Published in final edited form as:

J Biotechnol. 2009 January 1; 139(1): 12–18. doi:10.1016/j.jbiotec.2008.09.010.

Identification of amino acid residues involved in 4-chloroindole 3-hydroxylation by cytochrome P450 2A6 using screening of random libraries

Zhi-Gang Zhang^a, Yan Liu^a, F. Peter Guengerich^b, Johannes H. Matse^b, Jun Chen^c, and Zhong-Liu Wu^{a,*}

^aChengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, Sichuan 610041, People's Republic of China

^bDepartment of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146, USA

^cInstitute of Burns, Third Military Medical University, Chongqing, 400038, People's Republic of China

Abstract

Cytochrome P450 (P450) 2A6 is able to catalyze indole hydroxylation to form the blue dye indigo. The wild type P450 2A6 enzyme was randomly mutated throughout the whole open reading frame and screened using 4-chloroindole hydroxylation, a substituted indole selected from 30 indole compounds for enhanced color development. Mutants with up to 5-fold increases of catalytic efficiency (k_{cat}/K_m) and 2-fold increases in k_{cat} were selected after two rounds of screening. Important residues located both in (e.g., Thr305) and outside the active site (e.g., Ser224) were identified. The study utilized a better substrate for "indigo assay" to obtain new information on the structure-functional relationship of P450 2A6 that was not revealed by previous mutagenesis studies with this enzyme.

Keywords

cytochrome P450; P450 2A6; CYP2A6; monooxygenase; 4-chloroindole; indigo assay; indole hydroxylation; random mutagenesis; high-throughput screening

Cytochrome P450¹ (also termed "heme thiolate P450") [1] enzymes comprise a superfamily of monooxygenases that are of considerable interest as the most versatile redox enzymes known [2, 3]. Over the years, many aspects of this group of enzymes have been well addressed [2]. Molecular breeding and utilization in chemical synthesis of P450s are considered two of the emerging areas today [4–7]. In the case of human P450 2A6, a major

* Address correspondence to: Prof. Zhongliu Wu, Chengdu Institute of Biology, Chinese Academy of Sciences, 9 South Renmin Road, 4th Section, Chengdu, Sichuan 610041, P.R.China, Telephone:86-28-85221263, Fax:86-28-85222753, wuzhl@cib.ac.cn.

¹Abbreviations used: P450, cytochrome P450; NPR, NADPH-cytochrome P450 reductase; SRS, substrate recognition sites; PCR, polymerase chain reaction; epPCR, error-prone PCR; IPTG, *D*-isopropyl- β -thiogalactopyranoside; APCI, atmospheric pressure chemical ionization; WT, wild type; SM: P450 2A6 mutant N297Q; DM: mutant L240C/N297Q; TB, Terrific Broth; LB, Luria-Bertani; DMSO, dimethylsulfoxide.

enzyme involved in nicotine metabolism, extensive studies involving molecular breeding and novel protein kinase inhibitor synthesis have been done in one of our labs following the observation of blue pigment formation (indigo) from cultures that expresses bicistronic cytochrome P450 (P450) 2A6 [8–14]. Small libraries constructed from six randomized primers, each covering four positions of one of the proposed six substrate recognition sites (SRS) [15] were screened using a colorimetric reaction. Some mutants with enhanced activity were selected [11]. This screening method is generally called the "indigo assay" and considered as one of the few convenient solid phase high-throughput assays for oxygenases [16].

During this course of the work, we found that, for P450 2A6 wild type (WT), the blue color of indigo formation usually required additional 2–3 days at 4 °C to develop and was not well visualized in single colonies on plates. Mixed with the yellowish color of *Escherichia coli* cells, indigo blue usually appeared green or even brownish, which was unfavorable for high-throughput screening in the solid phase. Thus, an alternative substrate with more distinct color formation was desired.

Here we report for the first time the use of 4-chloroindole as the substrate in the "indigo assay" for considerably improved pigment formation and the search for enhanced mutants of P450 2A6 via another approach, random libraries constructed by error-prone PCR (epPCR) followed by staggered extension process (StEP) PCR [17]. Several mutants with improved catalytic activity were selected including some located in non-SRS regions.

Materials and methods

Chemicals

All of the substituted 1H-indoles used in this study were purchased from Aldrich Chemical Co. (Milwaukee, WI) or Acros Organics (Geel, Belgium). All the other chemicals and solvents were obtained from commercial suppliers and used without further purification.

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Analytical methods

UV-visible spectra were recorded using an Aminco DW2a/OLIS spectrophotometer.

HPLC was done on a Beckman Ultrasphere HPLC column (4.6 mm × 250 mm, 5 μm) with a CH₃CN/H₂O mobile phase. A linear gradient of 50 to 80 % CH₃CN (v/v, in H₂O) over 30 min was used with a flow rate of 1 ml/min. Detection was at 598 nm using an on-line HPLC/diode array detector system.

Mass spectra were obtained via direct infusion using a Finnigan TSQ 7000 triple quadrupole mass spectrometer (Thermo Electron Corp., San Jose, CA) equipped with a standard API-1 atmospheric pressure chemical ionization (APCI) source in the negative ion mode. N₂ was used as a sheath gas (50 p.s.i.). The vaporizer temperature was set to 500 °C, and the corona current was maintained at 5 μA. The capillary was set at 220 °C and –25 V. The tube lens

voltage was set to -96 V. Data acquisition and spectral analysis were conducted with Finnigan ICIS software on a Digital Equipment Alpha workstation.

NMR experiments were carried out at 298 K on Bruker Avance™ DRX 400 MHz instruments (Bruker, Billerica, MA). All samples were dissolved in d_6 -DMSO and semi-quantitative estimates of the amounts of samples were made by adding known amounts of $\text{CH}_3\text{CO}_2\text{H}$ and referencing to this internal $-\text{CH}_3$ signal. All NMR data was processed using XWIN-NMR™ software on an Octane workstation (Silicon Graphics, Mountain View, CA).

Strains and plasmids

E. coli DH5 α competent cells were prepared using a standard RbCl protocol [18]. The *trnA*[−] *E. coli* HME5 was developed by our lab using gene-replacement techniques [12] and designated as *E. coli* TRN5. *E. coli* DH10B ultracompetent cells were purchased from Invitrogen (Carlsbad, CA). All of the wild-type (WT) and mutant P450 2A6 enzymes were expressed together with human NADPH-cytochrome P450 reductase (NPR) using a “bicistronic” pCW' vector (pCW2A6bc) [19].

Nucleotide sequence analysis

Sequencing of the plasmid DNA encoding 2A6 mutants was performed using an Applied Biosystems Model 3700 fluorescence sequencing unit with a *Taq* dye terminator kit (Applied Biosystems, Foster City, CA) in the Vanderbilt facility. The mutated sequences were verified by comparing the sequences of the sense and antisense strands (entire open reading frame).

Dye formation from substituted indoles in liquid cultures

E. coli TRN5 cells expressing P450 2A6 were incubated for 5 h at 30 °C in 5 ml of Terrific Broth (TB) expression medium before being fortified with 0.1–1.0 mM of the indoles (each dissolved in DMSO). TB expression medium contained 48 g of Terrific Broth/liter (BD Biosciences), 2.0 g of Bacto-peptone/liter (BD Biosciences), 0.4% (v/v) glycerol, 0.5 mM 5-aminolevulinic acid, 100 μg of ampicillin/liter, 1.0 mM thiamin, 1.0 mM isopropyl- β -D-thiogalactopyranoside (IPTG), 0.025% (v/v) trace elements mixture (2.7 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.2 g of $\text{ZnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.2 g of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.2 g of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.1 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g of CuCl_2 , 0.05 g of H_3BO_3 , 10 ml of 30% HCl in a 100 ml aqueous solution), and 1% (v/v) starter cultures that had been grown with each newly transformed *E. coli* cells in LB medium (BD Biosciences) containing 100 μg of ampicillin/liter for 16 h at 32 °C with gyratory shaking at 200 rpm.

Dye formation from substituted indoles on plates

The *E. coli* TRN5, DH5 α or DH10B cells expressing P450 2A6 were spread on LB agar expression plates, incubated at 28°C or 30 °C for 48 h and stored at 4 °C. LB agar expression plates contained 25 g of LB Broth /liter (BD Biosciences), 15 g of Bacto agar/liter (BD Biosciences), 100 μg of ampicillin/liter, 0.4–1.0 mM IPTG, 0.025% (v/v) trace elements mixture (*vide supra*), 0.5 mM 5-aminolevulinic acid, and 0.1–0.5 mM substituted indoles.

CLB plates contained 25 g of LB Broth /liter, 15 g of Bacto agar/liter, 100 µg of ampicillin/liter, 0.4 mM IPTG, 0.025% (v/v) trace elements mixture (*vide supra*), 0.5 mM 5-aminolevulinic acid, and 0.1 mM 4-chloroindole.

Construction of P450 2A6 libraries

The library construction has been described previously [ref #]. Briefly, The open reading frame region (1.5 kb) of the P450 2A6 wild type or mutant J1 containing T305S mutation, was mutated using a low fidelity PCR method (Mutazyme®, Stratagene, La Jolla, CA) with forward primer 5'-TAGGAGGTCATATGGCTGCT-3' and reverse primer 5'-ATTTCTAGACCGGAAGGCTT-3'.

A StEP PCR method was performed for recombination of the mutants selected from epPCR library in a 50-µl PCR mixture containing 25 ng of plasmid mixture, 120 ng of forward and reverse primer (same as that in epPCR), 2.5 units of Taq DNA polymerase, 0.2 mM each dNTP, and 1× Taq reaction buffer, with a program of 5 min/95 °C, 80 cycles of 30 s/95 °C and 5 s/55 °C.

The 1.5-kb amplified PCR library fragment was purified by agarose gel electrophoresis and cloned into the pCW2A6bc vector using the *Nde* I and *Xba* I restriction sites. The ligation mixture was purified and transformed into *E. coli* DH10B ultracompetent cells by electroporation and library plasmid DNA was purified using a QIAprep® miniprep kit (Qiagen Inc.).

Phenotypic selection based on color formation

The bicistronic library DNA was transformed into *E. coli* TRN5, plated on CLB plates in a large bioassay Q tray (25×25 cm; Genetix, Hampshire, UK), and allowed to grow at 28 °C for 2 days. Individual colonies with blue color brighter than wild type P450 2A6 were picked and grown in 5 ml of LB medium containing ampicillin (100 µg/ml) for 15 h at 32 °C. Plasmid DNA was prepared from the cultures using a QIAprep® miniprep kit.

Expression of P450 2A6 mutants and preparation of membranes

E. coli TRN5 cells were incubated in TB expression medium at 28 °C for 14 h with gyratory shaking at 200 rpm and harvested with centrifugation at 5×10^3g for 30 min. Bacterial inner membranes containing P450 2A6 variants and NPR were isolated from 500 ml of TB expression medium of *E. coli* TRN5. The pellets were resuspended in a final volume of 10 ml of 50 mM Tris acetate (pH 7.4) buffer containing 250 mM sucrose and 0.25 mM EDTA and homogenized with a Dounce homogenizer. The P450 concentrations of the homogenized membrane fractions were measured using the reduced-CO versus reduced difference spectra [20], and the membranes were stored at -80 °C or used directly.

Steady-state kinetic assays for indole and 4-chloroindole hydroxylation

The reaction mixtures included 100 pmol of P450, 100 mM potassium phosphate buffer (pH 7.4), and varying concentrations of substrate in a total volume of 1.0 ml. Reactions (at 37 °C) were initiated by the addition of an NADPH-generating system composed of (final concentrations) 0.5 mM NADPH, 10 mM glucose 6-phosphate, and 1.0 IU yeast glucose 6-

phosphate dehydrogenase ml^{-1} [21] and were quenched by the addition of 0.1 ml of 0.5 M aqueous NaOH after 10 min. The formation of colored products was measured by recording absorbance spectra in the visible region, and the estimated concentrations were calculated based on the extinction coefficient of the major product [13]. Estimates of k_{cat} and K_{m} were made using nonlinear regression with GraphPad Prism 4.

Results

Screening system development

To find a compound more suitable as a substrate in "indigo assay", thirty substituted indoles (Fig.1) were subjected to biotransformation catalyzed by P450 2A6 expressed in *E. coli* TRN5. Among them, 4- and 7-nitroindoles could be tolerated at a concentration of 1 mM when being added at the beginning of the incubation, while 5- and 6-nitroindoles hindered the cell growth significantly at the same conditions and only worked when they were introduced 3–5 hours after incubation. All the halogen-substituted indoles showed some cytotoxicity towards the bacteria, with the potency of the groups following the halide order, $\text{I} > \text{Br} > \text{Cl} > \text{F}$. The working concentrations in liquid culture were 1 mM for fluoro-substituted compounds, 0.5 mM for chloro- and bromo-substituted compounds, and 0.2 mM for iodo-substituted compound when they were introduced 3–5 hours after incubation. Other compounds did not show cytotoxicity at a test concentration of 1 mM.

Cell pellets or extracts from the 30 indoles all showed colors with very different patterns [11]. Among them some were more intense, e.g. the 4- CH_3 , 4- CO_2CH_3 , 4- Cl , 4- Br , 7- F , 7- CH_3 indigoids (all of the above showed blue color), the 6- CH_3 , 6- F , 5- CN , 5- NO_2 indigoids (purple), 5- OCH_3 , 6- NO_2 (green), and the 7- NO_2 , 5- CO_2CH_3 indigoids (red). We then analyzed the behavior of these indoles directly on agar plates and inspected them visually. The color blue was easier to visualize than others because the rods in human eyes are more sensitive to blue-green photons and less sensitive to red photons. We picked 4-chloroindole over the others because of the vivid color of its products when mixed with yellowish-colored *E. coli* cells, the relatively small size relative to indole, lower toxicity than the bromo-substituted analogue, better solubility in water than methyl-substituted analogues, and relatively inert nature of the substituent compared with 4- CO_2CH_3 - or CN during whole-cell biotransformations.

E. coli strains TRN5, DH5a, and DH10B were tested in plate assays. All three strains were able to express both P450 2A6 and NPR and performed well in this assay. To prepare membranes without background color for later stage steady-kinetic assays, *E. coli* TRN5 (tryptophanase deleted [12]) was selected as the host strain in this work. Cell growth was hindered significantly if 4-chloroindole was used with a concentration 0.5 mM in the solid medium. A decreased concentration of 0.1–0.2 mM was found to be suitable for this assay. The resulting formula was designated CLB, indicating LB expression medium fortified with 4-chloroindole. Color formation on indigo (A) and CLB plates (B) is shown in Fig. 2. Single colonies on CLB plates developed blue color after two days incubation, while those on indigo plates required additional 2–3 days at 4 °C to develop visible blue color. The color difference was also very obvious in liquid medium fortified with indole or 4-chloroindole in 24-well plates (Figs. 2C and 2D).

Characterization of colored product generated from 4-chloroindole

The cell extracts from *E. coli* TRN5 cultures fortified with 4-chloroindole were analyzed by HPLC (598 nm). One main peak (>95% of total area, t_R 23 min) was detected (Fig. 3). The mass spectrum of the isolated product showed a base peak at m/z 330 (in the negative ion mode) and with an expected dichloride ratio of 9:6:1 for the peaks at m/z 330, 332, and 334. The NMR spectrum showed a symmetrical structure, typical of an indigo [STATE THE CHEMICAL SHIFTS, J VALUES IN THE TEXT]. This product, assigned as 4,4'-dichloroindigo, gave an absorbance maximum at 620 nm in DMSO and 640 nm in H_2O . With an extinction coefficient of $13.2 \text{ mM}^{-1}\text{cm}^{-1}$ at 640 nm, it was more than 3-fold darker compared to indigo, which has an extinction coefficient of $3.9 \text{ mM}^{-1}\text{cm}^{-1}$ in water [22].

The exclusive formation of an indigo from 4-chloroindole, instead of an indigo plus an indirubin as in the case of indole biotransformation, can be explained by the substituent group at 4-position. Indirubin was the unfavorable product isomer in this case because the substituent at the 4-position would cause a steric clash with the carbonyl group in the 3'-position on indirubin skeleton [12, 23]. Such specific product formation contributed to good color development and the consistency of the color pattern, reduced the complexity of the reaction and is an advantage when quantitative analysis was required.

First-round library screening

epPCR was used to generate a randomized libraries with a mutation rate of 0–3 mutations per 10^3 bp using P450 2A6 WT as a template. A total number of $\sim 2 \times 10^4$ colonies were screened on CLB plate, and 24 blue colonies more intense than wild type were picked and sequenced as listed in Table 1. A single mutant (N297Q), designated as SM and obtained from previous work by one-round of mutagenesis, was also included in this table for comparison purpose (Table 1, entry 2). Among the mutants picked in this work, 16 had at least one residue change in one of the six SRS regions and 8 had none. Thirty-four mutation sites were identified, with some appearing more than once. A T305S mutation, located in SRS region 4, was selected for six times, yielding mutants H2, H4, J1, J2, J10, and J13 (Table 1, entries 13, 14, 18–20 and 22) with the nucleotide sequence changing from ACC to TCC (4 times) or ACC to AGC (twice); V87I, located in a non-SRS region, was selected for three times with mutants I4, J12, and J14 (Table 1, entries 17, 21, and 23); L206Q and F209I, located in the SRS region 2 were both selected twice with mutants D2, D5 and E7, H5 (Table 1, entries 6, 7, 10, and 15). This did not seem to indicate a result of saturated screening because they were picked out as different variants containing the corresponding changes, not absolutely equal.

The intensity of the dye formed on the plates was correlated to both the catalytic activity and the expression level of the mutants. Fe^{2+} -CO vs Fe^{2+} difference spectroscopy indicated that whole cell expression levels of P450 2A6 varied among the mutants (Table 1). The expression levels of mutants selected in this paper were generally higher than wild type, which might result in more product formation and an increased chance for selection. Another possibility was that some positions might be more sensitive to mutation under epPCR conditions and being mutated more frequently.

Steady-state kinetic analysis of mutants selected from first-round metagenesis

Catalytic activity for 11 of the mutants, mutant SM, and wild type P450 2A6 were analyzed with membrane preparations. The rates of indole and 4-chloroindole 3-hydroxylation by P450 2A6 and the mutants were measured and normalized for P450 content (Table 2, Fig. 4). The catalytic efficiency of 4-chloroindole hydroxylation was similar to that of indole hydroxylation for wild type 2A6, but not for all the mutants. In most cases, a lower K_m was found for 4-chloroindole hydroxylation instead of indole.

For indole hydroxylation, mutant SM, containing an N297Q mutation in the SRS 3 region (Table 2, entry 2), had the highest k_{cat}/K_m value, ~ a 10-fold increase due to the dramatic decrease of K_m , but the k_{cat} was similar to that of wild type P450 2A6. However, this mutant had low 4-chloroindole hydroxylation activity. Only a moderate decrease of K_m was achieved, resulting in less than a two-fold increase in catalytic efficiency. Mutant J1, containing a T305S mutation in the SRS4 region (Table 2, entry 8), had the highest k_{cat} for both indole and 4-chloroindole, 2-fold over wild type and 2-fold and 3-fold increases in catalytic efficiency for indole and 4-chloroindole, respectively. The highest catalytic efficiency obtained in this work for indole hydroxylation was more than 4-fold compared with wild type P450 2A6, achieved with mutant J12 (containing mutations in the SRS 3 region, Table 2, entry 9), and more than a 3-fold increase for 4-chloroindole hydroxylation, achieved with mutant C6 (containing the mutation M18L in a non-SRS region, Table 2, entry 3) due to a decrease in K_m . Some mutations showed opposite effects on indole and 4-chloroindole hydroxylation. Mutants I4 and J14 (Table 2, entries 7 and 10), both containing only non-SRS mutations, had a 5-fold decrease in the K_m for 4-chloroindole—resulting in an increased catalytic efficiency—but for indole both had a decreased catalytic efficiency. Other mutations in non-SRS regions also affected the catalytic efficiency of P450 2A6. For indole hydroxylation, mutant J20 (Table 2, entry 12) showed ~ a 4-fold decrease in catalytic efficiency due to a significant increase in K_m , while mutants J17 and J22 (Table 2, entry 11 and 13), on the other hand, had > 2-fold increases in catalytic efficiency due to decreased K_m values.

Second-round library screening and steady-state kinetic analysis

Mutants selected from the first-round library were pooled and used as a template in a StEP PCR process to generate a second library. Mutant J1, containing mutation T305S, was also used as a template in the second-round epPCR. These two libraries were screened on CLB plates, and seven mutants were selected from a total of 8×10^3 colonies. The mutation positions and kinetic parameters for 4-chloroindole hydroxylation are shown in Table 3.

All of the mutants tested had decreased K_m values compared with WT and mutant DM P450 2A6 (L240C/N297Q), the best one selected from previous work [11] by two rounds of mutagenesis focused only on SRS regions. Their k_{cat} values were not necessarily higher, but their catalytic efficiencies were at least 2-fold > WT P450 2A6, or in some cases, >the DM mutant. Mutant St9, containing an L206Q mutation in the SRS 2 region and an S224P mutation in a non-SRS region, showed a 5-fold increase in catalytic efficiency compared with WT P450 2A6 due to a significant decrease in K_m (Table 3, entry 2). The addition of the non-SRS mutation S224P contributed to a 2.5-fold increase compared with mutation

L206Q alone (Table 2, entry 4). Other non-SRS mutations, e.g. M18I, S183C and Y287H (Table 3, entries 3 and 5) also appeared to contribute to a decrease of K_m and consequently an increase of catalytic efficiency. The effect was comparable with that caused by the SRS mutation L206Q (Table 3, entry 6).

Discussion

It is well documented that some monooxygenases and dioxygenases are able to catalyze the oxidation of indole to indoxyl and isatin, with subsequent coupling to produce indigo and indirubin pigments. The "indigo assay" is based on this principle and has been, in several cases, used as a general activity detection method for oxygenases, such as the high-throughput screening approaches (solid phase) in our laboratory and others for the direct evolution of monooxygenase P450s [11, 24, 25] and toluene dioxygenase [26]. It was also used to investigate the metabolic pathway of styrene degradation in a *Pseudomonas* strain [27]. Recently, a metagenome clone conferring indirubin and indigo production was isolated by Lim et al [28], and a novel styrene monooxygenase was also screened out from a metagenome library based on indigo formation [29].

Insofar as far as we know, in all the cases reported, only unsubstituted indole was used as the substrate except that in one specific case in our own work 5-benzyloxyindole was used instead for an expanded substrate specificity [13]. In the present paper we reported the screening of random libraries of P450 2A6 using 4-chloroindole hydroxylation, the substrate selected from thirty substituted indole compounds for better color development. The fact that some amino acid changes were selected several times with different variants indicates the consistency of the method. Among the mutants selected for increased 4-chloroindole hydroxylation activity, mutation positions were found not only in SRS region, but also throughout the open reading frame as shown in Table 1. Generally, all the mutants tested had increased catalytic efficiency for 4-chloroindole hydroxylation, but the results varied for indole hydroxylation (Table 2). These results could be well explained by a principle of directed evolution that "you get what you screen for" [30].

For 4-chloroindole hydroxylation, mutants with up to 5-fold increases of catalytic efficiency and 2-fold increases in k_{cat} were found. The performance exceeded the best mutant obtained in previous work [11]. For indole hydroxylation, mutants with > 3-fold increases of catalytic efficiency and 2-fold increases in k_{cat} were found in the first-round mutagenesis. The enhancement was, however, not as pronounced as that achieved in our previous work for mutant N297Q [11]. In the recently solved X-ray crystal structures of P450 2A6 (PDB: 1Z10)[31] and N297Q mutant (PDB: 2PG5)[32], Asn297 was found to be a functionally conserved residue in that the amide group acted as the only hydrogen bond donor in the active site. Hence, any mutation except Gln would probably result in a loss of function, at least for indoles or other substrates that hydrogen bond. The change of asparagine to glutamine requires a nucleotide change of AAC to CAA, a complex double transversion that would not likely be achieved by epPCR, explaining the inaccessibility of this position by libraries constructed from epPCR and demonstrating the complementary values of the alternate methods [11, 33, 34].

Another important residue revealed by X-ray crystal structure was Thr305, located on the N-terminal half of Helix I, as the only polar residue observed in the active site other than Asn297. It was well demonstrated in this study that T305S mutation was selected six times from epPCR-constructed library with different nucleotide sequence changes. This mutant had the highest k_{cat} for both indole and 4-chloroindole. We tried to test the double mutant constructed by site-directed mutagenesis that combined T305S (highest k_{cat}) and N297Q (lowest K_{m}), but failed to see additive or synergistic effect. Nevertheless, the importance of this residue was evident and the result of this study was in accordance with P450 2A6 crystal structure.

By screening epPCR-constructed library, more information was yielded on non-SRS regions not investigated in our previous work, which focused on SRS regions only [11]. After the first-round screening, the kinetic parameters were determined for mutants C6, I4, J14, J17, J20, and J22, which contained no SRS positions. Among them, some were found to have a significant decrease in K_{m} and increase in catalytic efficiency for 4-chloroindole (C6, I4, and J14) or indole (J17 and J22), but some had opposite effects (J20). Surprisingly, mutation M18L—located at the very beginning of the N-terminus of P450 2A6, which, in some instance, was truncated to facilitate expression [31]—played an important role in increasing catalytic efficiency for 4-chloroindole hydroxylation (e.g. mutant C6). The second round of screening also revealed some non-SRS positions (e.g. Ser224 and Try287) that attributed to increased catalytic efficiency. According to the crystal structure of P450 2A6, the locations of the predicted SRS regions were accurate with only minor shifts [31, 32]. The non-SRS residue changes selected in this work were all outside of the active site. Whether they are involved in the interaction with NPR, control protein motion, or otherwise interact indirectly with the active site is unclear at this point.

In conclusion, we have screened P450 2A6 random libraries constructed by epPCR and a sequent StEP PCR process with a higher-throughput "indigo assay" using 4-chloroindole as the substrate as instead of unsubstituted indole. The colony-based screening method was improved by enhanced color development. Kinetic analysis of 4-chloroindole hydroxylation activity revealed mutants with up to 5-fold increases of catalytic efficiency and 2-fold increases in k_{cat} . Several important residues effecting catalytic efficiency were identified, including those located in the non-SRS regions of the enzyme. The results shed light on another aspect of P450 2A6 structure-function relationships and thus may provide more information for drug development, chemical carcinogenesis, and related areas.

Acknowledgments

This work was supported by 100 Talents Program of the Chinese Academy of Sciences (Z.-L.W.) and U.S. Public Health Service Grants R37 CA90426 and P30 ES00267 (F.P.G.).

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R = H, 4-F, 5-F, 6-F, 7-F, 4-Cl, 5-Cl, 6-Cl, 7-Cl, 4-Br, 5-Br, 6-Br, 7-Br, 5-I, 4-CH₃, 5-CH₃, 6-CH₃, 7-CH₃, 7-CH₂CH₃, 4-OCH₃, 5-OCH₃, 6-OCH₃, 7-OCH₃, 4-NO₂, 5-NO₂, 6-NO₂, 7-NO₂, 4-CO₂CH₃, 5-CO₂CH₃, 5-CN

Fig. 1.
Substituted indoles tested in this work.

Fig. 2.
E. coli expressing P450 2A6 and human NADPH-P450 reductase on an indole plate (A) and a CLB plate (B), and in duplex biotransformation medium fortified with indole (C) or 4-chloroindole (D) on a 24-well plate.

Fig. 3.
HPLC profile of whole cell extract from biotransformation of 4-chloroindole and the structure of the product 4,4'-dichloroindigo.

Fig. 4. Steady-state kinetics of oxidation of indole (A) and 4-chloroindole (B) by P450 2A6 mutants. Each point presented is the mean $\pm$ S.D. (range) of duplicate assays.

Fig. 5.
(Structure with residues highlighted)

Table 1

Concentrations of functional active P450 in whole cells of P450 2A6 wild type and mutants.

Entry	Mutants	Mutation positions ^a	Regions	Expression (nmol/l)
1	WT	-	-	80
2	SM ^b	N297Q	SRS3	215
3	C3	D266G/K342M/F343Y	Non-SRS	90
4	C5	M205L	SRS2	110
5	C6	M18L	Non-SRS	178
6	D2	L206Q/A481V/H328L	SRS2&6	ND ^c
7	D5	L206Q	SRS2	192
8	D6	T308A/Q235R	SRS4	169
9	E5	I366N	SRS5	ND
10	E7	L199Q/F209I(ttc/atc)	SRS2	132
11	E8	F191Y/S474T	SRS6	ND
12	E10	P484L	Non-SRS	162
13	H2	L7F/S11R/G113C/T305S(acc/tcc)	SRS1&4	ND
14	H4	T305S(acc/age)	SRS4	273
15	H5	F209I(ttc/atc)	SRS2	225
16	I2	V9M/S119C/R129H	SRS1	ND
17	I4	V87I/G396S	Non-SRS	136
18	J1	T305S(acc/tcc)	SRS4	269
19	J2	T305S(acc/age)	SRS4	245
20	J10	T305S(acc/tcc)/L451I	SRS4	ND
21	J12	G243E/V87I	SRS3	239
22	J13	T305S(-acc/tcc)	SRS4	250
23	J14	V87I/Q126E/L317F	Non-SRS	143
24	J17	I132V/V140L/S224P/S225T	Non-SRS	120
25	J20	M18I/P75S	Non-SRS	272
26	J22	S183C	Non-SRS	175

^aPositions at SRS regions (SRS1:100–121; SRS2:202–211; SRS3:237–244; SRS4:290–308; SRS 5:363–373; SRS 6: 474–481) [15] are indicated.^bGenerated in previous work [11].^cND: not determined.

Table 2

Kinetic parameters for indole and 4-chloroindole hydroxylation by wild type P450 2A6 and mutants measured in bacterial membrane fractions.

Entry	2A6	Indole		4-Chloroindole				
		k_{cat} min ⁻¹	K_m μM	k_{cat}/K_m min ⁻¹ μM ⁻¹	k_{cat} min ⁻¹	K_m μM	k_{cat}/K_m min ⁻¹ μM ⁻¹	k_{cat}/K_m min ⁻¹ μM ⁻¹
1	WT	37±5	260±72	0.14±0.04	19±1	158±29	0.12±0.02	
2	SM ^a	35±1	26±6	1.3±0.3	25±2	116±20	0.21±0.04	
3	C6	56±18	316±183	0.18±0.12	19±0.4	48±4	0.39±0.03	
4	D5	22±1	58±11	0.39±0.08	8.4±0.3	36±5	0.24±0.03	
5	E7	36±4	167±56	0.21±0.08	ND ^b	ND	ND	
6	H5	30±2	196±27	0.15±0.02	ND	ND	ND	
7	I4	15±2	185±58	0.084±0.028	7±0.2	31±6	0.22±0.04	
8	J1	74±5	252±35	0.29±0.04	40±2	131±14	0.31±0.04	
9	J12	22±1	39±7	0.58±0.11	16±0.3	87±5	0.18±0.01	
10	J14	21±2	331±77	0.063±0.016	13±0.4	36±6	0.36±0.06	
11	J17	37±3	121±32	0.30±0.08	ND	ND	ND	
12	J20	27±8	871±375	0.031±0.016	ND	ND	ND	
13	J22	46±2	127±11	0.37±0.04	12±0.3	61±5	0.20±0.02	

^aGenerated in previous work [11].

^bND: not determined

Kinetic parameters for 4-chloroindole hydroxylation by P450 mutants generated from second-round mutagenesis, measured in bacterial membrane fractions.

Table 3

Entry	Mutants	Mutation positions ^a	k_{cat} min ⁻¹	K_m μM	k_{cat}/K_m min ⁻¹ μM ⁻¹
1	DM ^a	L240C/N297Q	45±3	138±19	0.33±0.05
2	St9	L206Q/S224P	13±0.4	21±4	0.61 ±0.1
3	St13	M18I/S183C/L206Q	12±0.3	29±4	0.42±0.06
4	St18	L206Q/T308A	22±1	73±7	0.31±0.03
5	Ja1	Y287H/T305S	41±3	99±18	0.41±0.08
6	Ja4	L206Q/T305S	38±2	90±15	0.42±0.08
7	Ja7	V92M/Y287N/T305S	31±2	122±16	0.25±0.04
8	Ja9	T305S/L462F	36±1	111±12	0.32±0.04

^a Generated in previous work [11].