（第３号様式）(Form No. 3)

学 位 論 文 要 旨
Dissertation Abstract

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学位論文題目：
Title of Dissertation

Study on crystal structures of two enzymes involved in degradation pathway I for pyridoxine in *Mesorhizobium loti*
（根粒菌が持つピリドキシン分解経路Ｉを構成する２種類の酵素の立体構造解析に関する研究）

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Introduction

Two different but related degradation pathways for pyridoxine, a free form of vitamin B_6, exist in bacteria that can use pyridoxine as a sole source of carbon and nitrogen. Degradation pathway I has been identified in *Pseudomonas sp.* MA-1 and *Microbacterium luteolum* YK-1. In this pathway, pyridoxine is degraded through eight enzyme-catalyzed steps. In degradation pathway II, which has been found in *Pseudomonas sp.* 1A and *Arthrobacter sp.* Cr-7, pyridoxine is degraded through five enzymatic steps. Recently, pathway I was discovered in a nitrogen-fixing symbiotic bacterium *Mesorhizobium loti* MAFF303099 and the genes encoding the enzymes identified. The genes occur as a cluster on chromosome. Pyridoxine 4-oxidase (PNOX) is the first-step enzyme in the pathway and is encoded by the gene *mll6785* while 5-formyl-3-hydroxy-2-methylpyridine 4-carboxylic acid (FHMPC) 5-dehydrogenase is the fifth enzyme and encoded by the gene *mlr*6793.

Crystal structure of Pyridoxine 4-oxidase

PNOX from *M. loti* is a monomeric, Glucose-methanol-choline (GMC) oxidoreductase family enzyme. It catalyzes flavin adenine denucleotide (FAD) dependent oxidation of pyridoxine into pyridoxal.

In this study, PNOX with a His_6-tag was prepared. The *mll6785* gene encoding PNOX on a plasmid pET-mll6785 was recloned to pTrc99A to obtain pTrc99A-mll6785, which contained His_6-tag on the C-terminus. *Escherichia coli* JM109 cells were co-transformed with pTrc99A-mll6785 and plasmid pKY206 carrying groEL/ES chaperonin genes. PNOX enzyme was purified with a Ni-NTA agarose column and QA52 column chromatographies. Crystallization was done by the sitting-drop

vapour-diffusion method at 277 K and the structure was solved by molecular replacement. The crystal structures of PNOX and PNOX with its substrate analogue pyridoxamine (PM) were determined at 2.2 Å and at 2.1 Å resolutions, respectively.

The overall structure consisted of FAD-binding and substrate-binding domains. PNOX had an opening socket for access of substrates. The opening was followed by a bottleneck and a tunnel. In the active site, His460, His462, and Pro504 were located on the re-face of the isoalloxazine ring of FAD. PM binds to the active site through several hydrogen bonds. The side chains of His462 and His460 are located at 2.7 and 3.1 Å from the N4′ atom of PM. The activities of H460A and H462A mutants of PNOX were very low, and the H460A/H462A double mutant exhibited no activity. His462 may act as a general base for abstraction of a proton from the 4′-hydroxyl of pyridoxine. The comparison of active site residues in GMC oxidoreductase shows that Pro504 in PNOX corresponds to Asn or His of the conserved His-Asn or His-His active site pair in other GMC oxidoreductases.

Crystal structure of 5-formyl-3-hydroxy-2-methylpyridine 4-carboxylic acid 5-dehydrogenase, an NAD^+- dependent dismutase

FHMPC dehydrogenase is an NAD^+ dependent dismutase. It catalyzes a dismutation reaction: the oxidation of FHMPC to 3-hydroxy-2-methylpyridine 4, 5 dicarboxylic acid (HMPDC) with NAD^+ and reduction of FHMPC to 4-pyridoxic acid with NADH. FHMPC dehydrogenase belongs to the L-3-hydroxyacyl-CoA dehydrogenase (HAD) family.

In this study, the plasmid designated as pET21a-mlr6793 was introduced into BL21 (DE3) cells and the cells were grown at 37 ºC. The enzyme was homogeneously purified by QA52 and Blue A column chromatographies. FHMPC dehydrogenase was crystallized by the sitting drop vapor diffusion method and the structure was determined by molecular replacement.

The crystal structure was refined to a resolution of 1.55 Å (R-factor of 16.4%, R_{free} = 19.4%). There were two monomers in the asymmetric unit. The overall structure of monomer consisted of N- and C-terminal domains connected by a short linker loop. The active site was located between the domains and highly conserved to that of human heart HAD (HhHAD). His-Glu catalytic dyad, a serine, and two asparagine residues of HhHAD were conserved.

Ser116, His137 and Glu149 in FHMPC dehydrogenase are connected by a hydrogen bonding network forming a catalytic triad. $N^{\delta 2}$ of Asn188 and O^{γ} of Ser116 are within hydrogen bond distance to wat577 at 3.1 and 2.9 Å, respectively. Wat577 occupies the putative substrate bind position. Asn140 is located near the nicotinamide ring of NAD^+. Thus, Ser116, His137, Glu149, Asn188 and Asn140 are important catalytic residues in FHMPC dehydrogenase.