Formation of Indigo by Recombinant Mammalian Cytochrome P450[1]

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The development of bicistronic systems for coexpression of recombinant human cytochrome P450 enzymes (P450s) with their redox partner, NADPH-cytochrome P450 reductase (NPR), has enabled P450 activity to be reconstituted within bacterial cells. During expression of recombinant P450 2E1 and some other forms, we observed the formation of a blue pigment in bacterial cultures. The pigment was extracted from cultures and shown to comigrate with standard indigo on TLC. UV-visible spectroscopy and mass spectrometric analysis provided further support for identification of the pigment as indigo. Indigo is known to form following the spontaneous oxidation of 3-hydroxyindole. Accordingly, we speculated that indole, formed as a breakdown product of tryptophan in bacteria, was hydroxylated by the P450 system, leading to indigo formation. Bacterial membranes containing recombinant P450 2E1 and human NPR were incubated *in vitro* with indole and shown to catalyze formation of a blue pigment in a time- and cofactor-dependent manner. These studies suggest potential applications of mammalian P450 enzymes in industrial indigo production or in the development of novel colorimetric assays based on indole hydroxylation. © 1999 Academic Press

P450 enzymes constitute a superfamily of hemoproteins catalyzing the monoxygenation of a diverse range of chemicals in a wide variety of organisms (2). The functional significance of P450-mediated reactions to individual organisms is similarly diverse; P450s are involved in clearance of xenobiotic chemicals, biosynthesis of hormones and other signaling molecules, homeostatic mechanisms, and other organism-specific phenomena such as pigmentation (3) and regulation of feeding behavior (4). Most P450 enzymes act in a concerted fashion with one or more redox partners. In the case of mammalian P450s involved in xenobiotic metabolism, the major accessory enzyme is NPR.

Our laboratories have been engaged in the study of human P450 enzymes expressed as recombinant enzymes in a bacterial host. Recently we have established systems in which individual P450 forms are coexpressed with hNPR using a bicistronic expression vector (5). In the course of work with several bicistronic P450 expression systems, we observed production of blue pigments in bacterial cultures. The following report describes the characterization of the major pigment produced in these cultures and its identification as indigo.

MATERIALS AND METHODS

Bacterial cultures. Bicistronic plasmids for expression of human P450 2E1 and NPR were prepared as described previously (5). The monocistronic expression construct for P450 2A6 was prepared by PCR amplification of the 2A6 coding sequence using primers encoding minor alterations to the 5′ nucleotide sequence of the P450 2A6 coding region (resulting in only a single change to the resultant amino acid sequence, namely substitution of Ala in the second position) and inserting a 5′ *Nde*I site and 3′ *Sal*I site flanking the P450 cDNA. The PCR product was then subcloned into the cognate sites of pCW′/NF14 (6) (replacing the P450 3A4 cDNA) and sequenced. One nucleotide difference was observed from the reported 2A6 wild type sequence, leading to mutation of Ile (ATA) at position 246 to Val (GTA) in the predicted amino acid sequence. The resultant mutant 2A6 protein appeared to retain cata-

lytic activity and to effectively incorporate heme, as judged by the demonstration of a typical Fe(II) · CO vs Fe(II) difference spectrum. The bicistronic P450 2A6 expression construct was prepared by subcloning the NdeI-SalI fragment from the monocistronic vector into the cognate sites of pCW'/2C10/hNPR (5) (Fig. 1).

Expression was done in *Escherichia coli* using TB media fortified with trace elements, thiamine, δ-aminolevulinic acid, ampicillin, and IPTG according to established protocols (6, 7). Bacterial membranes were prepared and incubated as described (5, 6). Yields of the novel recombinant P450 2A6 enzyme were approximately 200 nmol/l and 100 nmol/l using mono- and bicistronic expression systems, respectively.

Assay of pigment production in vitro. Bacterial membranes (0.10 μM P450, 0.095 μM hNPR or 0.27 mg protein/ml) were incubated in 0.10 M potassium phosphate buffer (pH 7.4) with 5 mM indole (Sigma, St. Louis, MO). Incubations were initiated by the addition of an NADPH-generating system containing (final concentrations) 1 mM NADP$^+$, 2.5 mM glucose 6-phosphate, and 0.5 U glucose 6-phosphate dehydrogenase/ml. Reactions were allowed to proceed with gentle agitation at 37°C for various times before aliquots were quenched by addition to an equal volume of a mixture of 1% sodium cholate (w/v) in 4 M urea. Relative pigment production was quantified by measuring A_{665}.

Isolation and characterization of pigments. For TLC analysis of pigments, 50 ml cultures of P450 2E1 (bicistronic with NPR) were grown for 48 h using the general conditions described. Centrifugation (1200g for 5 min) yielded dark blue pellets, which were collected and washed once with cold buffer containing 100 mM Tris (pH 7), 0.5 mM EDTA, and 500 mM sucrose. The pellets were resuspended in 2.5 ml of the same buffer and extracted three times with equal volumes of CHCl$_3$. The CHCl$_3$ layers were concentrated under a stream of Ar and then spotted on a silica gel G TLC plate. The plate was developed with CHCl$_3$-CH$_3$OH/50-1 (v/v).

For UV-visible spectral analysis, pigments were extracted from 2E1/hNPR cultures in a similar manner except that DMF was used to extract the pigments from cell pellets and record the spectra. UV-visible spectra were recorded in DMF using a BioSpec 1601 spectrophotometer (Shimadzu, Columbia, MD).

For mass spectrometric analysis of the blue pigment, a 1-liter culture of P450 2A6 was grown for 48 h using the general conditions described. Centrifugation (at 200g for 1 min) yielded a dark blue pellet, which was collected and washed 5 times with H$_2$O (resuspension and recentrifugation each time). The material was resuspended in 10 ml of DMF and subjected to repeated sonication with a microprobe. CHCl$_3$ (100 ml) was added and the organic phase was washed five times with an equal volume of H$_2$O. The CHCl$_3$ layer was dried over Na$_2$SO$_4$ and filtered (156 ml, A_{600} = 0.46). The material was then concentrated at 50°C *in vacuo* and streaked onto a 1 mm × 20 cm × 20 cm silica gel G TLC plate. The plate was developed with CHCl$_3$-CH$_3$OH/50-1 (v/v). Individual colored bands were excised and extracted with CHCl$_3$-CH$_3$OH (1-1, v/v) and then with acetone, followed by centrifugation and concentration under N$_2$.

Mass spectra were obtained in the positive ion mode using a Finnigan TSQ7000 instrument (Finnigan, Sunnyvale, CA) in the Vanderbilt facility, operating with an electrospray ionization interface, either with loop injection in 2% CH$_3$OH (v/v) in 10 mM NH$_4$CH$_3$CO$_2$ (pH 5.0) or eluting from a 4.6 × 150 mm C8 HPLC column with an increasing CH$_3$OH gradient in the same buffer.

RESULTS

Observation of blue cultures. In the course of other work with several bicistronic (P450/hNPR) expression systems for human P450 enzymes, the appearance of bluish cultures was observed in our individual laboratories (E.M.J.G., F.H.A., F.P.G.). Blue pigment was seen only in cultures co-expressing P450s with NPR, not in the absence of either or both proteins. The pigment appeared to form an opalescent layer and bubble-
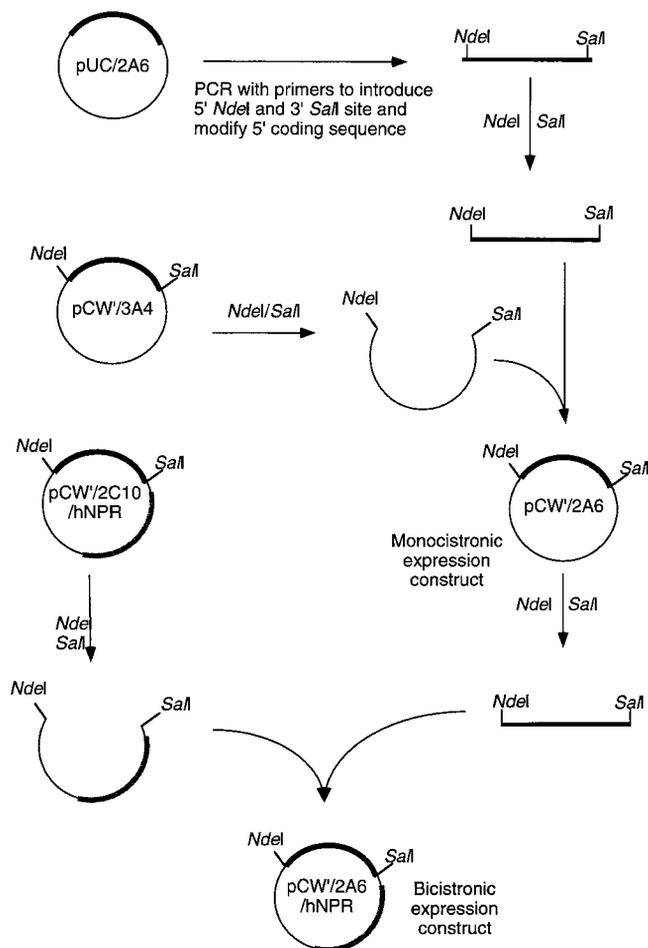

FIG. 1. Strategy used for generation of the bicistronic construct for coexpression of cytochrome P450 2A6 and hNPR.

like structures at the surface of cultures allowed to stand for several minutes (Fig. 2A). The blue color was retained in the bacterial pellet upon centrifugation of cultures, being concentrated in the lower part of the pellet, and membranes isolated by subcellular fractionation of bacteria were also colored blue.

Various hypotheses for the production of pigment were discounted after preliminary evaluations, including microbial contamination of recombinant *E. coli* cultures, degradation products of heme, precipitation of metal salts, or unusual flavin intermediates. The production of color by P450 2A6 cultures was IPTG induction-dependent but not stimulated further by the addition of 1 mM indole to the medium (Figs. 2B and 2C). Initial TLC with human P450 2E1 systems yielded evidence for indigo production (Fig. 3). A pink spot was observed on the TLC (Fig. 3) which may be indirubin, a structural isomer of indigo.

Characterization of products. The UV-visible spectra of the blue extracts from the P450 2E1 cultures were similar to that of indigo (Fig. 4). Preliminary mass spectrometric analysis of the extracellular blue pigment pro-
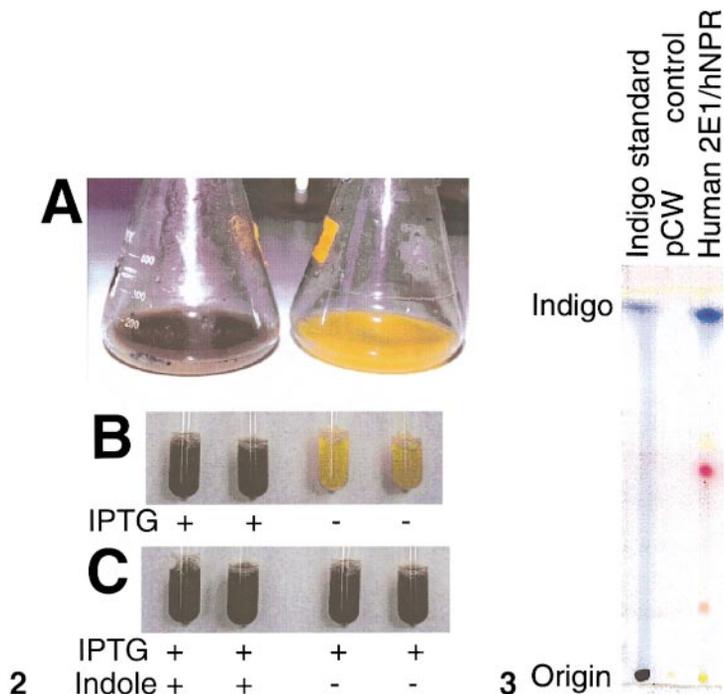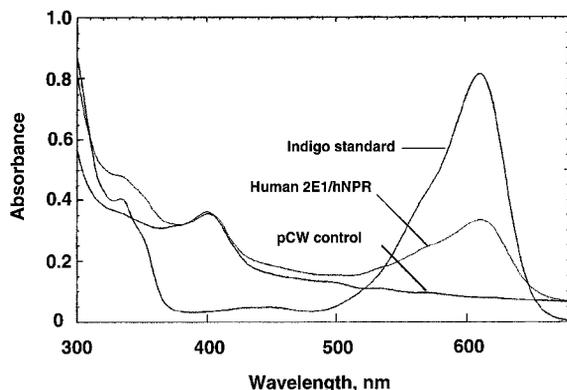
FIG. 2. Blue pigments in P450 cultures. (A) Flasks comparing P450 2A6 (left) and P450 3A4 (right) cultures. Note general dark color of medium and particles of blue pigment in the 2A6 culture compared to the usual color of cultures as represented by the P450 3A4 culture. (B) P450 2A6 cultures (2 ml) were grown with (+) and without (−) IPTG induction in TB media (48 h at 29°C) with vigorous shaking. (C) Expression of P450 2A6 was induced by IPTG with (+) or without (−) the addition of 1.0 mM indole to the culture.

FIG. 3. TLC of CHCl$_3$ extracts. Standard indigo (Aldrich Chemical Co., Milwaukee, WI) is included, along with an extract from the human P450 2E1/hNPR expression system and cells transformed with the empty expression vector alone (pCW control).

duced in a P450 2A6 culture (and purified by TLC) yielded a base peak at m/z 263.2, interpreted as MH$^+$ (molecular mass 262.1). UV-visible spectra of this P450 2A6 product were also similar to the spectra of indigo.

Formation of blue pigment in vitro. Bacterial membranes containing recombinant human P450 2E1 and hNPR were prepared and incubated with indole (5 mM) and NADPH. Control incubations were conducted


FIG. 4. UV-visible spectra of pigments formed by *E. coli* cells expressing P450 2E1 and hNPR compared to cells transformed with the empty expression vector alone (pCW control). Spectra were recorded in DMF.

in parallel using membranes isolated from bacteria transformed with the monocistronic vectors for expression of hNPR alone, and from bacteria containing no expression vector. A time-dependent increase in A$_{665}$ was observed with incubations containing 2E1/hNPR membranes, which was significantly enhanced over that observed in the absence of either P450 2E1 or hNPR (Fig. 5). Similar results were seen in experiments with P450 2A6 (results not presented).

DISCUSSION

The bacterial expression of some recombinant mammalian P450s under conditions in which they were capable of productive electron transport led to the production of blue cultures. This phenomenon had been observed independently in three of our laboratories. The blue pigment is identified as indigo, on the basis of its UV-visible and mass spectra and its co-migration with authentic material on TLC. The presence of additional blue pigments in the extracts cannot be ruled out at this time, however. A pink component was also found in material extracted from the P450-expressing *E. coli* cultures (Fig. 3). This may be indirubin, an isomer of indigo that is often seen in microbial preparations of indigo.

A likely precursor of indigo (and the putative indirubin) is indole, which is an intermediate in tryptophan
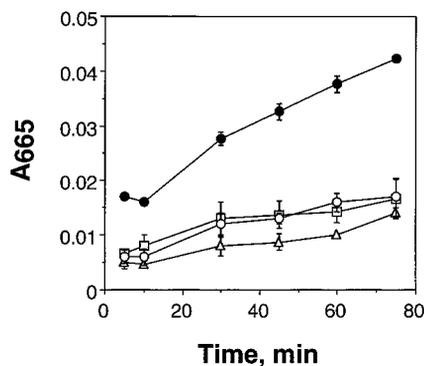
FIG. 5. *In vitro* production of blue pigments by membranes from bacteria coexpressing P450 2E1 and hNPR. The individuals plots show incubations with membranes containing: recombinant P450 2E1 and hNPR (●) (0.10 μM and 0.095 μM respectively; 0.27 mg membrane protein/ml); only recombinant P450 2E1 (○) (0.10 μM); only recombinant hNPR (△) (0.095 μM); membranes from cells transformed with the expression vector pCW, containing no recombinant cDNAs (□) (0.27 mg protein/ml). Results are presented as means ± SD of three independent determinations.

metabolism. *In vitro* experiments with bicistronic *E. coli* membranes containing both P450 2E1 and NPR showed that indole could be converted to blue pigment (Fig. 5), in a reaction shown to be NADPH-dependent (results not shown). Although the *E. coli* cultures produced indigo in the absence of supplemental indole (Fig. 2C), it is likely that the rich expression media used provides sufficient indole as substrate (via tryptophan degradation) such that supplemental indole does not lead to further improvement in indigo yields. Moreover, excess indole may be toxic to bacterial cells (8). On the basis of what is known about indigo synthesis in other systems, we speculate that some P450s may be capable of catalyzing the oxidation of indole to 3-hydroxyindole (indoxyl), which is known to be oxidized non-enzymatically to indigo (9). This chemistry is the basis of the commonly used "X-gal" and "X-glc" reactions, with substrates 5-bromo-4-chloro-3-indolyl-β-D-galactoside and with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, which use β-galactosidase or β-glucuronidase, respectively, to generate a 3-hydroxyindole (10). Precedent for oxidation of indoles by P450s is seen in the activation of the pneumotoxin 3-methylindole by P450 2F enzymes (11). The ability of P450s 2A6 and 2E1 to oxidize indole is consistent with their known propensity for small substrates (12, 13).

Indigo has a long history and is considered to be the oldest dye known to man (14, 15). The original source was plants, and the total synthesis of the dye was considered a major development in the 19th century chemical industry (15). More recently, several dioxygenases have been reported to oxidize indole to indigo (14, 16, 17), and a biotechnology process involving a naphthalene dioxygenase has been shown to be commercially viable (18, 19). Monooxygenases of microbial origin have also been re-

ported to produce indigo (20, 21). Exactly how the levels of indigo produced by mammalian P450s in these bacterial heterologous expression systems compare with these others remains to be determined.

The ability of mammalian P450s to utilize an endogenous component of a heterologous bacterial expression system as a substrate for production of a dyestuff was unexpected but is consistent with the wide substrate ranges of these enzymes. What is unclear is exactly what other indole oxidations are catalyzed by these (and other?) P450s and if any of these occur in mammalian systems *in vivo*. The production of dyestuffs by P450s may have potential for industrial and agricultural applications as well as use in more fundamental studies of how P450s catalyze substrate oxidations.

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