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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Regioselective oxidation of indole- and quinolinecarboxylic acids by cytochrome P450 CYP199A2

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Abstract CYP199A2, a bacterial P450 monooxygenase from *Rhodopseudomonas palustris*, was previously reported to oxidize 2-naphthoic acid and 4-ethylbenzoic acid. In this study, we examined the substrate specificity and regioselectivity of CYP199A2 towards indole- and quinolinecarboxylic acids. The *CYP199A2* gene was coexpressed with palustrisredoxin gene from *R. palustris* and putidaredoxin reductase gene from *Pseudomonas putida* to provide the redox partners of CYP199A2 in *Escherichia coli*. Following whole-cell assays, reaction products were identified by mass spectrometry and NMR spectroscopy. CYP199A2 did not exhibit any activity towards indole and indole-3-carboxylic acid, whereas this enzyme oxidized indole-2-carboxylic acid, indole-5-carboxylic acid, and indole-6-carboxylic acid. Indole-2-carboxylic acid was converted to 5- and 6-hydroxyindole-2-carboxylic acids at a ratio of 59:41. In contrast, the indole-6-carboxylic acid oxidation generated only one product, 2-indolinone-6-carboxylic acid, at a rate of 130 mol (mol P450)$^{-1}$ min^{-1}. Furthermore, CYP199A2 also oxidized quinoline-6-carboxylic acid, although this enzyme did not exhibit any activity towards quinoline and its derivatives with a carboxyl group at the C-2, C-3, or C-4 positions. The oxidation product of quinoline-6-carboxylic acid was identified to be 3-hydroxyquinoline-6-carboxylic acid, which was a novel compound. These results suggest that CYP199A2 may be a valuable biocatalyst for the regioselective oxidation of various aromatic carboxylic acids.

Keywords CYP199A2 · Cytochrome P450 · Hydroxylation · Indole · Oxidation · Quinoline

Introduction

Cytochrome P450 monooxygenases (P450s) are a superfamily of heme-containing enzymes that introduce a single oxygen atom derived from molecular oxygen into an organic molecule. P450s are found in animals, plants, and microorganisms and catalyze a variety of reactions such as hydroxylation of aliphatic and aromatic carbons, oxidation of organic nitrogen and sulfur, epoxidation, and Baeyer–Villiger oxidation (Bernhardt 2006; Chefson and Auclair 2006). P450-catalyzed reactions play numerous physiological roles in oxidative metabolism of endogenous and exogenous compounds (Furuya et al. 2008; Ullrich and Hofrichter 2007; Urlacher and Eiben 2006).

Several P450s have been reported to oxidize *N*-heterocyclic aromatic compounds including indole and quinoline. For example, human CYP2A6 oxidized indole at the C-2, C-3, or C-6 positions to produce oxindole (2-indolinone), indoxyl (3-hydroxyindole), and 6-hydroxyindole, respectively, and the subsequent oxidation and dimerization of indoxyl resulted in pigments, indigo, and indirubin (Gillam et al. 2000). These pigments were produced by several P450s, including bacterial P450s such as $P450_{cam}$ and P450 BM-3 mutants (Celik et al. 2005; Li et al. 2000). Human CYP2A6 also oxidized quinoline to quinoline *N*-oxide, 3-hydroxyquinoline, 5-hydroxyquinoline, and 8-hydroxyquinoline (Dowers et al. 2004; Reigh et al. 1996). As for bacterial P450s, $P450_{cam}$ was reported to oxidize quinoline to 5-hydroxyquinoline

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(Dowers et al. 2004), and a P450 BM-3 mutant was reported to oxidize 8-methylquinoline to 5-hydroxy-8-methylquinoline (Appel et al. 2001).

N-heterocyclic aromatic compounds are important building blocks for the synthesis of valuable chemicals including biologically active compounds (Kiener 1995; Petersen and Kiener 1999; Yoshida and Nagasawa 2000). Especially, *N*-heterocyclic compounds with both carboxyl and hydroxyl groups are valuable, since the functional groups play important roles in biological activities and also can be utilized for further modification. However, regioselective synthesis of the compounds is difficult to perform by chemical methods. In contrast, several microorganisms can catalyze regioselective hydroxylation of *N*-heterocyclic carboxylic acids. For example, an industrial process for 6-hydroxylation of nicotinic acid was established using *Achromobacter xylosoxidans* cells (Kiener 1995). 2-Hydroxynicotinic acid, 6-hydroxypicolinic acid, and 5-hydroxypyrazine-2-carboxylic acid were also synthesized using microorganisms (Petersen and Kiener 1999; Yoshida and Nagasawa 2000). In addition to these hydroxylated *N*-hetero-monocyclic carboxylic acids, hydroxylated indole- and quinolinecarboxylic acids are potential intermediates for the preparation of pharmaceuticals, because indole and quinoline derivatives find use in numerous medicinal applications (Michael 2008; Sugden and Yoloye 1978). For example, 5-hydroxyindole-2-carboxylic acid amides are of great potential as histamine-3 receptor inverse agonists for the treatment of obesity (Pierson et al. 2009). A 6-methoxycarbonyl-substituted indolinone (BIBF 1120) is a triple angiokinase inhibitor currently in phase III clinical trials for the treatment of non-small cell lung cancer (Roth et al. 2009). Also, hydroxylated quinolinecarboxylate derivatives were reported to exhibit anti-hepatitis B virus activities (Liu et al. 2008). The P450s that catalyzes the insertion of oxygen into indole- and quinolinecarboxylic acids might provide a facile synthetic approach to hydroxylated or oxygenated indole- and quinolinecarboxylic acids and their derivative bioactive compounds. So far, however, there have only been a few reports concerning P450s exhibiting such activities, although several P450s were reported to oxidize indole and quinoline with no functional group as described above. CYP73A1, a plant P450 responsible for the hydroxylation of cinnamic acid in the phenylpropanoid pathway, has been reported to oxidize indole-2-carboxylic acid to 5-hydroxyindole-2-carboxylic acid and an unidentified compound (Schoch et al. 2003).

In this paper, we report the substrate specificity and regioselectivity of CYP199A2, a bacterial P450 from *Rhodopseudomonas palustris*, towards indole- and quinolinecarboxylic acids. Previously, we found that CYP199A2 oxidized 2-naphthoic acid to 7- and 8-hydroxy-2-naphthoic acids (Furuya and Kino 2009). CYP199A2 was also reported to oxidize 4-ethylbenzoic acid to 4-(1-hydroxyethyl)-benzoic acid and 4-vinylbenzoic acid (Bell et al. 2008). Here, we investigated the application of CYP199A2 in synthesis of oxygenated indole- and quinolinecarboxylic acids.

Materials and methods

Materials

2-Naphthoic acid, indole, indole-2-carboxylic acid, indole-3-carboxylic acid, indole-5-carboxylic acid, indole-6-carboxylic acid, quinoline, quinoline-2-carboxylic acid, quinoline-3-carboxylic acid, quinoline-4-carboxylic acid, and quinoline-6-carboxylic acid were purchased from Wako Pure Chemicals (Osaka, Japan). All other chemicals were of analytical grade.

Cloning of a physiological ferredoxin gene

Palustrisredoxin (Pux, GenBank accession number, CAE27313) is encoded by the gene (ORF number, RPA1872) next to the *CYP199A2* gene (RPA1871) in the *R. palustris* genome sequence (GenBank accession number, NC_005296). The *pux* gene was amplified from the genomic DNA of *R. palustris* (ATCC BAA-98) by polymerase chain reaction (PCR) using two oligonucleotide primers 5′-TTCCAT**ATG**CCCAGTATCACGTTCATTCTT-3′ (the *Nde*I restriction site is underlined and the initiation codon is indicated by boldface type) and 5′-CACAAGCTT**TCA**GACCTGACGATCCGGAAT-3′ (the *Hin*dIII restriction site is underlined and the termination codon is indicated by boldface type), and cloned into the vector pET21a (Novagen, Darmstadt, Germany) to construct pETpux. Previously, we constructed the plasmid pMWpdRpdx, which includes putidaredoxin (Pdx) and putidaredoxin reductase (PdR) genes from *Pseudomonas putida* in the vector pMW218 (Furuya and Kino 2009). In this study, we replaced the *pdx* gene in the plasmid with the *pux* gene. A fragment containing the *pux* gene was prepared from pETpux by digestion with *Xba*I and *Hin*dIII and inserted into pMW218 carrying the *pdR* gene to construct pMWpdRpux.

Preparation of whole cells and reactions

For expression of the genes, two plasmids, pET21a carrying the *CYP199A2* gene (pETCYP199A2), which was previously constructed (Furuya and Kino 2009), and pMWpdRpdx or pMWpdRpux were simultaneously introduced into *Escherichia coli* BL21 Star (DE3) cells (Invitrogen, Carlsbad, CA, USA). Whole cells of the recombinant *E. coli* strains were

prepared as described previously (Furuya and Kino 2009). The *CYP199A2* gene was expressed under the control of the T7 promoter and the redox partner genes under the control of the *lac* promoter. The expression of these genes was induced by addition of isopropyl-β-D-thiogalactopyranoside. The reaction mixture (250 μl) contained whole cells of a recombinant *E. coli* strain (50 g of wet cells per liter), a substrate (1 mM), and potassium phosphate buffer (50 mM, pH 7.5) containing glycerol (10% *v*/*v*). In a previous study, although we had added NADH to the reaction mixture (Furuya and Kino 2009), we confirmed that NADH had no effect on oxidation activity of the whole cells. Thus, in this study, NADH was excluded from the reaction mixture. Whole-cell reactions were performed at 30°C with shaking.

Measurement of protein and P450 concentration

The cells were suspended in the buffer and were disrupted with an ultraoscillator. After centrifugation at 15,000×*g* for 30 min at 4°C, the resulting cell-free extracts were used to measure protein and P450 concentration. Protein concentration was measured using a Coomassie protein assay kit (Pierce, Rockford, IL, USA) with a bovine serum albumin standard (Bradford 1976). P450 concentration was measured based on CO-reduced difference spectra using an extinction coefficient of 91 mM^{-1} cm^{-1} at 450 nm (Omura and Sato 1964).

Product analysis

High-performance liquid chromatography (HPLC) analysis was performed using a HPLC system (1100 series, Agilent, Palo Alto, CA, USA) with an XTerra MS C18 IS column (4.6 mm × 20 mm; particle size, 3.5 μm; Waters, Milford, MA, USA) as described previously (Furuya and Kino 2009). In brief, the reaction mixture was acidified by the addition of HCl (pH 1.5–2) and was extracted with ethyl acetate (1 ml). The mixture following the reaction with quinoline-6-carboxylic acid was treated with an ultraoscillator to disrupt the cells before the extraction. The extract (800 μl) was evaporated and the resulting residue was dissolved in a water/methanol mixture at a ratio of 50:50 (400 μl). The samples (10 μl) were injected into the HPLC system. Mobile phases A and B were composed of an acetonitrile/methanol/potassium phosphate buffer (10 mM, pH 2.7) mixture at a ratio of 2.5:2.5:95 and acetonitrile, respectively. The samples were eluted with 0% B for 3 min, followed by a linear gradient of 0% to 70% B for 9 min at a flow rate of 1 ml min^{-1}. Compounds were detected spectrophotometrically at 220 nm. The reaction products were isolated using the HPLC system with a fraction collector (1200 series, Agilent) and an XTerra MS C18 column (4.6 mm × 250 mm; particle size, 3.5 μm; Waters) as described previously (Furuya and Kino 2009). All reaction products were commercially unavailable and were identified by mass spectrometry and NMR spectroscopy. The amounts of reaction products were calculated from standard calibration curves that were made using the compounds isolated in this study. Mass analysis was performed using a Thermo Finnigan LTQ FT (Waltham, MA, USA) with an electrospray ionization source (Furuya and Kino 2009). NMR analysis was performed using a Bruker ADVANCE600 (Billerica, MA, USA; Furuya and Kino 2009).

Results

Coexpression of the *CYP199A2* gene with the physiological ferredoxin gene

Previously, we found that CYP199A2 oxidized 2-naphthoic acid to 7- and 8-hydroxy-2-naphthoic acids (Fig. 1a). In whole-cell assays, the *CYP199A2* gene was coexpressed with the *pdx* and *pdR* genes from *P. putida* to provide the redox partners of CYP199A2 in *E. coli*. In this study, instead of the *pdx* gene, the physiological ferredoxin gene, *pux*, which exists next to the *CYP199A2* gene in the *R. palustris* genome sequence, was cloned and coexpressed with the *CYP199A2* and *pdR* genes.

The quantity of CYP199A2 produced in *E. coli*, as determined by CO difference spectroscopy, was almost the same between the recombinant *E. coli* strain carrying the *CYP199A2*, *pdx*, and *pdR* genes (0.16 μmol per liter of culture) and that carrying the *CYP199A2*, *pux*, and *pdR* genes (0.17 μmol per liter of culture). In addition, the expression levels of the ferredoxin (Pdx or Pux) and PdR, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, were almost the same between the two recombinant *E. coli* strains (data not shown). The whole cells (50 g of wet cells per liter) of the recombinant *E. coli* strain carrying the *CYP199A2*, *pux*, and *pdR* genes converted 1 mM 2-naphthoic acid to 0.29 mM 7-hydroxy-2-naphthoic acid and 0.44 mM 8-hydroxy-2-naphthoic acid in 90 min (Fig. 2). The initial rate of 2-naphthoic acid hydroxylation, which was estimated for the first 10 min reaction, was 13 mol (mol P450)$^{-1}$ min^{-1} (290 nmol (g of wet cells)$^{-1}$ min^{-1}). This rate was approximately 1.5 times higher than that obtained by the whole cells carrying the *pdx* gene instead of the *pux* gene (Fig. 2), which was 9.4 mol (mol P450)$^{-1}$ min^{-1} (190 nmol (g of wet cells)$^{-1}$ min^{-1}; Furuya and Kino 2009). Thus, the recombinant *E. coli* strain carrying the *pux* gene as ferredoxin gene was used hereafter.

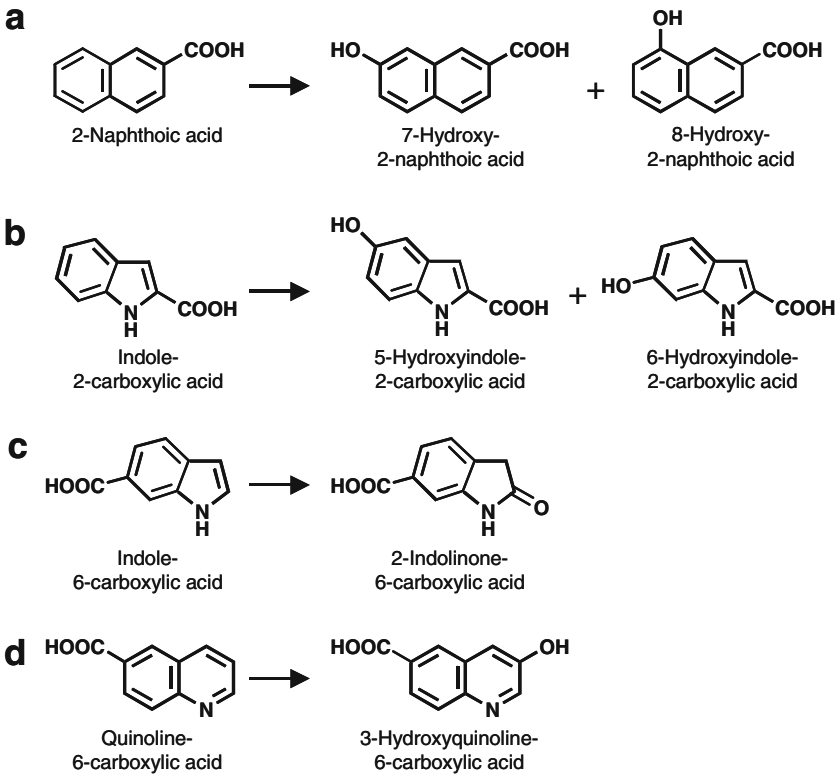

Fig. 1 Biocatalytic oxidation of 2-naphthoic acid (**a**), indole-2-carboxylic acid (**b**), indole-6-carboxylic acid (**c**), and quinoline-6-carboxylic acid (**d**) by CYP199A2

Substrate specificity and regioselectivity of CYP199A2 towards indolecarboxylic acids

We examined the substrate specificity of CYP199A2 towards indolecarboxylic acids. Whole-cell assays were performed with indole, indole-2-carboxylic acid, indole-3-carboxylic acid, indole-5-carboxylic acid, and indole-6-carboxylic acid as substrates.

As shown in Fig. 3a, HPLC analysis of the reaction of CYP199A2 with indole-2-carboxylic acid showed two major peaks (retention times, 3.0 min and 5.6 min) besides the substrate peak (7.1 min). These new peaks were not detected following the reaction with the recombinant *E. coli* cells carrying the void vector pET21a and pMWpdRpux. The compounds corresponding to the two peaks were isolated using a HPLC fraction collection system. These two compounds were confirmed to be monooxygenation products of indole-2-carboxylic acid based on the precise determination of their mass values (calcd for $C_9H_6O_3N$ $[M\text{-}H]^-$: 176.0348, found: 176.0348 and 176.0350 for the compounds at 3.0 min and 5.6 min, respectively). Furthermore, the products were analyzed by ^1H NMR, ^{13}C NMR, and two-dimensional NMR spectroscopy. In the correlation spectroscopy (COSY) spectrum of the product at 3.0 min (supplementary material, Table S1), only one ^1H–^1H coupling between H-6 and H-7 was observed, suggesting that the substrate was oxidized at the C-5 position. In the heteronuclear multiple bond correlation (HMBC) spectrum (Table S1), ^1H–^{13}C long-range couplings from H-7 to C-3a and C-5 were observed. Based on these observations, this product was identified to be 5-hydroxyindole-2-carboxylic acid (Fig. 1b). All other information from the spectral data was consistent with the molecular structure. Similarly, in the COSY spectrum of the product at 5.6 min (supplementary material, Table S2), only one ^1H–^1H coupling between H-4 and H-5 was observed, suggesting that the substrate was oxidized at the C-6 position. In the HMBC spectrum (Table S2), ^1H–^{13}C long-range couplings from H-4 to C-3, C-7a, and C-6 were observed. Based on these observations,

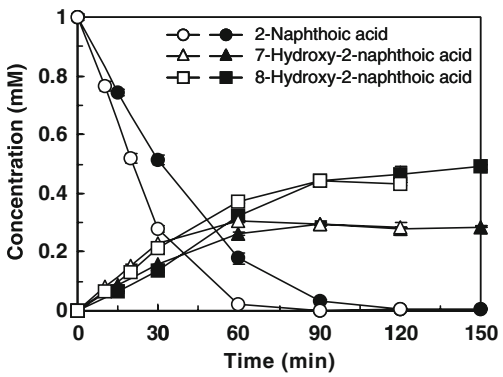

Fig. 2 2-Naphthoic acid oxidation by whole cells of the recombinant *E. coli* strain coexpressing Pux (*open symbols*) or Pdx (*closed symbols*, Furuya and Kino 2009) with CYP199A2 and PdR. The plot represents the average of three independent experiments, with *error bars* representing the standard deviation from the mean

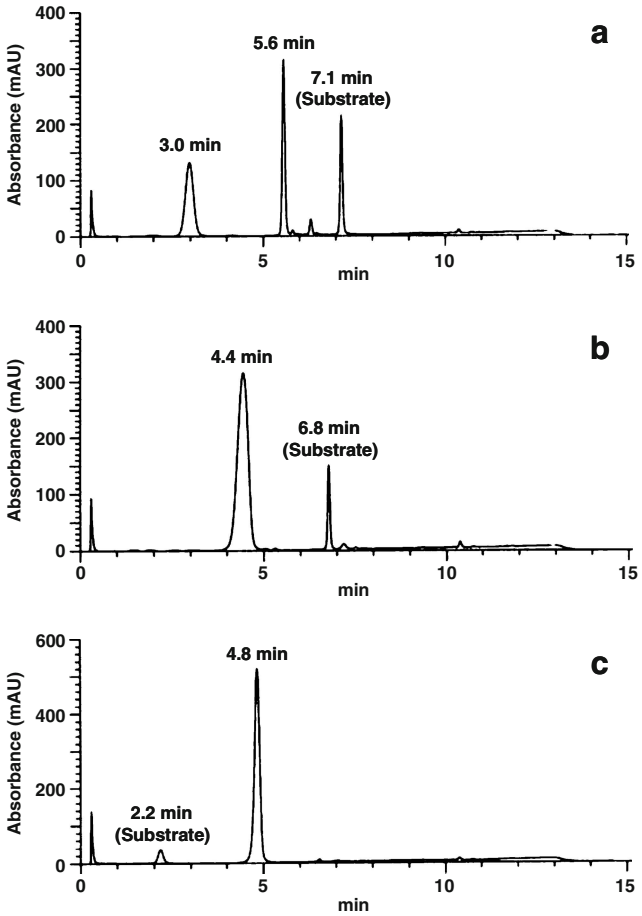

Fig. 3 HPLC chromatograms of the reactions of CYP199A2 with indole-2-carboxylic acid (**a**), indole-6-carboxylic acid (**b**), and quinoline-6-carboxylic acid (**c**). In **a**, peaks at 3.0 min and 5.6 min were found to correspond to 5- and 6-hydroxyindole-2-carboxylic acids, respectively. In **b**, a peak at 4.4 min was found to correspond to 2-indolinone-6-carboxylic acid. In **c**, a peak at 4.8 min was found to correspond to 3-hydroxyquinoline-6-carboxylic acid

this product was identified to be 6-hydroxyindole-2-carboxylic acid (Fig. 1b).

Indole-6-carboxylic acid was also transformed by CYP199A2. HPLC analysis of the reaction of CYP199A2 with indole-6-carboxylic acid showed one major peak (retention time, 4.4 min) besides the substrate peak (6.8 min; Fig. 3b). The compound corresponding to the new peak was confirmed to be a monooxygenation product of indole-6-carboxylic acid by mass spectrometry (calcd for $C_9H_6O_3N$ $[M\text{-}H]^-$: 176.0348, found: 176.0350). In the COSY spectrum (supplementary material, Table S3), only one ^1H–^1H coupling between H-4 and H-5 was observed, suggesting that the substrate was oxidized at the C-2 or C-3 position. In the HMBC spectrum (Table S3), the correlations of H-3 with C-2, C-3a, C-4, and C-7a were observed. Furthermore, the ^1H NMR spectrum showed the presence of two protons at the C-3 position and the upfield shift of the H-3 protons (3.56 ppm), whereas the ^{13}C NMR spectrum exhibited the downfield shift of C-2 (184.3 ppm) and the upfield shift of C-3 (44.0 ppm). These observations indicated that the substrate was oxidized at the C-2 position to form a carbonyl group. Thus, this product was identified to be 2-indolinone-6-carboxylic acid (Fig. 1c).

CYP199A2 exhibited low activity towards indole-5-carboxylic acid, since a small peak corresponding to a monooxygenation product was detected by HPLC-mass spectrometry (data not shown). In contrast, CYP199A2 did not exhibit any activity towards indole and indole-3-carboxylic acid.

Substrate specificity and regioselectivity of CYP199A2 towards quinolinecarboxylic acids

We examined the substrate specificity of CYP199A2 towards quinolinecarboxylic acids. Whole-cell assays were performed with quinoline, quinoline-2-carboxylic acid, quinoline-3-carboxylic acid, quinoline-4-carboxylic acid, and quinoline-6-carboxylic acid as substrates.

As shown in Fig. 3c, HPLC analysis of the reaction of CYP199A2 with quinoline-6-carboxylic acid showed one major peak (retention time, 4.8 min) besides the substrate peak (2.2 min). The compound corresponding to the new peak was confirmed to be a monooxygenation product of quinoline-6-carboxylic acid by mass spectrometry (calcd for $C_{10}H_6O_3N$ $[M\text{-}H]^-$: 188.0348, found: 188.0350). In the COSY spectrum (supplementary material, Table S4), only one ^1H–^1H coupling between H-7 and H-8 was observed, suggesting that the substrate was oxidized at the C-3 position. In the HMBC spectrum (Table S4), the correlations of H-2 with C-3, C-4, and C-8a and H-4 with C-2, C-3, C-4a, C-5, and C-8a were observed. Based on these observations, this product was identified to be 3-hydroxyquinoline-6-carboxylic acid (Fig. 1d).

In contrast, CYP199A2 did not exhibit any activity towards quinoline, quinoline-2-carboxylic acid, quinoline-3-carboxylic acid, and quinoline-4-carboxylic acid.

Synthesis of oxygenated indole- and quinolinecarboxylic acids by CYP199A2 whole-cell catalyst

We found that CYP199A2 catalyzed the regioselective oxidation of indole-2-carboxylic acid, indole-6-carboxylic acid, and quinoline-6-carboxylic acid. We investigated the application of CYP199A2 whole-cell catalyst in synthesis of the oxygenated indole- and quinolinecarboxylic acids.

As shown in Fig. 4a, the CYP199A2 whole-cell catalyst (50 g of wet cells per liter) converted 1 mM indole-2-carboxylic acid to 0.54 mM 5-hydroxyindole-2-carboxylic acid and 0.38 mM 6-hydroxyindole-2-carboxylic acid at a ratio of 59:41 in 180 min. The ratio between the two products was constant and independent of reaction time.

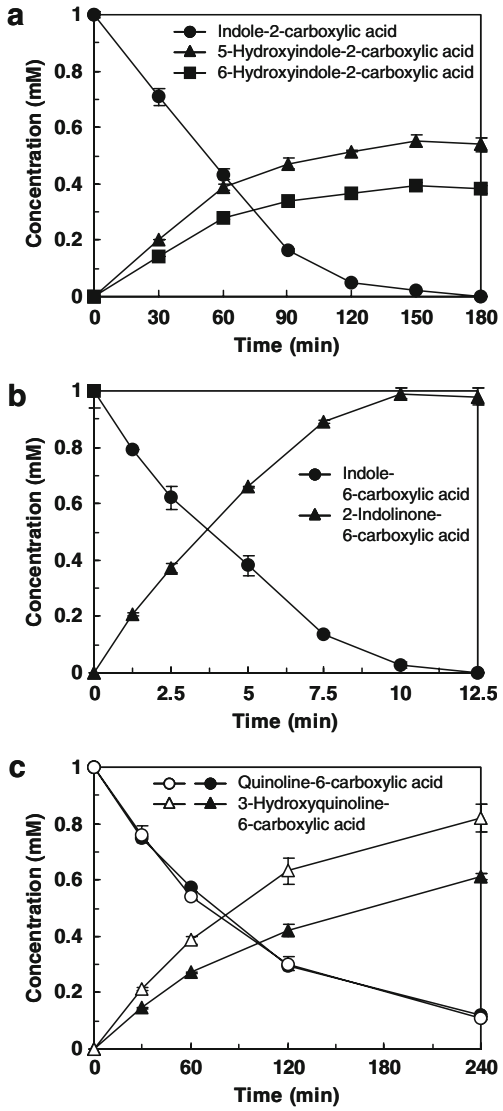

Fig. 4 Oxidation of indole-2-carboxylic acid (**a**), indole-6-carboxylic acid (**b**), and quinoline-6-carboxylic acid (**c**) by CYP199A2 whole-cell catalyst. In **c**, samples prepared with or without disruption of the cells (*open* and *closed symbols*, respectively) are shown. The plot represents the average of three independent experiments, with *error bars* representing the standard deviation from the mean

The initial rate of indole-2-carboxylic acid hydroxylation was estimated to be 10 mol (mol P450)$^{-1}$ min^{-1} (230 nmol (g of wet cells)$^{-1}$ min^{-1}).

In contrast to indole-2-carboxylic acid, the indole-6-carboxylic acid oxidation generated only one product. The CYP199A2 biocatalyst stoichiometrically converted 1 mM indole-6-carboxylic acid to 2-indolinone-6-carboxylic acid in 10 min (Fig. 4b). The initial rate of the oxidation was estimated to be 130 mol (mol P450)$^{-1}$ min^{-1} (3,000 nmol (g of wet cells)$^{-1}$ min^{-1}). The oxidation rate of indole-6-carboxylic acid was 13 times higher than that of indole-2-carboxylic acid.

Similarly, the CYP199A2 biocatalyst consumed 0.89 mM quinoline-6-carboxylic acid to produce 0.82 mM 3-hydroxyquinoline-6-carboxylic acid in 240 min (Fig. 4c). The initial rate of the hydroxylation was estimated to be 6.5 mol (mol P450)$^{-1}$ min^{-1} (140 nmol (g of wet cells)$^{-1}$ min^{-1}). When the whole-cell reaction mixtures were directly extracted with ethyl acetate, the conversion yields appeared to be low (Fig. 4c). However, by disrupting the cells before the extraction, the product was sufficiently recovered. There are some possibilities that 3-hydroxyquinoline-6-carboxylic acid produced in the cells might encounter difficulties in permeating cell membranes outside or interact with cell components with high affinity.

Discussion

CYP199A2, a bacterial P450 from *R. palustris*, was first reported by Bell et al. (2006) to exhibit activity towards *para*-substituted benzoic acids. They found that CYP199A2 oxidized 4-ethylbenzoic acid to 4-(1-hydroxyethyl)-benzoic acid and 4-vinylbenzoic acid (Bell et al. 2008). On the other hand, we previously screened bacterial P450s for hydroxylation activity towards 2-naphthoic acid and discovered CYP199A2 as 2-naphthoic acid monooxygenase (Furuya and Kino 2009). Characterization of the reaction products revealed that CYP199A2 oxidized 2-naphthoic acid to 7- and 8-hydroxy-2-naphthoic acids (Fig. 1a). In this study, we examined the substrate specificity of CYP199A2 towards indole- and quinolinecarboxylic acids and found that the enzyme catalyzed the regioselective oxidation of indole-2-carboxylic acid, indole-6-carboxylic acid, and quinoline-6-carboxylic acid. This is, to our knowledge, the first report concerning oxidation of indole- and quinolinecarboxylic acids by a bacterial P450. CYP73A1 from plants was reported to exhibit activity towards some indole- and quinolinecarboxylic acids, although the reaction products were hardly characterized (Schalk et al. 1997; Schoch et al. 2003).

In a previous study, we confirmed that Pdx and PdR from *P. putida* functioned as the redox partners of CYP199A2 (Furuya and Kino 2009). In this study, the physiological ferredoxin, Pux, from *R. palustris* was used instead of Pdx. The oxidation rate of 2-naphthoic acid by the recombinant *E. coli* cells coexpressing Pux with CYP199A2 and PdR was approximately 1.5 times higher than those coexpressing Pdx (Fig. 2). These results indicated that the physiological ferredoxin Pux coupled with CYP199A2 more effectively than Pdx. It was also reported that the oxidation rate of 4-ethylbenzoic acid by CYP199A2 with Pux was much higher than that with Pdx in vitro (Bell et al. 2008).

CYP199A2 oxidized indole-2-carboxylic acid to generate two products, 5- and 6-indole-2-carboxylic acids (Fig. 1b), as this enzyme generated two products, 7- and 8-hydroxy-2-naphthoic acids, from 2-naphthoic acid. In contrast, the oxidation of indole-6-carboxylic acid and quinoline-6-carboxylic acid generated only one product, respectively. The oxidation product of indole-6-carboxylic acid was identified to be 2-indolinone-6-carboxylic acid (Fig. 1c), which is the more stable tautomer of 2-hydroxyindole-6-carboxylic acid. Quinoline-6-carboxylic acid was oxidized to 3-hydroxyquinoline-6-carboxylic acid (Fig. 1d), which was a novel compound. On the other hand, indole and quinoline with no carboxyl group were not oxidized by this enzyme. Overall, CYP199A2 seems to recognize *N*-hetero-bicyclic aromatic compounds with a carboxyl group at the positions corresponding to C-2, but not C-1, on the 2-naphthoic acid molecule, and oxidize a carbon atom on the rings with no carboxyl group. Furthermore, it is interesting to note that quinoline-2-carboxylic acid and quinoline-3-carboxylic acid, which include a carboxyl group on the pyridine rings, were not recognized as substrate by CYP199A2, although the molecular structures are very similar to 2-naphthoic acid and quinoline-6-carboxylic acid. It is likely that the nitrogen atom on the quinolinecarboxylic acid molecules would significantly affect the interactions between the compounds and CYP199A2.

The whole cells expressing CYP199A2 efficiently catalyzed the oxidation of the indole- and quinolinecarboxylic acids. The oxidation rates of indole-2-carboxylic acid, indole-6-carboxylic acid, and quinoline-6-carboxylic acid were estimated to be 10, 130, and 6.5 mol (mol P450)$^{-1}$ min^{-1}, respectively (Fig. 4). For comparison, the oxidation rates of indole by a P450 BM-3 mutant and quinoline by $P450_{cam}$ were reported to be 164 and 1.9 mol (mol P450)$^{-1}$ min^{-1}, respectively (Dowers et al. 2004; Li et al. 2000). The CYP199A2 whole-cell catalyst probably used endogenous NADH as electron donor in the recombinant *E. coli*. Construction of an NADH regeneration system using a dehydrogenase in the cells will be effective in enhancing the productivity for practical applications (Chefson and Auclair 2006).

In conclusion, we found that CYP199A2 catalyzed the regioselective oxidation of two indole- and one quinoline-carboxylic acids, besides *para*-substituted benzoic acids and 2-naphthoic acid. Recently, the crystal structure of CYP199A2 was reported by Bell et al. (2008). Among the interesting features, it was found that the substrate binding pocket was hydrophobic, with Ser97 and Ser247 being only polar residues. Computer docking of 4-ethylbenzoic acid into the active site suggested that the Ser97 and Ser247 side chains interacted with the substrate carboxylate oxygens to anchor the substrate. These findings concerning the substrate specificity and the crystal structure suggest that CYP199A2 should be an efficient biocatalyst for the oxyfunctionalization of various aromatic carboxylic acids.

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