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Enzymatic properties of cytochrome P450 catalyzing 3′-hydroxylation of naringenin from the white-rot fungus *Phanerochaete chrysosporium*

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ABSTRACT

We cloned full-length cDNAs of more than 130 cytochrome P450s (P450s) derived from *Phanerochaete chrysosporium*, and successfully expressed 70 isoforms using a co-expression system of *P. chrysosporium* P450 and yeast NADPH-P450 reductase in *Saccharomyces cerevisiae*. Of these P450s, a microsomal P450 designated as PcCYP65a2 consists of 626 amino acid residues with a molecular mass of 68.3 kDa. Sequence alignment of PcCYP65a2 and human CYP1A2 revealed a unique structure of PcCYP65a2. Functional analysis of PcCYP65a2 using the recombinant *S. cerevisiae* cells demonstrated that this P450 catalyzes 3′-hydroxylation of naringenin to yield eriodictyol, which has various biological and pharmacological properties. In addition, the recombinant *S. cerevisiae* cells expressing PcCYP65a2 metabolized such polyaromatic compounds as dibenzo-*p*-dioxin (DD), 2-monochloroDD, biphenyl, and naphthalene. These results suggest that PcCYP65a2 is practically useful for both bioconversion and bioremediation.

White-rot fungi can degrade a wide variety of recalcitrant aromatic compounds, including polymeric lignin and environmentally persistent pollutants. Cytochrome P450 (P450)-mediated oxygenation reactions play an important role during the fungal metabolism of recalcitrant xenobiotic compounds as previously described [1]. *Phanerochaete chrysosporium* is the most extensively studied white-rot fungus with regard to ligninolysis and xenobiotic metabolism. The gene diversity of fungal P450s was recently suggested by a whole genomic sequence, where as many as 148 P450 genes have been found in the *P. chrysosporium* genome [2]. In previous studies, we demonstrated that *P. chrysosporium* P450s have diverse and unique functions using a series of well-characterized P450 substrates known for other organisms [3], and revealed the enzymatic properties of *P. chrysosporium* PcCYP1f using the *Pichia pastoris* heterologous expression system [1].

Recently, we cloned the full-length cDNAs of more than 130 *P. chrysosporium* P450s and successfully expressed 70 isoforms from these P450s using a co-expression system of *P. chrysosporium* P450 and yeast NADPH-P450 reductase in *Saccharomyces cerevisiae*. To elucidate the enzymatic properties of those *P. chrysosporium* P450s, we examined various substrate candidates including steroid hormones, several drugs, and such polycyclic aromatic hydrocarbons as dioxins using the whole cell of the recombinant yeast expressing each of *P. chrysosporium* P450.

In this study, we focused on *P. chrysosporium* P450s to metabolize flavonoids that exhibit an array of pharmacological properties, including antianxiety effects [4], improvement of cardiac function after ischemia [5], and antiestrogenic effects in breast cancer cell cultures [6]. Their basic chemical structure consists of two benzene rings linked through a heterocyclic pyrone or pyran ring in the middle. This structure allows multiple patterns and substitutions that give rise to various subclasses such as isoflavonoids, flavones, catechins, and anthocyanins. Despite the great similarity in overall structure between subgroups and within members of the subgroups, the biochemical and biological properties vary considerably with only minor modifications of the flavonoids structure. For instance, the number and the specific position of the hydroxyl groups on the three-ring structure determine whether the compound exhibits estrogenic activity [7], or functions as an antioxidant [8,9]. Flavonoids exist only in a relatively small food group that includes parsley, thyme, celery, and sweet red pepper [10]. Thus, biosynthesis of these compounds from microorganisms, similar to the biosynthesis previously described in Leonard et al. [11]

Abbreviations: CYP, cytochrome P450; DD, dibenzo-*p*-dioxin; 2-MCDD, 2-monochloro-dibenzo-*p*-dioxin.

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and references therein, can be a useful method instead of extraction from plants.

In this study, we demonstrate that the recombinant *S, cerevisiae* cells expressing PcCYP65a2 catalyze 3′-hydroxylation of naringenin to yield eriodictyol that has pharmacologically useful properties. In addition, we demonstrate the degradation of dibenzo-*p*-dioxin, 2-monochloroDD, biphenyl, and naphthalene by the recombinant *S. cerevisiae* cells, suggesting a possibility of their application to the biodegradation of toxic pollutants.

Material and methods

Materials. Naringenin and eriodictyol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dibenzo-*p*-dioxin (DD), biphenyl, and naphthalene were purchased from Wako Pure Chemical Industries (Osaka, Japan). 2-MonochloroDD (2-MCDD) was purchased from AccuStandard (New Haven, CT). NADPH was purchased from Oriental Yeast (Tokyo, Japan). All other chemicals were purchased from standard commercial sources and were of the highest quality available.

cDNA cloning of PcCYP65a2. PcCYP65a2 cDNA was obtained from *P. chrysosporium* cDNA library by PCR using primers (i) 5′-CGCTCTAGACCATCATGCCCCTGCTCT-3′ and (ii) 5′- GCAAAGCTTATTCAAGACACTGTCCGGGGA-3′ for its entire coding region on the basis of PcCYP65a2 genome sequence (data not shown). Nucleotide sequence of PcCYP65a2 cDNA was confirmed by DNA sequencing.

Construction of the expression plasmid for PcCYP65a2. The expression plasmid for PcCYP65a2 was constructed as follows. The PCR fragment (1899 bp) encoding PcCYP65a2 with *Hin*dIII sites at each sides was cloned into the co-expression vector for P450 and yeast NADPH-P450 reductase, pGYR with *Zygosaccharomyces rouxii* GAPDH promoter, digested with the same restriction enzymes [12]. *S. cerevisiae* AH22 cells were transformed by the resultant plasmid named pG65a2 as described previously [12].

Metabolism of naringenin, DD, 2-MCDD, biphenyl, and naphthalene in the culture of the recombinant S. cerevisiae cells expressing PcCYP65a2 and S. cerevisiae NADPH-P450 reductase.

The recombinant *S. cerevisiae* cells expressing PcCYP65a2 and NADPH-P450 reductase were cultivated in a synthetic minimal medium containing 8% glucose, 5.4% yeast nitrogen base without amino acids, and 160 mg/L histidine at 30 °C. The substrates naringenin, DD, 2-MCDD, biphenyl, and naphthalene were each dissolved in ethanol. Twenty-two hours after addition of each substrate to the culture at a final concentration of 0.1 mM, the cell culture was vigorously mixed with four volumes of chloroform/methanol (3:1 v/v). The organic phase was recovered and dried in a vacuum evaporator centrifuge (Sakuma Seisakusyo, Tokyo, Japan). The resultant residue was dissolved in acetonitrile and applied to HPLC under the following conditions: column, YMC-Pack ODS-AM (5 μm; 4.6 mm × 300 mm) (YMC Co., Kyoto, Japan); UV detection, 290 nm for naringenin, 227 nm for DD and 2-MCDD, 254 nm for biphenyl, and 286 nm for naphthalene; flow-rate, 1.0 mL min^{-1}; column temperature, 40 °C; mobile phase, a linear gradient of 20–100% acetonitrile aqueous solution containing 0.01% trifluoroacetic acid per 25 min followed by 100% acetonitrile for 10 min.

LC–MS analysis of the metabolite produced by PcCYP65a2 and authentic standard of eriodictyol. Isolated metabolites from HPLC effluents and authentic standard of eriodictyol were each subjected to mass spectrometric analysis, using a Finnigan LCQ ADVANTAGE MIX (ThermoFisher SCIENTIFIC, Waltham, MA, USA) with atmospheric pressure chemical ionization, positive mode. The conditions of LC were described below: column; reverse phase ODS column (2 mm × 150 mm, Develosil RPAQUEOUS-AR-5, Nomura Chemical Co. Ltd., Aichi, Japan); 30–80% methanol; flow-rate, 0.2 mL/min; temperature, 40 °C; UV detection, 290 nm.

^{1}H NMR analysis of the metabolite produced by PcCYP65a2. The 400-MHz ^{1}H NMR spectra of the metabolite of naringenin were measured on a BRUKER-400 (^{1}H, 399.9 MHz). The metabolite of naringenin (15 μg) and authentic standard of eriodictyol were each dissolved in 500 μL of MeOD and transferred into a probe. The time of domain data was multiplied with a squared sine-bell function.

Preparation of microsomal fractions from the recombinant S. cerevisiae cells. The recombinant *S. cerevisiae* AH22/pG65a2 cells expressing PcCYP65a2 were cultivated in a synthetic minimal medium containing 8% glucose, 5.4% yeast nitrogen base without amino acids, and 160 mg/L histidine. Microsomal fractions of AH22/pG65a2 cells were prepared as described previously [12].

Measurement of reduced CO difference spectra. The reduced CO difference spectra were measured with a Shimadzu UV-2200 spectrophotometer (Kyoto, Japan) according to the following procedure as described previously [13]. The concentration of PcCYP65a2 was determined from the reduced CO difference spectrum using a difference of the extinction coefficients at 452 and 490 nm of 91 mM^{-1} cm^{-1} [14].

Measurement of naringenin 3′-hydroxylation in the microsomal fractions prepared from the recombinant S. cerevisiae cells. Membrane fractions prepared from *S. cerevisiae* AH22/pG65a2 cells expressing PcCYP65a2 and yeast NADPH-P450 reductase were used for metabolism of naringenin. The substrate naringenin was dissolved in dimethyl sulfoxide. The reaction mixture contains PcCYP65a2 (50 nM) and 50–800 μM naringenin in a final volume of 0.25 mL of 50 mM potassium phosphate buffer, pH 7.4. The reaction was initiated by the addition of 1 mM NADPH, and the reaction mixture was incubated at 37 °C for 10 min. Aliquots of the reaction mixture were extracted with four volumes of chloroform/methanol (3:1 v/v) and analyzed by HPLC as mentioned above. The kinetic parameters, K_m and k_{cat}, were calculated by the nonlinear regression analysis according to the following equation using the Kaleida–Graph (Synergy Software).

$$v/E_0 = k_{cat} \cdot S/(K_m + S)$$

where S and E_0 are the substrate concentration and P450 contents in the microsomal fraction, respectively.

Other methods. Protein concentration was determined by the method of Lowry et al. [15], using bovine serum albumin as standard. A sequence alignment between PcCYP65a2 and human CYP1A2 was produced with ClustalW software (http://align.genome.jp/sit-bin/clustalw).

Results

Expression of PcCYP65a2 in the recombinant S. cerevisiae cells

The reduced CO difference spectra of whole cell and microsomal fraction of the recombinant *S. cerevisiae* cells expressing PcCYP65a2 showed a peak at approximately 452 nm indicating the presence of an active PcCYP65a2 (Fig. 1). The expression level of PcCYP65a2 was approximately 1 nmol/L culture. The content of PcCYP65a2 in the microsomal fraction was estimated to be 40 pmol/mg protein.

Metabolism of naringenin by recombinant yeast cells expressing PcCYP65a2, and identification of the metabolite

Metabolism of naringenin was examined by the addition of naringenin to the culture of the recombinant AH22/pG65a2 cells.

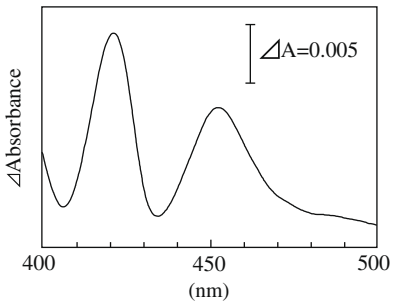

Fig. 1. Reduced CO difference spectrum of membrane fractions prepared from the recombinant *S. cerevisiae* AH22/pG65a2 cells. This spectrum showed a peak at approximately 452 nm indicating the presence of PcCYP65a2 hemoprotein.

Fig. 2A shows HPLC profile of naringenin and its metabolite by PcCYP65a2. The retention time of the metabolite was identical to that of authentic standard of eriodictyol. LC–MS analysis revealed that the molecular weight of the metabolite was 288 (naringenin + 16) (Fig. 2B), identical to that of authentic eriodictyol.

To confirm that the metabolite of naringenin by PcCYP65a2 is eriodictyol, we performed ^{1}H NMR analysis of naringenin and its metabolite. The peak pattern of the metabolite was identical to that of authentic standard of eriodictyol and similar to that reported previously [16]. Based on LC–MS and NMR analyses, the metabolite of naringenin was identified as eriodictyol.

Metabolism of DD, 2-MCDD, biphenyl, and naphthalene by recombinant S. cerevisiae cells expressing PcCYP65a2

Metabolism of DD, 2-MCDD, biphenyl, and naphthalene was examined by the addition of each substrate to the culture of the recombinant yeast cells expressing PcCYP65a2 as shown in Fig. 3. Judging from its retention time, the metabolite of DD by PcCYP65a2 appears to be 2-hydroxy-DD which is a major metabolite by human CYP1A2 as described in our previous report [17]. The peak of the metabolites of 2-MCDD appears to contain two hydroxylated compounds at 3 or 7 or 8 position, based on our previous study on human CYP1A2-dependent metabolism of 2-MCDD [17]. The HPLC profiles of biphenyl, naphthalene, and their metabolites formed by PcCYP65a2 were almost the same as those by human CYP1A2. Biphenyl was supposed to be hydroxylated at positions 2 and 4 as described by Haugen et al. [18]. Naphthalene metabolites were supposed to be 1-naphthol as a major metabolite and 2-naphthol as a minor one (Fig. 3D) [19].

Kinetic analysis of naringenin 3′-hydroxylation activity of PcCYP65a2

Kinetic analysis of PcCYP65a2-dependent 3′-hydroxylation of naringenin demonstrated that the k_{cat} and K_m values of PcCYP65a2 were 0.29 ± 0.02 min^{-1} and 391 ± 27 μM, respectively. The k_{cat} and K_m values represent means ± SD from three separate experiments.

Alignment of the sequences of PcCYP65a2 and human CYP1A2

Full-length PcCYP65a2 cDNA encodes 626 amino acid residues with a calculated molecular weight of 68.3 kDa which is the largest

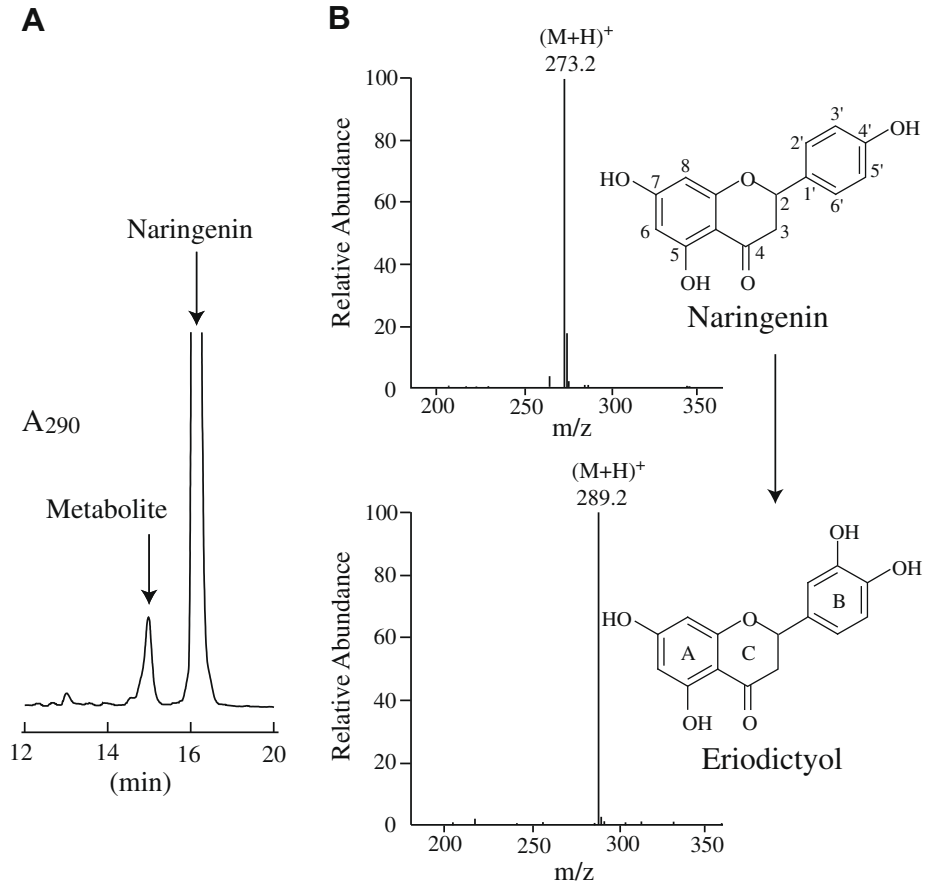

Fig. 2. HPLC profiles of naringenin and its metabolite formed in the culture of the recombinant *S. cerevisiae* AH22/pG65a2 cells expressing *Phanerochaete chrysosporium* PcCYP65a2 (A). Mass spectra of naringenin and its metabolite by PcCYP65a2 (B). Based on LC–MS and NMR analyses, the metabolite of naringenin was identified as eriodictyol.

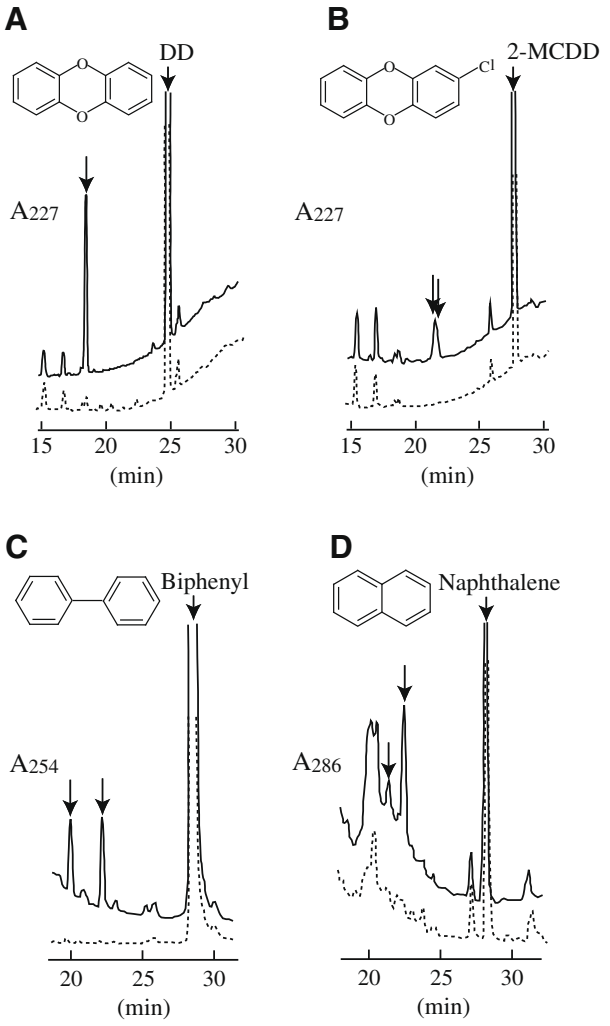

Fig. 3. HPLC profiles of substrates and their metabolites formed in the culture of the recombinant *S. cerevisiae* AH22/pG65a2 cells expressing PcCYP65a2. (A) DD, (B) 2-MCDD, (C) biphenyl, (D) naphthalene. The solid lines represent HPLC profiles formed in the culture of the recombinant *S. cerevisiae* AH22/pG65a2 cells. The dotted lines represent HPLC profiles formed in the culture of the control AH22/pGYR cells. Arrows represent metabolites.

in microsomal type *P. chrysosporium* P450s (Fig. 4). PcCYP65a2 and human CYP1A2 have less than 15% amino acid sequence identity. PcCYP65a2 contains typical motifs of P450 including distal Thr, proximal Cys, heme-binding domain, several helices and sheets. It is noted that PcCYP65a2 is 110 amino acid residues larger than human CYP1A2, and 6 insertions consisting of more than 8 amino acids are observed on the basis of human CYP1A2 sequence. Helices B and K contain long insertions consisting of 9 and 13 amino acid residues, respectively. The insertion between D and E helices consisting of 22 amino acid residues contains 4 proline residues, suggesting that it forms a loop structure at the molecular surface of PcCYP65a2. On the other hand, the insertion consisting of 14 amino acid residues between E and F helices contains one proline residue at a center of the insertion. Judging from amino acid sequence, both E and F helices of PcCYP65a2 are considerably longer than those of CYP1A2. Sansen et al. [20] revealed that human CYP1A2 has a compact and closed active site cavity to adapt a large planar substrate. Judging from enzymatic properties of PcCYP65a2, it seems likely that the active site cavity of PcCYP65a2 is similar to that of CYP1A2. It is noted that Phe 226 of CYP1A2, which plays an important role in substrate recognition, corresponds to Phe 290 of PcCYP65a2.

Discussion

In this study, we demonstrated that PcCYP65a2 converts naringenin to eriodictyol. To our best knowledge, this is the first report demonstrating the potentiality of *P. chrysosporium* P450 for the production of a useful food factor. NMR and LC–MS analyses strongly suggest that the metabolite is 3′-hydroxy-naringenin which is known as eriodictyol. Furthermore, we examined the activity by PcCYP65a2 using a microsomal fraction prepared from the recombinant yeast cells, and confirmed that PcCYP65a2 can produce eriodictyol from naringenin. Interestingly, human CYP1A2, CYP3A4 [21], and *Catharanthus roseus* CYP75 catalyze the same reaction [22].

Eriodictyol (5,7,3′,4′-tetrahydroxyflavanone), which is present in several fruits and vegetables, is particularly abundant in lemon [23]. These plants probably have P450 species to convert naringenin to eriodictyol as *C. roseus* has CYP75. A number of papers demonstrated that eriodictyol has various biological and pharmacological properties. Eriodictyol has an inhibitory effect toward several proteins ranging from enzymes, e.g. cytochrome P450 and myeloperoxidase, to ion channel, e.g. plasma membrane calcium pump [24]. Besides, it was demonstrated that eriodictyol had a lipid peroxidation inhibitory effect [25], and a protective effect on oxidative stress [23,24,26]. Flavonoids are generally regarded as one of the most important groups of naturally occurring antioxidants in our diet. Evans et al. [27] reported that the major metabolites of flavonoids have been found to exhibit an increased antioxidant capacity compared to parent compounds, especially when the metabolites have the dihydroxylated 3′,4′-moiety (catechol) of the B-ring. The catechol moiety does confer a higher stability to aroxy radicals and obviously participates in electron delocalization [28]. Moreover, it was reported that some flavonoids protected L-929 cells from TNF-induced cell death, and that eriodictyol was the most potent protective agent of all the flavonoids tested, while naringenin showed enhancement of TNF cytotoxicity [29]. These facts strongly suggest a great difference in the biological effect between naringenin and eriodictyol, and that the *o*-dihydroxy structure in the B-ring should be the active center for metal chelation, scavenging, and antioxidant activity. This is a major reason why we focused on the P450 species which has an ability to convert naringenin to eriodictyol in this study. It is noted that only minor alterations of the flavonoids structure can impact on the associated biological properties. Although many microbial enzymes show broad substrate specificity, they catalyze reactions with high degree of regio-and stereospecificity. Therefore, using microorganisms to introduce hydroxyl groups at specific positions on the flavonoid nucleus seems quite useful. However, from the viewpoint of practical applications, the activity of the recombinant *S. cerevisiae* cells expressing PcCYP65a2 must be increased by enhancing its activity and expression level.

It should be noted that PcCYP65a2 metabolizes DD, 2-MCDD, biphenyl, and naphthalene (Fig. 3). These results may suggest that PcCYP65a2 is involved in ligninolysis, and a physiologically important CYP in *P. chrysosporium*. On the other hand, the activity of this P450 toward polyaromatic hydrocarbons might be practically useful for bioremediation. The HPLC profiles of the metabolites by PcCYP65a2 shown in Fig. 3 were almost the same as those by human CYP1A2 (data not shown). Note that human CYP1A2 converts naringenin to eriodictyol as mentioned above. These facts strongly suggest that the substrate specificity of PcCYP65a2 closely resembles human CYP1A2. Thus, we compared the amino

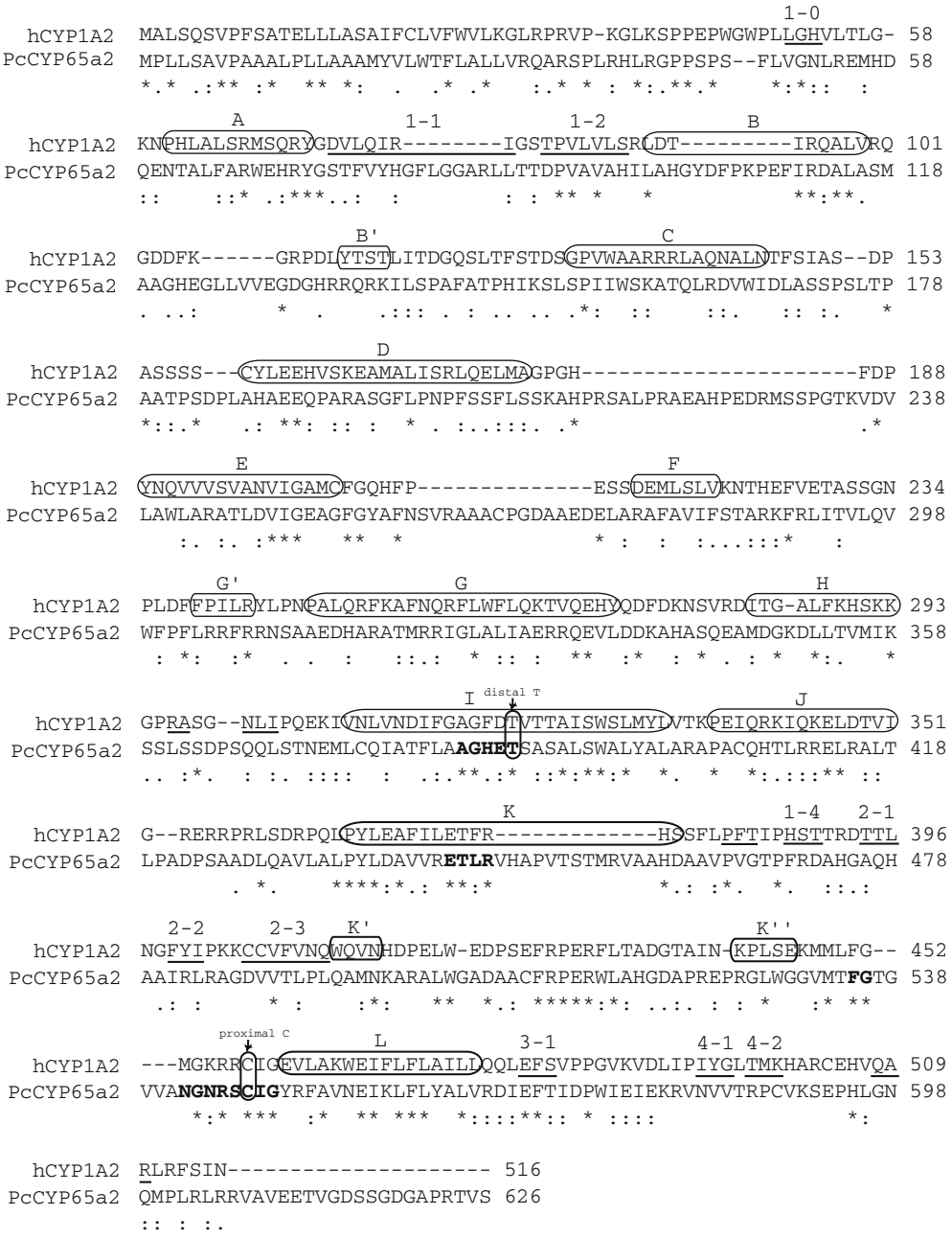

Fig. 4. Sequence alignment of human CYP1A2 and PcCYP65a2. CYP1A2 secondary structure elements are marked with circles (helices) and underline (sheets) and designated with letters (helices) and numbers (sheets) above the alignment. Well-conserved heme-binding, helix-I, and helix-K regions are shown in boldface.

acid sequence of PcCYP65a2 to that of human CYP1A2 whose crystal structure has already been determined [20]. Surprisingly, PcCYP65a2 consists of 626 amino acids. To our best knowledge, this is the largest CYP among the CYPs whose cDNAs are functionally expressed. As shown in Fig. 4, PcCYP65a2 contains typical motifs of P450 including distal Thr, proximal Cys, heme-binding domain, several helices, and sheets, but several insertions were observed as compared to human CYP1A2. Helices B and K contain long insertions, and the insertion of 5 amino acids (TGVVA) inside the heme-binding domain is uncommon, suggesting that PcCYP65a2 has a unique structure. Although we cannot explain the meanings of these insertions, some insertions might play an important role in the catalytic activity or interaction with NADPH-P450 reductase.

In conclusion, we revealed that PcCYP65a2 with its unique structure has a potential for the production of useful flavonoids and the degradation of polyaromatic pollutants.

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