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Protein engineering of toluene *ortho*-monooxygenase of *Burkholderia cepacia* G4 for regiospecific hydroxylation of indole to form various indigoid compounds

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Abstract Previous work showed that random mutagenesis produced a mutant of toluene *ortho*-monooxygenase (TOM) of *Burkholderia cepacia* G4 containing the V106A substitution in the hydroxylase α-subunit (TomA3) that changed the color of the cell suspension from wild-type brown to green in rich medium. Here, DNA shuffling was used to isolate a random TOM mutant that turned blue due to mutation TomA3 A113V. To better understand the TOM reaction mechanism, we studied the specificity of indole hydroxylation using a spectrum of colored TOM mutants expressed in *Escherichia coli* TG1 and formed as a result of saturation mutagenesis at TomA3 positions A113 and V106. Colonies expressing these altered enzymes ranged in color from blue through green and purple to orange; and the enzyme products were identified using thin-layer chromatography, high performance liquid chromatography, and liquid chromatography–mass spectroscopy. Derived from the single TOM template, enzymes were identified that produced primarily isoindigo (wild-type TOM), indigo (A113V), indirubin (A113I), and isatin (A113H and V106A/A113G). The discovery that wild-type TOM formed isoindigo via C-2 hydroxylation of the indole pyrrole ring makes this the first oxygenase shown to form this compound. Variant TOM A113G was unable to form indigo, indirubin, or isoindigo (did not hydroxylate the indole pyrrole ring), but produced 4-hydroxyindole and unknown yellow compounds from C-4 hydroxylation of the indole benzene ring. Mutations at V106 in addition to A113G restored C-3 indole oxidation, so along with C-2 indole oxidation, isatin, indigo, and indirubin were formed. Other TomA3 V106/A113 mutants with hydrophobic, polar, or charged amino acids in place of the Val and/or Ala residues hydroxylated indole at the C-3 and C-2 positions, forming isatin, indigo, and indirubin in a variety of distributions. Hence, for the first time, a single enzyme was genetically modified to produce a wide range of colors from indole.

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Introduction

Indigo (Fig. 1a) is one of the oldest dyes (Ensley et al. 1983) and is still used worldwide for textiles, with an annual production of 22×10^3 t, worth U.S. $ 200×10^6 (Wick 1995; Maugard et al. 2002). The production of indigo is primarily by chemical syntheses, such as the Adolf von Baeyer chemical synthesis of 1890 (Gillam et al. 2000), which resulted in the fifth Noble prize in chemistry. More recently, bacterial systems for commercial indigo production have been developed (Murdock et al. 1993), which were inspired by the discovery that recombinant *Escherichia coli* expressing naphthalene dioxygenase from *Pseudomonas putida* PpG7 in rich medium resulted in the formation of indigo (Ensley et al. 1983). Indigo is formed as the result of the cloned enzyme oxygenating C-3 of the indole pyrrole ring (Fig. 1), and indole is produced from tryptophan via tryptophanase in *E. coli* (Ensley et al. 1983). Although limited to the production of a single hue, various monooxygenases and dioxygenases growing on aromatic hydrocarbons have been identified that are capable of indole oxidation to form indigo (Ensley et al. 1983; O'Connor et al. 1997; O'Connor and Hartmans 1998; Bhushan et al. 2000; Gillam et al. 2000), and these biological processes are inherently safer than the chemical processes since they do not produce such toxins as aromatic amines (bladder carcinogens) and cyanide (Frost and Lievense 1994; Wick 1995).

Indirubin (Fig. 1a), a pink pigment, has important therapeutic applications (Hoessel et al. 1999). It is the active ingredient of a traditional Chinese medicine used to

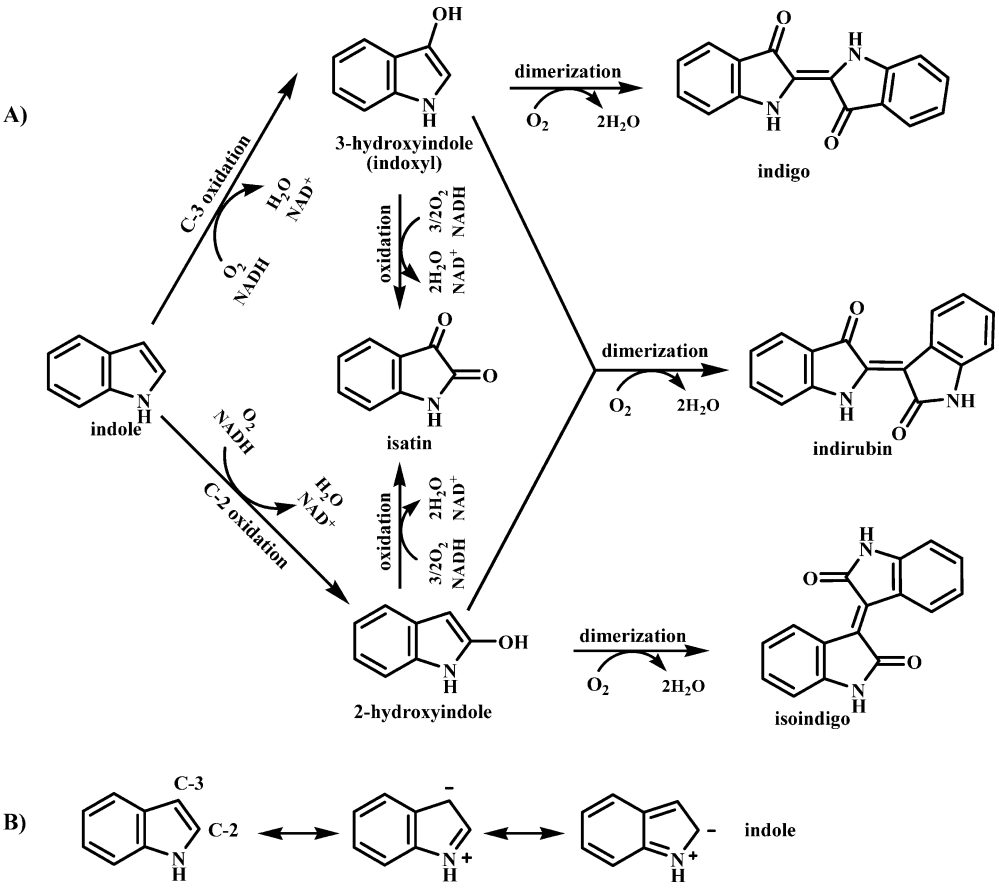

Fig. 1 **a,b** Indole. **a** Proposed pathway for converting indole to indigoid compounds (adapted from Eaton and Chapman 1995; Maugard et al. 2001) and **b** resonance structure of indole (Sundberg 1996)

treat diseases such as chronic myelocytic leukaemia and is a potent inhibitor of cyclin-dependent kinases, it therefore belongs to a group of promising anticancer compounds (Hoessel et al. 1999; Buolamwini 2000).

Burkholderia cepacia G4 was isolated as the first pure strain that degrades trichloroethylene (TCE; Nelson et al. 1986), and toluene *ortho*-monooxygenase (TOM) has been shown to oxidize mixtures of chlorinated compounds, including TCE (Shim and Wood 2000). TOM catalyzes the regiospecific hydroxylation of toluene to form 3-methylcatechol via the intermediate *o*-cresol (Shields et al. 1989; Rui et al. 2004) and catalyzes three successive hydroxylations of benzene to form 1,2,3-trihydroxybenzene (Tao et al. 2004b). The subunits of TOM are similar to the corresponding components of crystallographically characterized soluble methane monooxygenase (sMMO) from methanotrophic bacteria (Newman and Wackett 1995), with their α-subunits sharing about 20% amino acid sequence identity. TOM is a three-component complex consisting of a 211-kDa hydroxylase (encoded by *tomA1A3A4*) with two binuclear iron centers in the $(\alpha\beta\gamma)_2$ quaternary structure, a 40-kDa NADH-oxidoreductase (encoded by *tomA5*), and a 10.4-kDa cofactor-less regulatory protein (encoded by *tomA2*) involved in the electron transfer between the hydroxylase and reductase (Newman and Wackett 1995). The $(\alpha\beta\gamma)_2$ component contains the active site for substrate binding and hydroxylation reaction and is capable of a peroxide-shunt mechanism (Newman and Wackett 1995) like sMMO (Wallar and Lipscomb 1996).

TOM originally was not considered as an indigo-forming enzyme (Nelson et al. 1987; Shields et al. 1989), but our laboratory found it was responsible for color development and indole hydroxylation (Luu et al. 1995). During growth in complex medium, recombinant *E. coli* expressing TOM forms brown colonies on rich agar plates and a brown color in broth, whereas typical indole-oxygenating enzymes in whole cells form blue colonies on agar plates and blue, water-insoluble pigments in liquid medium. One TOM variant with a single amino acid change (V106A) in the hydroxylase α-subunit (TomA3) was created by us (first DNA shuffling of a non-heme monooxygenase) and was identified as a potential indigo-forming enzyme based on the green color of its colonies on agar plates and in culture (Canada et al. 2002). In this variant, a single mutation was responsible for the cell color change, presumably due to the alteration in the hydroxylation of indole.

The aims of this study were to create different color-producing TOM variants via DNA shuffling, to discern the important residues that cause the formation of these various colored products, to explore the altered patterns of indole hydroxylation by *E. coli* TG1 expressing TOM with amino acid variations at positions V106 and A113 of the hydrolase α-subunit (TomA3), and to seek an explanation at the molecular level for why indole was oxidized in three possible ways (hydroxylation of indole at C-3, C-2, and at

both C-2 and C-3; Fig. 1). Since site-directed saturation mutagenesis can provide much more comprehensive information than can be achieved by single-amino-acid substitutions (Brannigan and Wilkinson 2002), it was used to introduce all possible mutations at two key catalytic sites that were identified by us for the first time via DNA shuffling. It was discovered that small changes in the enzyme structure lead to distinctive TOM enzyme variants with profound changes in indole oxidation, resulting in the synthesis of different indigoid compounds that produce a rainbow of different colors.

Materials and methods

Chemicals and synthesis of isoindigo

Indigo, indirubin, isatin, 4-hydroxyindole, 5-hydroxyindole, and oxindole (Fig. 1a) were purchased from Fisher Scientific Co. (Pittsburgh, Pa.). 6-Hydroxyindole was obtained from Matrix Scientific (Columbia, S.C.). Lacking a commercial source, isoindigo (Fig. 1a) was prepared as described by Hoessel et al. (1999), by reacting 10.19 mmol isatin (Fig. 1a) with 10.14 mmol oxindole (Fig. 1a) under acidic conditions in a mixture of 30 ml glacial acetic acid and 0.5 ml concentrated hydrochloric acid, with stirring at 95°C for 3 h. The resulting dark brown precipitate was filtered and washed with methanol and diethyl ether and identified as isoindigo both by its UV-visible absorbance spectrum and by mass spectroscopy (MS), using a HP 1090 series II liquid chromatograph (LC; Hewlett–Packard, Palo Alto, Calif.) with a diode array detector coupled to a Q-TOF2 MS (Micromass, Beverly, Mass.). The major ion at *m/z* 263.1 (MH^+) matched the molecular weight of 262 and the UV-visible spectrum also matched the published data for isoindigo (Maugard et al. 2001). MS was also used to study two of the indigoid products of TomA3 variant A113G.

Bacterial strains and growth conditions

E. coli strain TG1 {*supE hsd*Δ5 *thi* Δ(*lac-proAB*) F′ [*traD*36 *proAB*$^+$*lacI*^{q}*lacZ*ΔM15]; Sambrook et al. 1989} was utilized as the host for gene cloning and expression. TG1 was routinely cultivated by inoculation from fresh, single colonies at 37°C in Luria–Bertani (LB) medium with 100 μg/ml kanamycin (Kan) added to maintain the plasmid pBS(Kan)TOM (Canada et al. 2002), which expresses *tomA012345* from a constitutive *lac* promoter.

DNA shuffling of TOM

DNA shuffling was performed to generate TOM-Green as reported by Canada et al. (2002), which was an adaptation of the methods of Stemmer (1994) and Zhao and Arnold (1997). A 3.5-kb fragment was subjected to DNA shuffling; and this fragment was flanked by the naturally occurring *Avr*II and *Ppu*MI restriction sites, which were used to clone the shuffled fragment in pBS(Kan)TOM. Effectively shuffled were all of *tomA2A3A3*, 57% of *tomA1*, and 56% of *tomA5*. Cells were screened based on colony color (e.g., blue, green) on LB-Kan plates.

Saturation mutagenesis and DNA sequencing

Saturation mutagenesis was performed at sites N14 and A113 and simultaneously at sites V106 and A113 of TOM-Green TomA3 (GenBank accession no. AF349675). By replacing the target codon with NNN via overlap-extension polymerase chain reaction (PCR), all 64 codons were created at the corresponding positions (Sakamoto et al. 2001). Degenerate primers N14 Front (GAAGAAGAAACTCGGTTTAAAG*NNN*CGCTACGCAG) and N14 Rear (GCGGGTCATCGCTGCGTAGCG*NNN*CTTTAAACCGA) were designed to randomize position N14 in the nucleotide sequence, degenerate primers A113 Front (GTGTGACACCGTTGGAGTATATG*NNN*CACCGAGG) and A113 Rear (CAATGTGGGCAAAACCTCGGTG*NNN*CATATACTCC) were designed to randomize position A113; and degenerate primers V106 + A113 Front (ATCCAGGGT*NNN*ACACCGTTGGAGTATATG*NNN*CACCGAGG) and V106 + A113 Rear (AACCTCGGTG*NNN*CATATACTCCAACGGTGT*NNN*ACCCTGG) were used to randomize positions V106 and A113 at the same time. The two additional primers for cloning were *Bcl*I Front (TCGAAGACCGGATCGGCATGAAGTTCG) and *Sph*I Rear (GTTGTAGTGCGAGAGA*GCATGC*ATTTC), where the two restriction enzyme sites, *Bcl*I and *Sph*I, occur naturally in TomA3 upstream and downstream from positions N14 and A113, respectively.

For saturation mutagenesis of TomA3 A113, plasmid pBS(Kan)TOM was used as the template with *Pfu* DNA polymerase (Stratagene Corp., La Jolla, Calif.) in the PCR to minimize random point mutations. A 499-bp DNA degenerate fragment was amplified using primers *Bcl*I Front and A113 Rear, and a 152-bp DNA degenerate fragment was amplified using *Sph*I Rear and A113 Front. After purification from agarose gels, the two PCR fragments were combined at a 1:1 ratio as templates to obtain the full-length PCR product with the *Bcl*I Front and *Sph*I Rear primers. The PCR program used was: 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1.5 min, with a final extension of 72°C for 10 min. The resulting randomized PCR products (603 bp) were cloned into pBS(Kan)TOM after double-digestion with *Bcl*I and *Sph*I and replaced the corresponding fragment in the original plasmid. The resulting plasmid library was electroporated into *E. coli* TG1 competent cells using a GenePulser/Pulse Controller (Bio-Rad Laboratories, Hercules, Calif.) at 15 kV/cm, 25 μF, and 200 Ω. An analogous procedure was used for saturation mutagenesis at N14 (PCR products of 201, 449, 603 bp) and for simultaneous saturation mutagenesis for V106/A113 (PCR products of 486, 160, 603 bp). Cells were screened based on colony color (e.g.,

blue, green, yellow) on LB-Kan plates. DNA sequencing of the sub-cloned region (603 bp) in the TOM color variants was performed using the *Bcl*I Front and *Sph*I Rear primers, as described by Rui et al. (2004).

Identification of indigoid compounds

Fifty milliliters of *E. coli* TG1 variants expressing TOM were grown overnight (13–14 h) at 37°C and the entire culture was then extracted once with an equal volume of chloroform. For wild-type TOM, the cell culture was started from a fresh pre-culture [0.4% inoculum, at an optical density (OD) of 1.2], and after 3 h incubation at 37°C, 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added for 15–16 h at 37°C for additional TOM expression. The chloroform layer was separated by centrifugation and dried with anhydrous sodium sulfate. Chloroform-extracted products were concentrated to 4 ml and qualitatively analyzed by thin-layer chromatography (TLC) on activated silica gel plates (Selecto Scientific, Suwanee, Ga.), which were developed with toluene: acetone (4:1).

To confirm that indole is the precursor of the indigoid compounds, exponentially growing TG1 expressing wild-type TOM, TOM-Green (V106A), or TOM A113G was harvested at OD 1.2 (note color does not develop until ca. OD 2.0, so these cultures were uncolored), washed, and resuspended in Tris-HCl buffer to OD 2.5 and incubated with 0.5 mM indole for 3 h. Then the whole cells were extracted once with an equal volume of chloroform [TG1/pBS(Kan) was the negative control]. After centrifugation, the chloroform layer was dried with anhydrous sodium sulfate.

To investigate whether the unknown colored compounds produced by TomA3 A113G in TG1 were from hydroxylation of the indole benzene ring, exponentially grown cultures of TG1 expressing A113G (OD 1.2) were washed and resuspended in 40 ml of Tris-HCl buffer at OD 2.5 and incubated with 0.5 mM 4-hydroxyindole, 5-hydroxyindole, 6-hydroxyindole (3.5 h for 5-hydroxyindole, 20 h for 4-hydroxyindole, 6-hydroxyindole) and extracted once with an equal volume of chloroform. As controls, TG1 expressing wild-type TOM, TG1/pBS(Kan), and Tris-HCl buffer were also incubated with the three substrates. The chloroform layer was further separated and concentrated in the same way as above for analysis by high performance liquid chromatography (HPLC). To determine that 4-hydroxyindole was produced by variant A113G, cells were grown from single colonies to OD 0.12 and 0.5 mM IPTG was then added. At OD 0.9 (100 min later), the cells were washed three times, then indole was added (0.5 mM), and the cells were incubated for 1.5 h or 12 h. The products were extracted with an equal volume of chloroform.

Quantitative analysis and the separation of pigments and intermediates were conducted using a HPLC system from Waters Corp. (Milford, Mass.), including 515 HPLC pumps, a 996 photodiode array detector, and Millenium32 chromatography manager software. Analysis was done using a Zorbax C8 reverse-phase column (4.6×250.0 mm, 5 μm) from Agilent Technologies (Palo Alto, Calif.), with a flow rate of 1.0 ml/min and gradients composed of 0.1% formic acid in H_2O (HPLC grade) and acetonitrile. The gradients were: 0–16 min from 0% to 60% acetonitrile, 16–22 min at 60% acetonitrile, 22–32 min from 60% to 0% acetonitrile. UV/visible spectra were acquired online using a diode array detector (scanning from 200 nm to 700 nm) to characterize and quantify the indole oxidation and dimerization products. Products were identified by comparing the retention times and UV/visible spectra with authentic standards and were confirmed by co-eluting with the standards. The indigoid compounds formed by wild-type TOM were analyzed by LC-MS using a HP 1090 series II LC (Hewlett–Packard) coupled to a Q-TOF2 MS (Micromass).

Homology structure modeling

Those residues of TOM which share the highest homology (ca. 30% identity, residues 95–250) with the sMMO hydroxylase α-subunit sequence from *Methylococcus capsulatus* (Bath) were modeled into the known three-dimensional structure of sMMO (Protein DataBase accession code 1MTY), using the SWISS-MODEL server as described by Rui et al. (2004).

Results

Blue colonies via DNA shuffling of TOM

By cloning the shuffled TOM DNA into pBS(Kan)TOM, a library of mutants with a variety of different colored colonies was generated, including a distinct blue colony (*E. coli* TG1 cells expressing wild-type TOM are brown) with mutations N14D, A113V, and F465S in the α-subunit of the hydroxylase (TomA3, 519 amino acids). Based on the homology model, A113V was predicted to be responsible for the cell color variation, since N14 and F465 are located far from the active site.

Saturation mutagenesis creates various colored colonies

Saturation mutagenesis at positions N14 and A113 of wild-type TOM TomA3 confirmed that A113 was responsible for the blue color, since the colonies created from N14 saturation mutagenesis showed the uniform brown color of wild-type TOM, while saturation mutagenesis at position A113 generated a library of 1,000 colonies of various colors, including blue. Since V106A of TomA3 created green-colored colonies (Canada et al. 2002), saturation mutagenesis was also performed simultaneously at both TomA3 V106 and A113, which also yielded 1,000 transformants containing a variety of

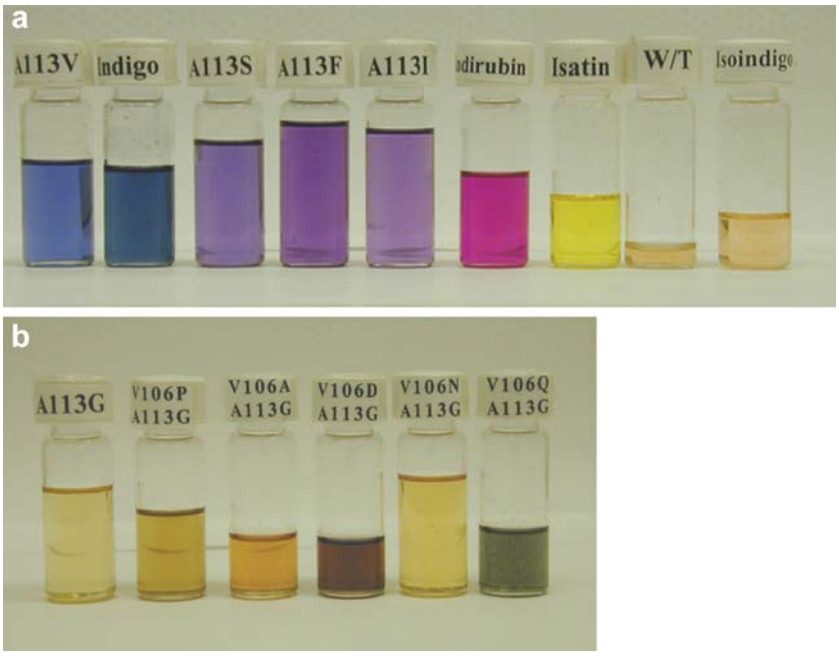

Fig. 2 **a,b** Colored compounds produced by TOM variants. **a** Colored chloroform culture extracts of TOM variants with mutations at position TomA3 A113, along with standards (indigo, indirubin, isatin, isoindigo). **b** Cell color extracts of TomA3 A113G mutants

different-colored colonies. Visual screening was used to identify catalytically active variants after about 18 h incubation at 37°C. About 42% inactive mutants (white colonies) resulted from the double mutagenesis, while 19% white colonies were found for the single mutation at position A113. Orange, purple, and various shades of blue colonies appeared on the agar plates in addition to the original brown color of wild-type TOM, green color of TOM-Green, and blue color of the variant A113V. The color arises due to the various hydroxylation products of indole (formed as a breakdown product of tryptophan in *E. coli*) that further dimerize into indigoid compounds (Mermod et al. 1986; Fig. 1a). Based on the calculation that 292 independent clones from saturation mutagenesis at one site and 342 for two sites need to be sampled for a 99% probability that each possible codon has been tested (Rui et al. 2004), we screened over 400 colonies from each library, and all variants with distinct cell colors (six from saturation mutagenesis at position A113, 20 from saturation mutagenesis at positions V106, A113) were sequenced (Table 1). Three white colonies devoid of TOM catalytic activity were randomly picked from each saturation mutagenesis library for sequencing to investigate

Table 1 Hydroxylation of indole by TOM variants. Note that chloroform extract colors did not always match those of the colonies/broth exactly. *Dashes* (–) Product not detected

Mutations in TomA3	Color of chloroform extract	Isatin		Indigo		Indirubin		Isoindigo		Unknown colored compounds
		μM	%	μM	%	μM	%	μM	%	
Wild type (V106/A113)	Brown	11	6	12.5	7	–		146	86	–
A113V	Blue	14	5	267	89	18	6	–		–
V106S/A113V	Purple	313	52	136	23	152	25	–		–
A113H	Purple	335	58	126	22	118	20	–		–
V106H/A113S	Purple	80	29	114	41	86	31	–		–
V106I/A113S	Purple	92	35	104	39	69	26	–		–
A113S	Purple	34	13	87	33	145	54	–		–
A113F	Purple	17	5	121	35	203	60	–		–
A113I	Purple	25	10	67	27	160	64	–		–
V106F	Purple	3.4	1	153	44	190	55	–		–
V106A	Purplish blue	127	38	175	52	34	10	–		–
V106P	Purplish blue	71	21	241	73	21	6	–		–
A113G	Yellow	–		–		–		–		4
V106P/A113G	Yellow	–		–		–		–		6
V106A/A113G	Yellow	25	63	15	37	–		–		5
V106D/A113G	Dark pink	23	32	39	54	10	14	–		5
V106N/A113G	Yellow	29	36	38	48	14	17	–		4
V106Q/A113G	Green	139	5	388	71	21	4	–		3

what kind of amino acid changes are responsible for inactivating the TOM enzyme.

Sequence changes

Altogether, 15 active variants from saturation mutagenesis at positions V106/A113 and five active variants from saturation mutagenesis at position A113 were unique in sequence (Table 1). For the inactive variants, three mutations at position A113 and/or V106 lead to inactivity (A113R, V106R/A113W, V106Q/A113Q) and these were only representative of a small portion of the inactive enzyme sequence.

Mutation A113G and its derivatives are very important as they added a great variety to color development, including purple and orange in various shades, both of which are unique to our original color reservoir of TOM variants. In addition, it was found that active mutations at position A113 are relatively conservative (involving mutations primarily from hydrophobic to hydrophobic or neutral residues), compared with position V106, which can accommodate a variety of mutations (even residues like proline) and still remain active. Notably residue A113 is closer then V106 to the diiron center and this may explain the more diverse colors seen from mutagenesis at this position.

Identification of indigoid compounds from TOM variants

Extraction and HPLC analysis identified the major compounds produced by *E. coli* TG1 expressing TOM color variants. The concentration of each compound was determined by making calibration curves under the same HPLC conditions. The concentration of the colored compounds produced by each TOM variant are listed in Table 1 and some of the extracts are shown in Fig. 2.

Wild-type TOM expressed in TG1 is unique in its brown color, seen both on LB agar plates and in LB liquid medium, and its HPLC spectrum (including co-elution) indicated that the brown color is from the formation of isoindigo as the primary product (146 μM), which was not found with other variants (Table 1). Both TLC and LC-MS analysis corroborated the HPLC data that isoindigo is the primary indigoid compound formed by wild-type TOM, since the major peak matched the orange isoindigo spot on silica gel plates and had the predominant ion of *m*/*z* 263.1 (MH^+) that appears with the retention time and absorbance spectrum of isoindigo. To our knowledge, no other indole-hydroxylating oxygenase has been reported to produce isoindigo.

TG1 expressing mutant TomA3 A113V hydroxylated indole into indigo mainly (89%) and indirubin accounted for only a very minor part (6%; Table 1). Mutants V106S/A113V and A113H produced isatin as the major compound (>50%), while indigo and indirubin ranked as minor products with similar amounts. This product distribution may contribute to their vivid blue color on agar plates. Mutants V106H/A113S and V106I/A113S may also fall into this category, based on the indigoid compounds produced. Another category includes the mutants A113S, A113F, and A113I, which share the common feature that they produced indirubin as their major product (ca. 60%). They also produced 30% indigo and 10% isatin. Both mutants V106A and V106P were green on agar plates; and their cell color stems from the mixture of indigo (52% and 73%, respectively) and isatin (38% and 21%, respectively).

TOM A113G did not produce isatin, indirubin, or isoindigo, which means that it was not capable of indole C-3 or C-2 hydroxylation. Instead, it produced orange-colored compounds. These compounds are the result of hydroxylation of the indole benzene ring (rather than the pyrrole ring), since TOM A113G formed 4-hydroxyindole from indole as shown by HPLC and co-elution. This compound undergoes subsequent rearrangement since two of the four unknown yellow-colored compounds (Table 1) that were produced by TG1 expressing A113G were found when these cells were incubated with 4-hydroxyindole (4-hydroxyindole itself is not colored) and had a molecular weight of 222 as determined by LC-MS. These compounds were not found with TG1 expressing wild-type TOM with 4-hydroxyindole, nor in the absence of monooxygenase with 4-hydroxyindole, nor in the absence of 4-hydroxyindole. The other two unknown colored compounds may be derived from 7-hydroxyindole, as A113G in TG1 incubated with 5-hydroxyindole or 6-hydroxyindole did not yield any of the unknown compounds.

Substitution at position V106 of TomA3 (V106A, V106D, V106N, V106Q) in addition to the A113G mutation restored the ability of the enzyme to produce isatin, indigo, and indirubin in addition to the original unique-colored compounds associated with TOM A113G (Table 1). For example, variant V106Q/A113G produced the highest amount of indigo (388 μM) in vivo.

When the cells expressing TOM variants were incubated with indole in buffer, the indigoid compounds obtained were the same as in cells growing in complex medium. The compounds were identified by HPLC (data not shown); and the results are exemplified by TOM-Green (V106A) which produced isatin, indigo, and indirubin as the major colored compounds after incubation with indole, the same products generated from LB medium (Table 1). No colored products were produced in the absence of indole. These results indicate that the colored compounds originate from indole. Further, the monooxygenase is responsible for color formation since no colored compounds were produced from indole by the negative control TG1/pBS(Kan) (which lacks the monooxygenase). The qualitative analysis of colored compounds by TLC (data not shown) also corroborated our HPLC results.

Discussion

These results show clearly that random mutagenesis of TOM led to the identification of the key sites V106 and A113 in TomA3 that are responsible for the different indole hydroxylation patterns and that saturation mutagenesis at these key sites resulted in the discovery of more diversified indole hydroxylation which allows a single enzyme for the first time to make a diverse range of indigoid compounds. Wild-type TOM (which was discovered to form primarily isoindigo) and TOM variants were created that produced primarily indigo (A113V), isatin (V106S/A113V, A113H), indirubin (A113S, A113F, A113I), and orange compounds presumably from benzene ring oxidation (A113G; Table 1). These TOM mutants produce diverse colors that range from blue through olive green and purple to orange, compared with the brown color of wild-type TOM. These results reveal that a single or double amino acid change can create catalytically distinct enzyme variants that hydroxylate indole in different regiospecific positions on the pyrrole and benzene rings (e.g., TOM A113G, 4-hydroxyindole production). As revealed from the changes in product profiles in HPLC analysis and with the exception of the TOM A113G mutant, the color changes result from a variation in the relative amounts of colored compounds, including indigo, indirubin, isatin, and isoindigo.

Indole (Fig. 1b) is a π-excessive, planar, heteroaromatic compound (Sundberg 1996). The reactivity of its C-3 carbon towards various electrophiles is due to significant delocalization of electron density from nitrogen to carbon atoms in the pyrrole ring, particularly at C-3 (resonance structure of indole; Fig. 1b), while the C-2 position is the second most reactive site toward oxygenating reagents (Sundberg 1996). Hence, a hydroxyl is preferentially added to C-3 of indole. This can be shown from the well established fact that 3-hydroxyindole (indoxyl, hydroxylation at C-3; Fig. 1a) is the major product from indole in biological systems (Sundberg 1970; Damani and Crooks 1982; Gillam et al. 2000). The 2-hydroxylated compound (oxindole, the more stable carbonyl tautomer of 2-hydroxyindole) usually forms as a minor product (Damani and Crooks 1982). However, here it was found that wild-type TOM produces isoindigo; and since isoindigo forms via the dimerization of oxindole (Maugard et al. 2001; Fig. 1a), this enzyme is unique as the only reported oxygenase that produces the dimerized indole C-2 hydroxylation product. TOM also produces a small amount of indigo (10% of isoindigo), which means indole-hydroxylation catalyzed by TOM occurs also at the C-3 position. However, it primarily occurs at the C-2 position.

Whole cells expressing TOM with the TomA3 A113V variation produce 90% indigo with isatin and indirubin as minor impurities. Hence, this may have value in the commercial production of indigo dye. It shows that, along with metabolic engineering (Bialy 1997; Berry et al. 2002), tailoring the oxygenase may itself may be beneficial for indigoid production. Other TOM variants were found to produce primarily isatin (such as mutant A113H), indigo (mutant A113V), and indirubin (such as mutant A113F), and all three compounds may have potential as pharmaceuticals. Indirubin is a potent inhibitor of cyclin-dependent kinases (Hoessel et al. 1999), indigo and indirubin are high-affinity (nanomolar concentration) agonists of the aryl hydrocarbon receptor in human (Adachi et al. 2001), and isatin is known as a potent inhibitor of monoamine oxidase (Glover et al. 1988).

The ratios of pigments differ greatly among the different TOM variants, which may be the result of different rates of indoxyl and oxindole production. We propose that this may also be related to the fact that TOM may partly, if not completely, control the oxidative coupling of indole derivatives to form dimeric conjugates, although indoxyl is normally believed to form indigo by non-enzymatic condensation and oxidation (Ensley et al. 1983; Meyer et al. 2002). Since these dramatic effects on indole hydroxylation are caused by A113 and/or V106 substitution, it may be interesting to explore the mechanism of dimerization. Although there is no direct evidence, it seems that the identity of residues V106 and A113 not only determines the binding orientation of the substrate and its regiospecific hydroxylation but also influences the subsequent dimerization. As seen in Table 1 with V106F and V106P, these mutants produced similar overall concentrations of colored compounds (346 μM and 333 μM, respectively), which implies they have similar indoxyl and oxindole formation rates. But V106F dimerized 99% of the indole hydroxylation products into indigo and indirubin, while V106P only converted 79% of the hydroxylation products into dimers (the rest formed isatin). In addition, while C-2 hydroxylation clearly occurred in the variants that produced indirubin (dimerization product of 2-hydroxyindole and 3-hydroxyindole), only wild-type TOM dimerized this intermediate into isoindigo. Further, TomA3 variant A113V produced yellow-colored derivatives from 4-hydroxyindole (itself uncolored) that were not seen in the absence of enzyme or with the wild-type enzyme. Along with the diversified products distributions in Table 1 that are not easily explained by different hydroxyindole formation rates, it appears the enzyme may be controlling both dimerization and the position of hydroxylation of the benzene or pyrrole ring.

Variation of the analogous two residues in the related enzymes toluene 4-monooxygenase and toluene-o-xylene-monooxygenase caused substantial alterations in regiospecificity towards various aromatic compounds (Tao et al. 2004a; Vardar and Wood 2004). The introduction of large polar or charged residues at position V106, such as V106E discovered in our previous mutagenesis experiment (Rui et al. 2004), does not prevent substrate access. However, substitution of large charged residues at position A113 (A113R) or at both positions (V106R/A113W, V106Q/A113Q) appears to fill the active site and occlude substrate binding, since there is no indole oxidation.

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