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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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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)

学位論文要約 :
Dissertation Summary

GENERAL INTRODUCTION

Vitamin B_6 consists of six forms in nature, *i.e.* pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), pyridoxine 5′ phosphate (PNP), pyridoxal 5′ phosphate (PLP) and pyridoxamine 5′ phosphate (PMP). Two different but related degradation pathways of free forms of vitamin B_6 (PN, PL, and PM) have been identified in bacteria that can use PN as sole sources 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 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.

Here, the tertiary structures of PNOX and FHMPC dehydrogenase, (Mugo *et al*., 2013; Mugo *et al*., 2014) were determined.

CRYSTAL STRUCTURE OF PYRIDOXINE-4-OXIDASE
FROM *MESORHIZOBIUM LOTI*

1. INTRODUCTION

Pyridoxine 4-oxidase (PNOX: EC 1.1.3.12) is the first enzyme in degradation pathway I for vitamin B_6 (Burg *et al.,* 1960). PNOX containing FAD catalyzes the oxidation of pyridoxine (PN) to pyridoxal (PL), as shown in scheme 1.1.
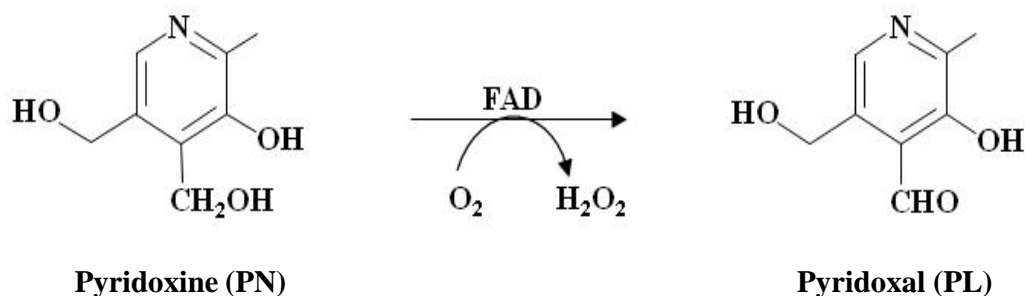
Scheme 1.1. Reaction catalyzed by PNOX.

PNOX shows activity only toward PN, but no reactivity toward pyridoxine 5′-phosphate (PNP), which is a precursor of pyridoxal 5′-phosphate (PLP), a coenzyme form of vitamin B_6. In contrast, an FMN-dependent PNP [pyridoxamine 5′-phosphate (PMP)] oxidase shows similar activities towards PNP and PMP to produce PLP (Choi *et al.,* 1983), which is a coenzyme form of vitamin B_6. It has been shown that HS on C4′ in PNP or PMP is transferred to N5 of FMN as the hydride ion in the catalysis of the PNP (PMP) oxidase (PNPOX) (Di Salvo *et al.,* 2002). In contrast, the reaction mechanism of PNOX has not been elucidated. It is interesting to find out the oxidation mechanism of PNOX, which makes the selective activity towards PN.

In this study, the tertiary structure of PNOX was determined by molecular replacement, and the roles of amino acid residues located in the active site of PNOX were examined by means of site-directed mutagenesis.

2. MATERIALS AND METHODS
2.1. Overexpression and purification of pyridoxine 4-oxidase
PNOX with a His_6-tag was prepared and used for crystallization. *E. coli* JM109 cells were co-transformed with pTrc99A-mll6785 containing a His_6-tag and plasmid pKY206 carrying chaperonin genes (Mizobata *et al.,* 1992). The transformed cells were grown in LB medium at 23 $^{\circ}$C. Purification was done by Ni-NTA agarose column (QIAGEN) and QA52 column (Whatman) chromatographies. The purified enzyme solution was dialyzed at 4 $^{\circ}$C against the crystallization buffer.

2.2. Crystallization and x-ray diffraction
The purified enzyme was concentrated and crystallized by the sitting-drop vapour-diffusion method. The diffraction data were collected at the BL38B1 station of SPring-8 (Hyogo, Japan). The structure of PNOX was solved by molecular replacement using the MOLREP program (Vagin *et al*., 2010). The atomic coordinates of PNOX and the PNOX-PM complex were deposited in the PDB under accession codes 3T37 and 4HA6, respectively.

3. RESULTS AND DISCUSSION
3.1. Amino acid sequence comparison with other GMC oxidoreductase family members
The identities of PNOX with choline oxidase (CLOX, PDB code: 2JBV) from *A. globiformis* (Finnegan *et al.,* 2010), aryl alcohol oxidase (AAO, 3FIM) from *Pleurotus eryngii* (Fernandez *et al.,* 2007), and glucose oxidase (GOX, 1CF3) from *Aspergillus niger* (Wohlfahrt *et al.,* 1999) were 30%, 28%, and 27%, respectively. One histidine residue (His462, His466, His502, and His526 in PNOX, CLOX, AAO, and GOX, respectively) is conserved.

3.2. Quality of the model of PNOX and PNOX-PM complex
The structure of PNOX and PNOX-PM were refined to R factor of 18.5 % (R_{free} = 21.9 %) at 2.2 Å

resolution, and of 18.8 % (R_{free} = 23.2%) at 2.1 Å, respectively. There is one subunit in the asymmetric unit of PNOX. PNOX consists of two domains: an FAD-binding domain and a substrate-binding domain, as shown in Fig 1.1.
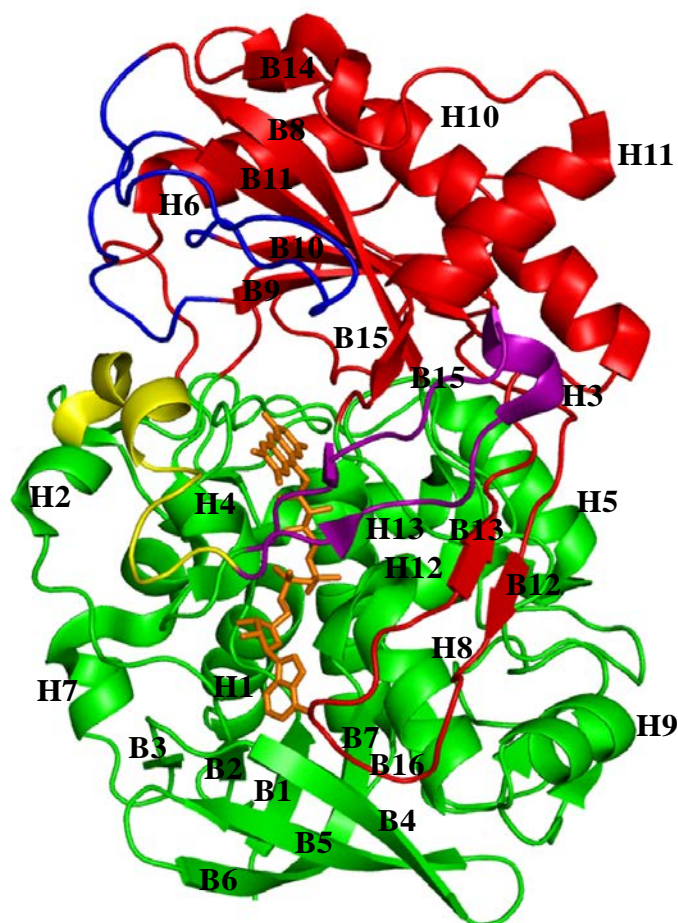

Figure 1.1. Tertiary structure of PNOX. Cartoon view of PNOX. The substrate binding-domain is colored red; the FAD-binding domain green; and FAD orange. Secondary structure elements, α-helix and β-sheet, are shown as H and B, respectively. The residues forming the entrance socket are colored yellow (residues 53-71), purple (72-93), and blue (320-330 and 355-370).

3.3. The environment of FAD

The FAD molecule is non-covalently bound to PNOX. The FAD molecule in the FAD-binding domain is in an extended conformation with the adenine and isoalloxazine moieties distal to each other: the planar isoalloxazine moiety lies in the active site cavity, which lies at the interface of the two domains.

3.4. Access of substrates to the active site

The surface structure of the PNOX molecule shows that it has an entrance socket. Because part of the residues (60-73 and 324-327) in the entrance socket show high temperature factors, they are flexible and may serve as a lid to prevent the access of extra substrate to the active site, and to allow exit of the product, as reported for some GMC family enzymes (Quaye *et al.,* 2008; Hallberg *et al.,* 2002; Yue *et al.,* 1999).

3.5. Histidine residues in the active sites of PNOX and CLOX

The overall structures of PNOX and the Ser101Ala mutant of CLOX were quite similar. His462 and His466 in PNOX and CLOX, respectively, completely overlap. Pro504 in PNOX occupies almost the same spatial position as that of Asn510 in CLOX. CLOX has catalytic residue, His351, which is not found in PNOX. However, PNOX contains a corresponding residue, His460, which is not found in CLOX. Among the GMC oxidoreductase family members; a pair of residues, either His and Asn or His and His, which is necessary for catalysis, is found in the active site (Hallberg *et al.,* 2002). PNOX is the first member that contains Proline at the spatial position corresponding to the Asn or His.

3.6. Binding mode of PM in the active site

PM binds to the active site of PNOX through several hydrogen bonds. The side chains of His460 and His462 are located at 2.7 and 3.1 Å, respectively, from the N-4' atom of PM, and at ~5 and ~6 Å, respectively, from the N5 atom of FAD. Thus, His462 can act as a general base for abstraction of a proton from the 4'-hydroxyl of PN. His460 may play important roles in the binding and positioning of PN for hydride ion transfer.

3.7. Site-directed mutagenesis

The catalytic efficiencies ($k$cat/Km) of the H460A and H462A mutant PNOXs were only 0.5% and 1.2%, respectively, of that of the wild-type PNOX. The double mutant PNOX showed no activity. These results confirm that His460 together with His462 plays an essential role in the catalysis of PNOX

### 3.8. Reaction mechanism of PNOX and PNP oxidase

The PNP (PMP) oxidase oxidizes both PNP and PMP. In contrast, PNOX cannot oxidize PM. The difference in the substrate specificity can be attributed to the difference in the arrangement of catalytic residues in the active site. The results suggest that the mutant PNOX with a high activity towards PNP would show no activity towards PMP.

CRYSTAL STRUCTURE OF 5-FORMYL-3-HYDROXY-2-METHYLPYRIDINE 4-CARBOXYLIC ACID 5-DEHYDROGENASE, AN NAD$^+$-DEPENDENT DISMUTASE FROM *MESORHIZOBIUM LOTI*

1. INTRODUCTION

FHMPC dehydrogenase participates in degradation pathway I for pyridoxine, (Burg *et al*., 1960; Yuan *et al,.* 2004). FHMPC dehydrogenase catalyzes the oxidation of 5-formyl-3-hydroxy-2-methylpyridine 4-carboxylic acid (FHMPC) to 3-hydroxy-2-methylpyridine 4, 5-dicarboxylic acid (HMPDC) with NAD$^+$ and the reduction of FHMPC to 4-pyridoxic acid with NADH as a hydrogen donor (Yokochi *et al.,* 2009; Lee *et al.,* 1986) as shown in scheme 2.1.
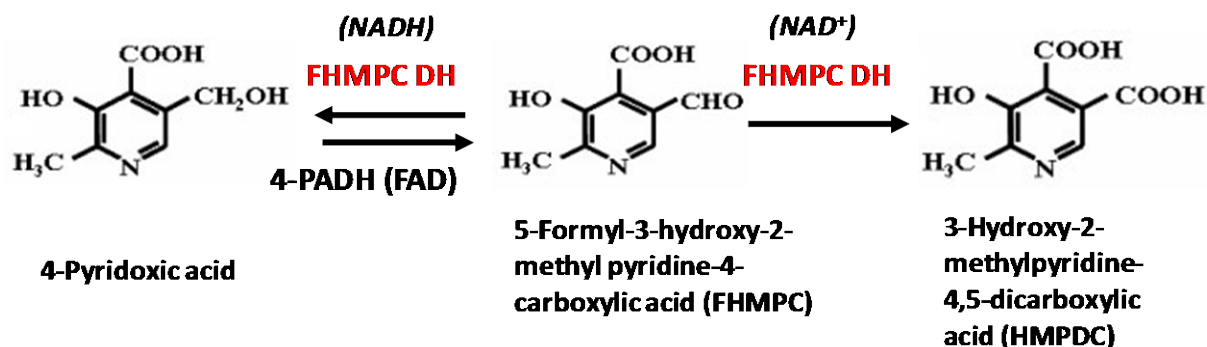
Scheme 2.1. Reaction catalyzed by FHMPC dehydrogenase. The enzyme catalyzes oxidation of FHMPC to HMPDC with NAD$^+$ and reduction of FHMPC to 4-pyridoxic acid with NADH.

The enzyme is a homodimer with subunit molecular mass of 34 kDa. FHMPC dehydrogenase belongs to the L-3-hydroxyacyl-CoA dehydrogenase (HAD) family. A conserved His-Glu dyad is vital for HAD catalytic mechanism. The His residue acts as a catalytic/general base (Barycki *et al.,* 1999; Barycki *et al.,* 2000; Barycki *et al.,* 2001; He *et al.,*1996; He *et al.,*1997). In addition to the His-Glu pair, a conserved serine and two asparagine residues are localized in the active site.

In this work, the tertiary structure of FHMPC dehydrogenase was solved to elucidate the catalytic mechanism of the enzyme. Five active site residues were identified.

2. MATERIALS AND METHODS
2.1. Cloning, expression, and purification of FHMPC dehydrogenase
The plasmid, designated as pET21a-mlr6793, was introduced into BL21 (DE3) cells. The transformed cells were aerobically grown in LB medium containing ampicillin at 37 °C. Purification was done by QA52 column (Whatman) and a Blue A column (Amicon) chromatographies. The purified enzyme was dialyzed at 4 °C against the crystallization buffer (50 mM Tris-HCl (pH 8.0), 10% (w/v) glycerol, 1 mM DTT and 0.01% (v/v) 2-mercaptoethanol).

2.2. Crystallization and structure determination
The purified enzyme was concentrated and NAD$^+$ added to a final concentration of 5 mM. Crystallization was done by the sitting drop vapor diffusion method. X-ray diffraction data sets were collected at the BL26B1 station of SPring-8 (Hyogo, Japan). The crystal belonged to space group *C2* with cell dimensions of a = 122.81, b = 44.21 and c = 109.34. The structure was solved by means of molecular replacement using the MOLREP program.

3. RESULT AND DISCUSSION
3.1. Amino acid sequence comparison with other enzymes
Sequence alignment of FHMPC dehydrogenase showed it`s highly identical to human heart 3-hydroxyacyl-CoA dehydrogenase (HhHAD) (32%), rabbit L-gulonate 3-dehydrogenase (RGDH) (30%), (Ishikura *et al.,* 2005); and diketoreductase (DKR) (30%), (Huang *et al.,* 2012; Wu *et al.,* 2009). The alignment showed that the catalytic His-Glu pair together with Ser and Asn residues corresponds to: His137, Glu149, Ser116 Asn188 and Asn140 in FHMPC dehydrogenase. The distribution of secondary structure elements in FHMPC dehydrogenase was highly similar to that in HhHAD (Barycki *et al.,* 1999)

3.2. Overall structure

The structure was refined to an R factor of 16.2 % (R_{free}=19.4 %) at 1.55 Å resolution. The structure contains two monomers in the asymmetric unit. The two subunit could form a dimer by symmetry operation.This is in good agreement with FHMPC dehydrogenase existing as a homodimer in solution (Yokochi *et al.,* 2009). The monomeric structure consists of the N-terminal and C-terminal domains. Both domains are made-up of residues 1-178 and 186-309, respectively. The C-terminal domain adopts entirely helical architecture. (Fig 1.1)
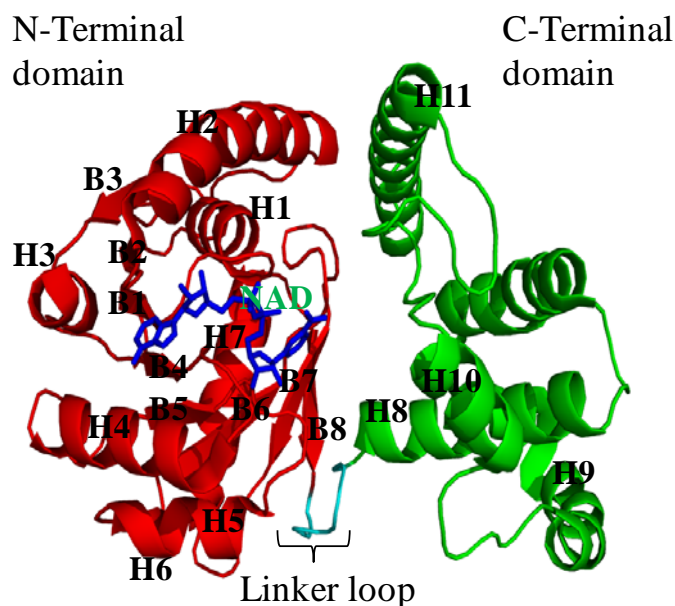

Figure 1.1. Tertiary structure of FHMPC dehydrogenase subunit. The N-terminal, C-terminal domains and linker loop are colored red, green and cyan, respectively. NAD$^+$ is shown as a stick in blue. Secondary structure elements, α-helix and β-sheet, are shown as H and B, respectively. N-terminus domain contains α-helix and β-sheet, C-terminal domain contains only α-helixes.

3.3. Co-factor binding

The structure contained one molecule of NAD$^+$ per subunit, which is bound in an extended conformation in a cleft within the N-terminal domain. NAD$^+$ is anchored by several hydrogen-bonding interactions between surrounding residues and water molecules.

3.4. Dimerization interface

Subunit dimerization is mediated by 56 interactions between the residues in C-terminal domains of two subunits. The helixes of one subunit in C-terminal domain runs nearly in antiparallel to helixes of the other subunit.

3.5. The putative substrate binding site and interaction of the active site residues

The substrate binding position is located between the N- and C-terminal domains. The catalytic and substrate-binding residues in HhHAD occupy almost the same positions as those (His137, Glu149, Ser116, Asn188 and Asn140) in FHMPC dehydrogenase. Ser116, His137 and Glu149 are connected by a hydrogen bonding network to form the catalytic triad.

3.6. Catalytic mechanism

The presence of Glu149 and Ser116 is necessary to drive the reaction at pH 8.0: the optimum pH of FHMPC dehydrogenase. Glu149 donates electrons to drive the oxidation reaction. Ser116 is likely to assist

protonation of His137 to drive the reduction reaction. Asn188 may be involved in substrate-binding and stabilization of the bound substrate during the catalysis while Asn140 may be involved in substrate anchoring.

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