論文の内容の要旨

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論文題目　Studies on enzymes involved in dissimilatory nitrogen metabolism in *Aspergillus oryzae*
（麹菌の窒素異化代謝に関わる酵素の研究）

Nitrogen is a basic element for life because it is an essential component of most bio-organic substances, such as amino acids, proteins, vitamins and nucleic acids. It exists in the biosphere in several redox states and interconversions of these nitrogen species are mostly attributed to biogeochemical nitrogen metabolism. The nitrogen metabolisms can be categorized into two types, assimilatory and dissimilatory metabolism, according the process and purpose in organisms. Denitrification is a typical form of dissimilatory nitrogen metabolism. In this study, we studied the enzymes involved in denitrification and nitric oxide (NO) detoxification as shown Figure 1.

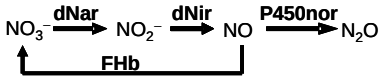

Figure 1. Enzymes involved in denitrification and NO detoxification in fungi.

Denitrification has been considered to be an exclusive process of bacteria until the fungal denitrification was discovered by Shoun *et al.* firstly at 1991. Denitrifying fungi can reduce nitrate (NO_3^-) or nitrite (NO_2^-) to nitrous oxide (N_2O) by dissimilatory nitrate reductae (dNar) or dissimilatory nitrite reducase (dNir) and nitrous oxide reductase (P450nor) (Figure 1). The acitivities of the enzymes involved in denitrification process were detected in some denitrifying fungi. However, the corresponding genes have not been identified and isolated except the one encoding p450nor. My predecessor, Master Nakanishi, isolated a copper-containing dNir (NirK) gene from *Aspergillus oryzae* (*A. oryzae*) and gave a characterization of its recombinant protein for the first time.

NO is a common product of enzymic and nonenzymic oxidation of reduced nitrogen compounds, which ubiquitously exists in environment. NO has the potential to damage a variety of biomolecules,

microorganisms living in such an environment required detoxification of NO. Flavohemoglobin (FHb) of bacteria and yeast is a main enzyme involved in NO detoxification under aerobic conditions by its NO dioxygenase (NOD) activity. Multiple FHb homologue genes are found in the genomes of fungi. But their physiological significant were not known. There is considerable interest in clarifying whether the fungal FHb homologues display NOD activities and whether the multiple proteins in the same fungus share the same properties.

In this work, I cloned and characterized two FHbs from *A. oryzae*, and elucidated their physiological significant. On the other hand, I kept on the study of NirK to investigate the relation between NirK and denitrification. In addition, I cloned a eucaryotic dissimilatory nitrate reductase (NapA-like) gene from *A. oryzae* for the first time.

1. Cloning and characterization of two FHbs.

Two FHb genes, *fhb*1 and *fhb*2, were cloned from *A. oryzae*. The deduced amino acid sequences of FHb1 and FHb2 showed high identity to other FHbs except the predicted mitochondrial targeting signal in the N-terminus of FHb2. Recombinant proteins of full length FHb1 and FHb2 without the putative mitochondrial targeting signal were expressed in *E. coli* and purified to homogeneity. The recombinant proteins displayed similar absorption spectra to other FHbs, but FHb2 did not contain FAD cofactor at all. FHb1 and FHb2 were estimated to be a monomer and a dimer in solution, respectively. Both of the two enzymes exhibit high NOD activities. FHb1 utilizes either NADH or NADPH as the external electron donor, whereas FHb2 can use NADH only. These results suggest that FHb1 and FHb2 are fungal counterparts of the bacterial FHbs and act as NO detoxification enzymes in cytosol and mitochondria, respectively.

2. *In vivo*, FHb1 and FHb2 are involved in NO detoxification in cytosol and mitochondria, respectively.

Expression responses of *fhb*1 and *fhb*2 to NO stress were investigated by real-time PCR. *fhb*1 in wild type was up-regulated to about 15 times by NO or NO generator, NO_2^-. However, the intracellular levels of *fhb*2 was too low to be detected under any conditions. The *fhb1*-deletion mutant (△*fhb1*) and *fhb2*-deletion mutant (△*fhb2*) were constructed to investigate their physiological functions. In the minimal agar medium (CD), under the normal conditions, the growth rate of △*fhb1* was obviously slower than △*fhb2* and wild type. In the liquid CD medium, △*fhb1* could not to form clumps. Whereas, there was no difference between △*fhb2* and wild type. It was suggested that the FHb1 affect the fungal morphology. On the other hands, in the rich medium (DPY), the three strains did not show any phenotypic differences in the absence of NO stress. So DPY medium was used to investigate the effects of *fhbs* disruption on NO stress. △*fhb1* became hypersensitive to NO stress, whereas, *fhb2* deficiency did not display any obvious effects compared with wild type. It may be attributed to the little effect of the externally added NO stress on mitochondria. To investigate the expression of *fhb2*, I used a *nirK*-over expression strain, which can promote mitochondria NO production by its high mitochondrial nitrite

reductase activity. As expected, the expression of *fhb2* became detectable by real-time PCR in this strain which indicated *fhb2* was induced by mitochondrial NO stress.

3. FHbs enhanced the oxidative stress of H_2O_2 probably dependent on Fenton reaction.

E. coli FHb (HMP) and *S. cerevisiae* FHb (YHb) were reported to participate in defense against oxidative stress. So FHbs of *A. oryzae* were investigated for their responses to oxidative stress from H_2O_2. It is interesting that both of *△fhb1* and *△fhb2* exhibited higher resistance than wild type to H_2O_2. Meanwhile, the strain harboring the *fhb2* over-expression plasmid also showed hypersensitivity to H_2O_2. These findings indicated FHb expression might have deleterious effects at the presence of oxidative stress, which is obviously conflicted the conclusion from HMP and YHb. The C-terminal portion of FHbs showed homology with *E. coli* flavin reductae (Fre) which possesses a ferric iron reductase activity. So when the NO is absent, FHbs can reduce FAD to $FADH_2$, which may in turn act as ferric iron reductant and drive the Fenton reaction to cause the DNA damage. The flavin reductase activities of the purified FHbs were confirmed experimentally. We are measuring and comparing the degree of DNA damage among *△fhb1*, *△fhb2* and wild type in the presence of H_2O_2.

4. Cloning of a periplasmic nitrate reductase (NapA) homologue gene from *A. oryzae* .

Bacterial NapA is a periplasmic dissimilatory nitrate reductase. Bacterial NapA is used to support anaerobic metabolism as an alternative to the NarGHI pathway when nitrate concentration is low in the culture or used as an electron sink to eliminate an excess of reducing equivalents accumulated in the cytoplasm as NADH and $FADH_2$. To date, no eukaryotic dissimilatory nitrate reductase has been isolated, yet. However, by Blast search on the fungal genomes, a *nap*A-homologue gene was found in some strains. So I tried to clone and characterize the *napA*-homologue gene from *A. oryzae*. The full-length cDNA of *napA*-homologue gene was cloned and the 990-amino acids sequence of the protein was deduced. Different from bacterial NapA, no putative targeting signal peptide was found at its N-terminus. So, it may be localized into cytosol. To characterize the recombinant protein, expression systems of *E. coli* and *A. oryzae* were used. But only *A. oryzae* system worked. The recombinant NapA in crude extract exhibited an obvious anaerobic nitrate reductase activity. Though the expression level was very low, NapA could be distinguished as a main band at about 110 kDa after the purification by nickel affinity column. But it became denatured before the following purification by gel filtration chromatography. So optimization of the conditions of expressing and purification remain to be done in future.

5. Study on the enzymes involved in denitrification of *A. oryzae*.

In this chapter, I revised and supplemented some data for the characterization of the recombinant NirK which had been done previously by Nakanishi. Then I mainly investigated the relation between the *nirK* gene and denitrification. I also confirmed the necessity of P450nor to denitrification of *A. oryzae*. The recombinant NirK showed robust anaerobic nitrite reductase activity, and many properties similar to bacterial NirK. The *A. oryzae* strains possessing a NirK over-expression plasmid, constructed by Nakanishi priviously, showed about 6 times higher in denitrification ability than the wide-type in my

experiment condition. The pellet of the homogenate from the *nirK* over-expression strain showed high NirK activity. So these results suggested that NirK should be anchored in mitochondria membrane by the action of extensional predicted mitochondrial targeting signal sequence existing in its N-terminus. I compared the effects of O_2 and NO_2^- on the expression level of *nirK* gene, *in vivo*. *nirK* was robustly induced under denitrification conditions. I constructed *nirK*-deletion mutant ($\triangle$ *nirK*) and P450nor-deletion mutant to compare their denitrification abilities with wide-type. P450nor deficient mutant could not denitrify any more, but $\triangle$*nirK* still kept denitrifying activity like wide-type. It has been reported that plant assimilatory nitrate reductase and yeast mitochondrial cytochrome oxidase are also involved in NO production. Both of the two enzymes exist in *A. oryzae*. So it is possible that some NO producing enzyme substitutes NirK to reduce NO_2^- to NO in the *nirK* deficient mutant. On the other hand, P450nor is vital to denitrification.

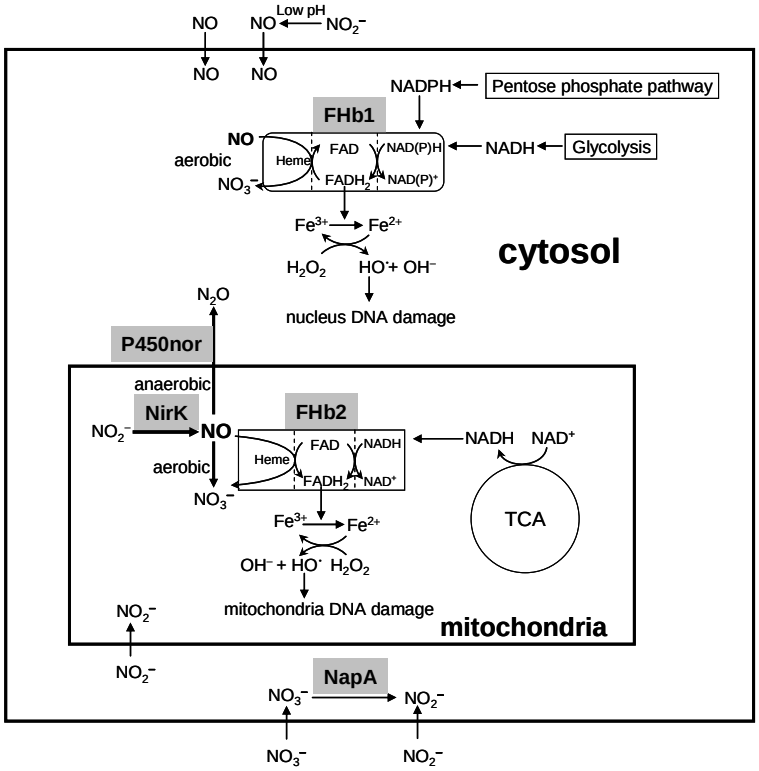

Figure 2. Enzymes involved in dissimilatory nitrogen metabolism studied in this work.

Conclusions:

The gene of a eukaryotic dissimilatory nitrate reductase (NapA-homolog) was isolated for the first time, and the recombinant protein exhibited an anaerobic Nar activity. NirK anchored in mitochondria membrane catalyzes the conversion of NO_2^- to NO in the denitrification pathway. P450nor reducing NO to N_2O at cytosol is vital to denitrification but there may be some isoenzyme of NirK exist in *A. oryzae*. FHb1 and FHb2 play a role of NO detoxification in cytosol and mitochondria, respectively. In the absence of NO stress, FHb1 and FHb2 would amplify the oxidative damage of H_2O_2 probably by driving Fenton reaction.