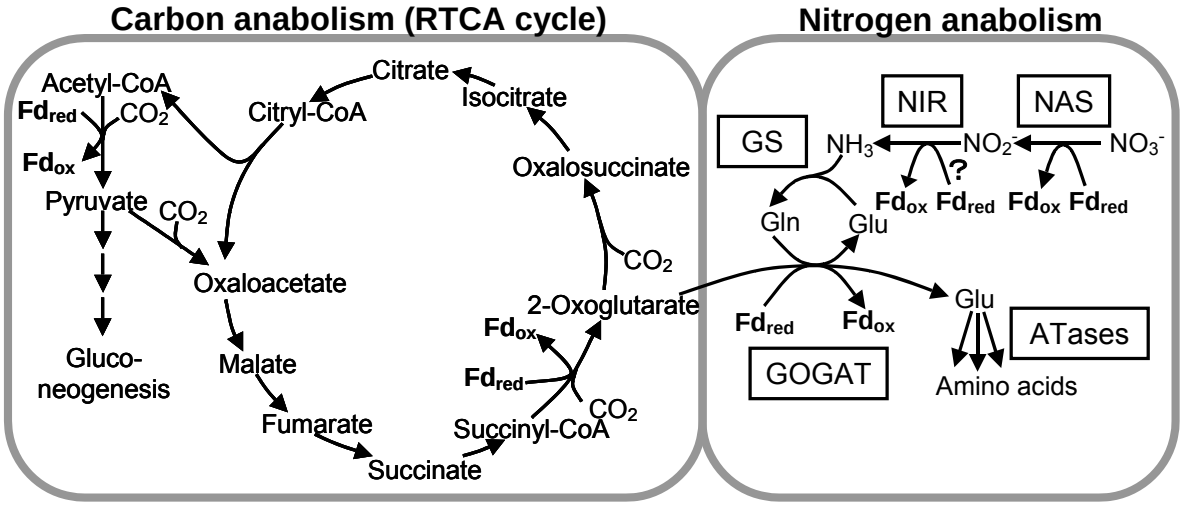

Fig. 5. Nitrogen and carbon anabolisms in *H. thermophilus*. ATases, aminotransferases; Fd_{ox}, oxidized ferredoxin; Fd_{red}, reduced ferredoxin.