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Cloning and characterization of two flavohemoglobins from *Aspergillus oryzae*

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ABSTRACT

Two flavohemoglobin (FHb) genes, *fhb1* and *fhb2*, were cloned from *Aspergillus oryzae*. The amino acid sequences of the deduced FHb1 and FHb2 showed high identity to other FHbs except for the predicted mitochondrial targeting signal in the N-terminus of FHb2. The recombinant proteins displayed absorption spectra similar to those of other FHbs. FHb1 and FHb2 were estimated to be a monomer and a dimer in solution, respectively. Both of the isozymes exhibit high NO dioxygenase (NOD) activity. FHb1 utilizes either NADH or NADPH as an electron donor, whereas FHb2 can only use NADH. These results suggest that FHb1 and FHb2 are fungal counterparts of bacterial FHbs and act as NO detoxification enzymes in the cytosol and mitochondria, respectively. This study is the first to show that a microorganism contains two isozymes of FHb and that intracellular localization of the isozymes could differ.

Flavohemoglobins (FHbs) are widely found in prokaryotic and eukaryotic microorganisms [1]. The FHb family is a very homologous group of proteins. They are fused proteins consisting of a hemoglobin-like domain with a noncovalently bound heme B and a reductase domain with binding sites for FAD and NAD(P)H [2]. For a decade, NO detoxification has been regarded as the main physiological role of FHb [3,4]. For several microorganisms including bacteria and yeast, it has been demonstrated that FHb plays an important role in protection against nitrosative stress [5–8]. Extensive studies of the purified native or recombinant FHb proteins from bacteria and yeast have revealed details of their structure, function, and reaction mechanism [9]. In the presence of molecular O_2, FHb catalyzes the NO dioxygenase (NOD) [3] reaction in which NO is stoichiometrically converted to innocuous nitrate ions. NOD activity has been identified in FHbs from many microorganisms [10–14].

To date, the sole fungal FHb sample has been isolated from denitrifying cells of *Fusarium oxysporum* [15]. Its UV–visible absorption spectra and prosthetic groups have been investigated, but other properties and the physiological functions of fungal FHbs remain uncharacterized. Enterobacteria such as *Escherichia coli* should frequently encounter nitrosative stress originating from their host mammals. However, it is unclear whether nitrosative stress often occurs in the natural environment of fungi. Interestingly, multiple FHb homologue genes are found in the genomes of fungi whose genome analyses have been completed. There is considerable interest in clarifying whether the fungal FHb homologues display NOD activity and whether the multiple proteins from the same cells share the same properties. In this work, we biochemically characterized recombinant proteins of two FHbs from *Aspergillus oryzae*.

Materials and methods

Cloning of the cDNAs. A. oryzae RIB40 was grown on DPY agar medium (2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH_2PO_4, 0.05% $MgSO_4{\cdot}7H_2O$, and 1.5% agar) with 10 mM $NaNO_2$ at 30 °C. Total RNA was isolated using TRIZOL Reagent (Invitrogen), and full-length cDNAs were obtained by 5′–3′ RACE using the GeneRacer Kit (Invitrogen). The forward and reverse gene specific primers for the *fhb1* gene are 5′-ccggccagtatatctccgtgcagg-3′ and 5′-gccgtggatgtagtgaatcttgcgc-3′, respectively. The forward and reverse gene specific primers for *fhb2* gene are 5′-ggccagttcgttagtgtgaagtgc-3′ and 5′-gcacgacaggcgtggtagcattaa c-3′, respectively. Amplified 5′- and 3′-RACE DNA was separately cloned using pT7Blue (Takara).

Subcloning, overexpression, and purification of the recombinant FHbs. To express the full-length FHb1 protein, the *fhb1* gene was subcloned using the forward primer, 5′-catatgatgccgctctcccctgaacaaatcc-3′ (NdeI site underlined), and the reverse primer, 5′-gcggccgcctaagcaggaacaccgccg-3′ (NotI site underlined). To express the FHb2 protein lacking the N-terminal 30 amino acid putative mitochondrial targeting signal, the *fhb2* gene was subcloned using

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the forward primer, 5′-gaattcaagctcacccccagcagatt c-3′ (EcoRI site underlined), and the reverse primer, 5′-ctcgagttattcagcccggagaacc gaac-3′ (XhoI site underlined). The PCR products were digested with the corresponding restriction enzymes and were inserted into pET28b (Novagen). *E. coli* BL21-CondonPlus(DE3)-RIL (Stratagene) cells harboring the vectors were grown in LB medium containing 20 μg/ml kanamycin and 100 μg/ml chloramphenicol at 37 °C to an OD of 0.5. Recombinant protein expressions were induced at 20 °C by 0.1 mM isopropyl-1-thio-β-D-galactopyranoside with the addition of 0.8 mM $FeCl_3$ and 0.5 mM 5-aminolevulinic acid for 16 h. Harvested cells were resuspended in 20 mM K-phosphate buffer (pH 7.2), 0.3 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 0.5 M NaCl and 10 mM imidazole. The sonicated cells were centrifuged, and the supernatant was applied to affinity chromatography with HiTrap Chelating HP (GE Healthcare). The column was washed with 20 mM Na-phosphate buffer (pH 7.2) and 10 mM imidazole, and the bound proteins were eluted with a gradient of 10–500 mM imidazole. Fractions containing FHbs were applied to gel filtration using HiLoad 16/60 (GE Healthcare) in 20 mM K-phosphate buffer with 0.15 M NaCl at a flow rate of 1 ml/min.

Analysis of prosthetic groups. Flavins were extracted and determined by the method described in [16] with a little modification. The FHb proteins or the flavin standard solutions (100 μl) were mixed with 190 μl of methanol–methylene chloride (9:10, v/v) and vortexed for 60 s. After addition of 90 μl of 0.05 M ammonium acetate buffer (pH 6.0), the mixture was again vortexed for 60 s and then centrifuged for 20 min at 17,400*g* at 4 °C. The upper phase was then filtered through a 0.45 μm filter and immediately injected into a Waters Model 600E HPLC System (Waters) equipped with a PEGASIL ODS column (4.6 × 250 mm**,** Senshu Kagaku). The samples were protected against light throughout the procedure. For separation and identification of flavins, the following elution method was performed. Elution in the isocratic mode at 20:80 (v/v) methanol–0.05 M ammonium acetate (pH 6.0) for 1 min, and then in linear gradient from 20:80 to 90:10 of methanol–ammonium acetate within 10 min at the flow rate 1 ml/min. Heme content was determined by the pyridine ferrohemochromogen method employing the molar absorption coefficient of the chromogen of protohemes as 34.4 mM^{-1} cm^{-1} at 557 nm [17].

NOD activity measurement and other methods. NOD activity was measured using an ISO-NO MARK II-type NO electrode (World Precision Instruments). The standard reaction mixture contained 2 ml of 50 mM citrate–NaOH buffer (pH5.5), 100 μM NAD(P)H, 1 μM flavin, purified FHb, and 3 μM 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene (NOC-18). Amino acid sequence alignment was done with ClustalW and illustrated using ESPript. The prediction of the mitochondrial targeting signal was performed using the iPSORT server. Protein concentration was determined by BCA Protein Assay Reagent (Pierce) using bovine serum albumin as a standard. UV–visible absorption spectra were recorded using a V-550 spectrophotometer (Jasco).

Results

Cloning of FHb homologues from A. oryzae

Two FHb-like genes, *fhb1* (locus tag AO090011000202) and *fhb2* (AO090012000171), were identified by a BLAST search on the DOGAN database (http://www.bio.nite.go.jp/dogan/) using the amino acid sequence of *E. coli* FHb (HMP) as a query. The *fhb1* and *fhb2* genes are located on chromosome 7 and chromosome 4, respectively. The full-length cDNAs of the two genes were cloned and sequenced, and there was no intron in either of the genes. Fig. 1 shows the deduced amino acids sequences from the two FHb genes. FHb1 consists of 416 amino acids, whereas FHb2 contains 30 amino acids of a putative mitochondrial targeting signal peptide within a total of 436 amino acids. The deduced fungal FHb sequences were compared with two well-characterized bacterial FHbs, HMP and *Alcaligenes eutrophus* FHb (FHP) (Fig. 1). FHb1, FHb2, HMP and FHP share sequence identities higher than 40%. The catalytic triad residues at the proximal site of heme (His-F8, Tyr-G5, and Glu-H23), the residues essential for O_2 binding (Tyr-B10 and Gln-E7), and the RQYS motif essential for FAD binding in the bacterial FHbs [3] are completely conserved in both of the FHb1 and FHb2 sequences.

Molecular mass determination of the recombinant FHbs

Recombinant proteins of full-length FHb1 and FHb2 without the putative mitochondrial targeting signal, both of which have an N-terminal $(His)_6$-tag, were expressed in *E. coli* and purified to homogeneity (Supplemental data Fig. 1). About 20 and 2 mg of purified proteins were obtained from 1 l of cell culture for FHb1 and FHb2 expression, respectively. The apparent molecular mass of both FHb1 and FHb2 on SDS-PAGE was estimated to be 51 kDa. The molecular mass of the FHb1 and FHb2 proteins in solution was approximately 51 and 98 kDa, respectively, as estimated by calibrated gel filtration chromatography. These results suggest that FHb1 and FHb2 exist as a monomer and a dimer in solution, respectively.

Absorption spectra and prosthetic groups

UV–visible absorption spectra of purified FHb1 and FHb2 in resting (Fe^{3+}), dithionite-reduced (Fe^{2+}), dithionite-reduced plus CO (Fe^{2+}–CO), and NADH-reduced (Fe^{2+}–O_2) forms are shown in Fig. 2, and their peak maxima are summarized in Table 1. The characteristics of the absorption spectra of both FHbs are similar to those of previously reported spectra of other FHbs [12,15,18,19]. Unlike FHb1 and other FHbs, however, the Soret peak maximum of FHb2 did not show a red-shift from the resting form to the NADH-reduced form (Fig. 2B), suggesting that the heme of FHb2 can not accept electrons from NADH via the flavin cofactor. The type of flavin in the purified FHb1 was analyzed by HPLC using FMN, FAD, and riboflavin as standards and identified as FAD (data not shown). The contents of FAD and heme in the purified preparation of FHb1 were estimated as 0.30 mol/mol protein and 0.39 mol/mol protein, respectively. We could not detect any flavin in the purified FHb2, but its heme content was estimated as 0.89 mol/mol protein. It has been shown that heme and FAD cofactors are often partially dissociated from FHb during the purification procedures [5,18,20,21]. When the absorption spectra of FHb2 were measured after incubation of 16 μM FHb2 protein with 32 μM FAD for 5 min at room temperature (Fig. 2C), a spectral shift of the Soret peak maximum and the appearance of a visible peak at 580 nm were observed (Table 1), indicating that the electron transfer from NADH to heme has been restored.

NOD activity

The NOD activities of the purified FHbs were measured with an NO electrode (Fig. 3). FHb1 exhibited NOD activity in both the presence and absence of externally added FAD. In contrast, FHb2 required external FAD for NOD activity. This result is consistent with the quantitative analysis of the FAD contents of these FHbs. It has been shown that FHbs from various microorganisms vary widely in their preference for the electron donor for NOD activity. For example, FHP does not utilize NADPH as an electron donor, whereas *Saccharomyces cerevisiae* FHb (YHb) and HMP utilize either NADH or NADPH [10]. The dependency of the NOD activities

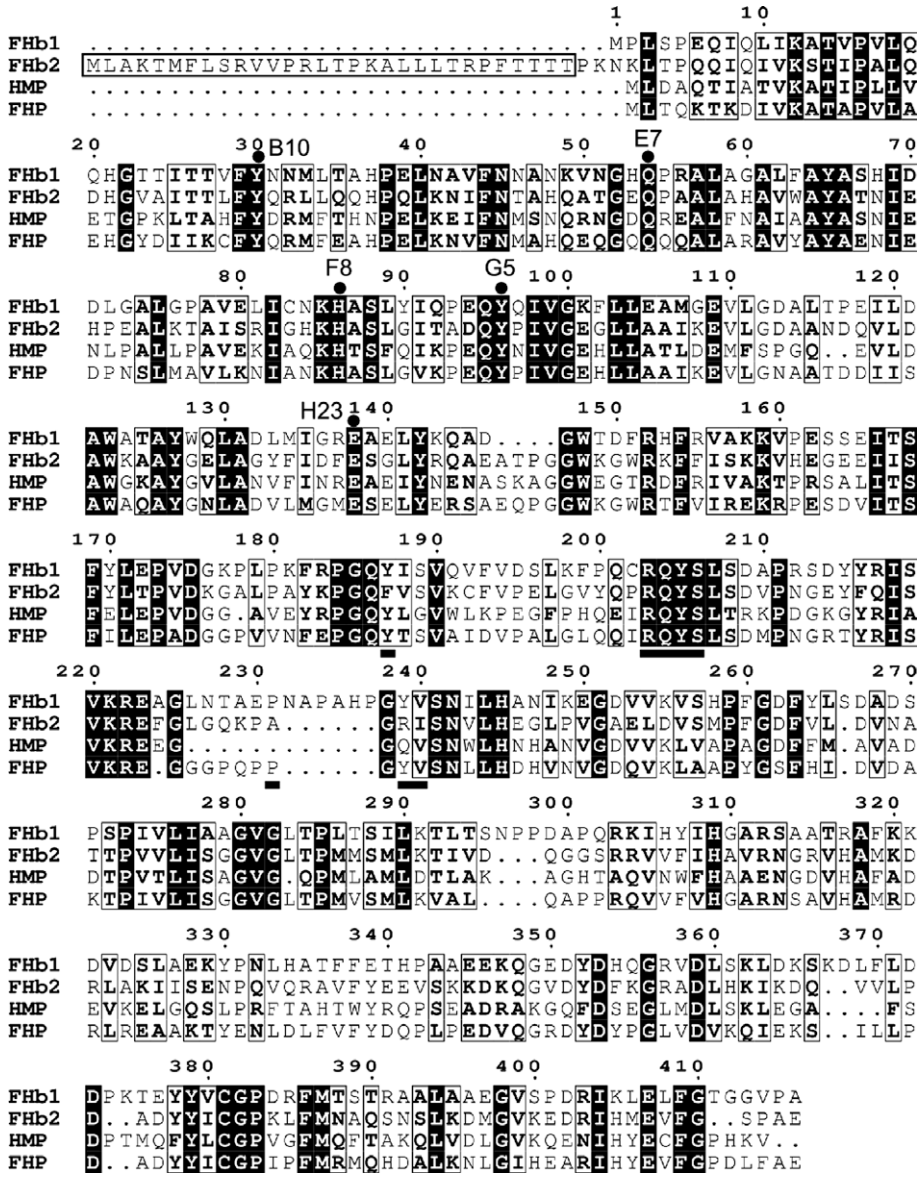

Fig. 1. Amino acid sequence alignment of FHb1, FHb2, *E.coli* FHb (HMP), and *A. eutrophus* FHb (FHP). The predicted N-terminal mitochondrial targeting signal sequence of FHb2 is shown in a box. Essential residues for heme binding and NO dioxygenase activity are indicated by closed circles above the sequences. Residues responsible for FAD binding in FHP are indicated by bars under the sequences.

of FHb1 and FHb2 on electron donors and flavin cofactors were compared (Table 2). Addition of flavin cofactors did not have a great effect on the NOD activity of FHb1, but FAD gave a slight activation effect. This result is consistent with the FAD to heme ratio (3:4) of the purified FHb1 protein. In contrast to the cases of YHb and HMP, FHb1 showed slightly higher activity against NADPH than NADH. With FHb2, addition of both FAD and NADH was required for full NOD activity, but FMN and NADPH were not effective. Steady-state kinetic parameters of the NOD activity of the two FHbs were measured with various NO concentrations at room temperature (25 °C). The K_m and V_{max} values of FHb1 in the presence of 100 μM NADPH were 2.0 μM and 9.4 s^{-1}, and the K_m and V_{max} values of FHb2 in the presence of 100 μM NADH and 1 μM FAD were 2.3 μM and 4.5 s^{-1}. The V_{max} values are within the range of reported V_{max} values of other FHbs at 37 °C [10] or at room temperature [11]. The K_m values of two FHbs against NO were not greatly different, but the V_{max} value of FHb1 was significantly higher than that of FHb2.

Discussion

Eukaryotic microorganisms often have multiple genes in their genomes that are homologous to FHb. For example, two FHb genes of *Dictyostelium discoideum* are located close to each other in the genome [22]. Both of their gene products are responsible for NO stress resistance, indicating that they have the same function, acting in a co-operative manner. However, the *Candida albicans* genome contains three FHb homologue genes, but only one of them (*CaYHb1*) is responsible for NO detoxification [23]. Essential amino acid residues for NOD activity are not conserved in the other two genes (*CaYHb4* and *CaYHb5*). In our study, both of the two FHb homologues from *A. oryzae* have been shown to exhibit robust NOD activities. One of the unique characteristics of FHb2 is its possible N-terminal mitochondrial targeting signal, since no typical signal sequence has been found in other FHbs to date. YHb, which is encoded by a nuclear gene of *S. cerevisiae*, is located in both the cytosol and the mitochondrial matrix although no targeting signal

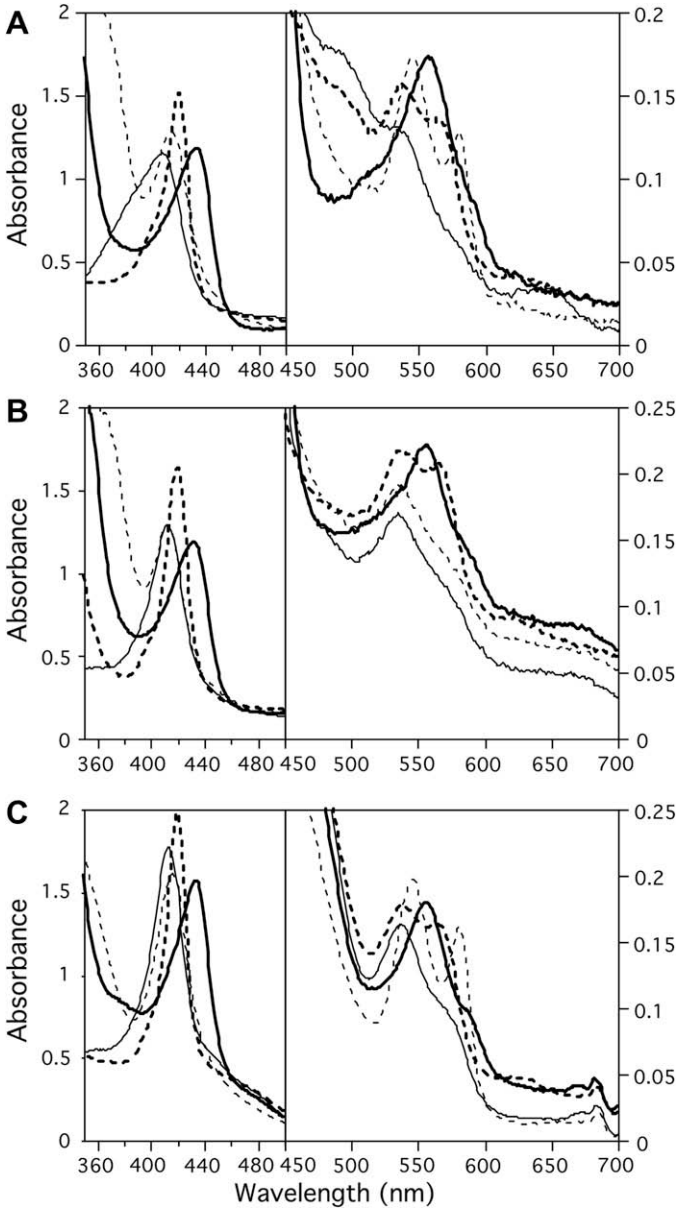

Fig. 2. UV–visible absorption spectra of purified FHb1 (A), FHb2 (B), and FHb2 after incubation with FAD (C). Left, γ-(Soret) band region; right, visible region. Resting (Fe^{3+}, thin lines), dithionite-reduced (Fe^{2+}, thick lines), dithionite-reduced plus CO (Fe^{2+}–CO, thick broken lines), and NADH-reduced (Fe^{2+}–O_2, thin broken lines). The spectra were measured for 0.7 mg/ml FHb1 or 0.5 mg/ml FHb2 in 20 mM Na-phosphate buffer (pH 7.2) at room temperature.

Table 1
Peak maxima of UV-visible spectra.

FHb	Form	Soret	Visible
FHb1	Fe^{3+}	407	540, 645
	Fe^{2+}	434	558
	Fe^{2+}–CO	421	538, 565
	Fe^{2+}–O_2	414	545, 579
FHb2	Fe^{3+}	412	535
	Fe^{2+}	432	556
	Fe^{2+}–CO	420	537, 560
	Fe^{2+}–O_2	412	535
FHb2 after incubation with FAD	Fe^{3+}	412	535
	Fe^{2+}	434	556
	Fe^{2+}–CO	420	539, 565
	Fe^{2+}–O_2	416	546, 580

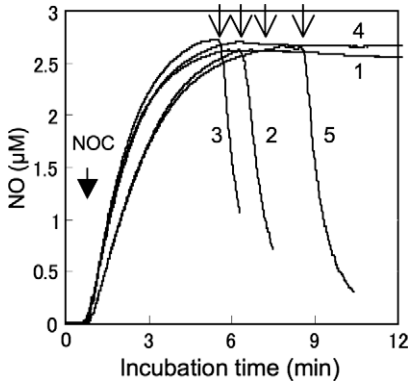

Fig. 3. NOD activities of FHb1 and FHb2. In all systems, NO-releaser (NOC-18) was initially added and the activities were measured at room temperature. Enzyme was added at the time indicated by the arrow. Line 1, no enzyme; line 2, FHb1; line 3, FHb1 plus 1 μM FAD; line 4, FHb2; line 5, FHb2 plus 1 μM FAD.

Table 2
Dependency of the NOD activity on electron donors and flavin cofactors.

FHb	Additives	NOD activity (s^{-1})
FHb1	NADH	1.71 ± 0.17
	NADH + FAD	2.26 ± 0.15
	NADH + FMN	2.04 ± 0.25
	NADPH	2.43 ± 0.10
	NADPH + FAD	2.66 ± 0.15
	NADPH + FMN	2.63 ± 0.12
FHb2	NADH	ND[a]
	NADH + FAD	2.92 ± 0.23
	NADH + FMN	0.74 ± 0.11
	NADPH	ND[a]
	NADPH + FAD	0.34 ± 0.08
	NADPH + FMN	0.08 ± 0.02

[a] Not detected.

sequence is present [24]. The way in which it is imported into the mitochondria is unclear, and its physiological function in mitochondria remains to be elucidated. The production of NO in mitochondria is thought to occur in several eukaryotes. Dissimilatory nitrite reductase in mitochondria produces NO in the denitrifying fungi, *F. oxysporum* and *Cylindrocarpon tonkinense* [25]. Additionally, in the mitochondria of *S. cerevisiae*, NO is produced by cytochrome oxidase under hypoxic conditions [26]. Therefore, the presence of mitochondrial FHb in eukaryotic cells may respond to the mitochondrial NO stress. In the mitochondria of denitrifying fungi, cytochrome P450 NO reductase (P450nor) is thought to detoxify NO under anaerobic conditions [27–30]. Interestingly, the deduced amino acid sequence of the sole P450nor gene in the *A. oryzae* genome lacks the mitochondrial targeting signal [31].

The recombinant FHb1 and FHb2 proteins were estimated to be monomer and dimer in solution, respectively. To our knowledge, all bacterial and yeast FHbs isolated so far are monomeric. However, it is proposed that HMP might exist as a dimer in vivo due to the observed cooperativity in cyanide binding [32].

FHbs prepared from bacteria and yeast contained only 24–44% FAD due to dissociation from the protein during the purification procedures [10]. In our preparation from recombinant *E. coli* cells, FHb1 contained 30% FAD, whereas FHb2 released it completely. The FAD binding domain of the FHb family is highly conserved, but FHb2 exhibits some differences in this region. Pro232, Tyr235, and Val236 in FHP are responsible for binding the pyrophosphate and adenosine moieties of FAD [33]. These residues are conserved in FHb1 (Pro231, Tyr239, and Val240), but not in

HMP and FHb2 (Fig. 1). A Tyr residue, located near from the isoalloxazine ring of FAD [34], is conserved in HMP (Tyr188), FHP (Tyr190), and FHb1 (Tyr187), but is replaced by Phe in FHb2.

FHb1 showed a slight preference for NADPH over NADH, but FHb2 was highly specific to NADH. Interestingly, two P450nor isozymes in *C. tonkinense* have similar characteristics; one isozyme with a mitochondrial targeting signal (P450nor1) is specific to NADH, whereas the other without a signal (P450nor2) utilizes both NADH and NADPH and prefers the latter [27,28]. Such preferences for electron donors may be related to the cellular distribution of these enzymes. NADH is present in both of the cytosol and mitochondria because it is produced from glycolysis and the citric acid cycle. In contrast, the major source of NADPH is the oxidative phase of the pentose phosphate pathway in the cytosol, and NADPH levels are usually very low in mitochondria. We are currently studying the distribution and functions of FHb1 and FHb2 in *A. oryzae* cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.01.112.

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