Periplasmic uroporphyrin formation catalysed by an exported CYP

Export of a heterologous cytochrome P450 (CYP105D1) in Escherichia coli is associated with periplasmic accumulation of uroporphyrin

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Abbreviations used: ALA, 5-aminolevulinic acid
CYP, cytochrome P450
TA, Tris-acetate (10mM) buffer (pH 8)
URO, uroporphyrin
UROgen, uroporphyrinogen

Running title: Exported CYP catalyses periplasmic production of uroporphyrin

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SUMMARY

This report suggests an important physiological role of a CYP in the accumulation of uroporphyrin I arising from catalytic oxidative conversion of uroporphyrinogen I to uroporphyrin I in the periplasm of *Escherichia coli* cultured in the presence of 5-aminolevulinic acid. A structurally competent *Streptomyces griseus* CYP105D1 was expressed as an engineered, exportable form in aerobically-grown *Escherichia coli*. Its progressive induction in the presence of 5-aminolevulinic acid-supplemented medium was accompanied by an accumulation of over a hundred-fold higher amount of uroporphyrin I in the periplasm relative to cells lacking CYP105D1. Expression of a cytoplasmic-resident engineered CYP105D1 at a comparative level to the secreted form was far less effective in promoting porphyrin accumulation in the periplasm. Expression at ten-fold molar excess over the exported CYP105D1 of another periplasmically-exported hemoprotein, the globular core of cytochrome b5, did not substitute the role of the periplasmically-localised CYP105D1 in promoting porphyrin production. This, therefore, eliminated the possibility that uroporphyrin accumulation is merely a result of increased hemoprotein synthesis. Moreover, in the strain that secreted CYP105D1, uroporphyrin production was considerably reduced by azole-based P450 inhibitors. Production of both holo CYP105D1 and uroporphyrin was dependent upon 5-aminolevulinic acid, except that at higher concentrations this resulted in a decrease in uroporphyrin. This study suggests that the exported CYP105D1 oxidatively catalyses periplasmic conversion of uroporphyrinogen I to uroporphyrin I in *Escherichia coli*. The findings have significant implications in the ontogenesis of human uroporphyria-related diseases.
INTRODUCTION

Porphyrin metabolism in humans has been extensively investigated, particularly in the occurrence of a group of clinically-related diseases known as porphyrias, arising from genetic defects (1) of the enzymes in heme biosynthesis causing accumulation of various heme precursors (2,3). The commonest porphyric disorder, porphyria cutanea tarda, and its related forms are characterized by massive hepatic accumulation and increased excretion of uroporphyrin (URO) and to a lesser extent other types of porphyrins such as coproporphyrins and isocoproporphyrins (4). A number of etiological factors such as iron overload (5), ascorbic acid deficiency (6), hepatitis c infection (7), alcohol abuse (8) and induction of CYP isoenzymes (9) have been suggested to be the cause of the sporadic form of the diseases. Of the many possible URO isomers, only type I and III occur in nature. Both isomers arise from spontaneous oxidation of the heme metabolic intermediates, uroporphyrinogen (UROgen) I and III. UROgen III synthase catalyzes the conversion of hydroxymethylbilane to UROgen III which involves rearrangement of the propionate and acetate groups of ring IV of the tetrapyrrole, whereas UROgen I is formed non-enzymatically. UROgen I and III can be further metabolized by UROgen decarboxylase to yield coproporphyrinogens I and III, respectively, which can also undergo spontaneous oxidation to coproporphyrins I and III.

Uroporphyric-like states which lead to a significant build-up of URO have been experimentally induced in eukaryotic cells exposed to CYP-inducers such as chlorinated biphenyls (10) and methylcholanthrene (11). Since such xenobiotics are classic inducers of CYP (12), a link has been suggested between susceptibility to uroporphyria (porphyrias characterized by URO and UROgen overload) and CYP-mediated oxidization of heme intermediates. The in vitro observation of CYP-catalyzed conversion of UROgen to URO (13) has been suggested as
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a major potential factor in the initiation of uroporphyrinia. Although there is strong, but indirect, evidence for the participation of a variety of CYPs in the etiology of uroporphyrinas, for example, CYP1A subfamily (14), CYP1A2 and particularly CYP3A (10), the metabolic contribution of the hemoprotein in vivo has not been firmly established.

Most recently, the soluble CYP105D1 of Streptomyces griseus has been successfully expressed in E. coli as an exportable form that was efficiently localised to the periplasm (15). Expression of the periplasmic CYP105D1 was coupled with generation of a highly fluorescent compound identified as URO I. We now show that the uroporphyrinic state induced in the engineered E. coli, which has a requirement for ALA to be present, is associated in vivo with the exported, catalytically-active CYP105D1 and arises from URO I accumulation. The formation of such products has not been observed before in prokaryotic cells heterologously-expressing CYP and has in turn important implications in the ontogenesis of the related diseases in human disorders.
EXPERIMENTAL PROCEDURES

Unless stated otherwise, all chemicals were purchased from Sigma Chemicals. Clotrimazole, Triadimefon and Miconazole were kindly donated by Dr E. I. Mercer (University of Wales, Aberystwyth). UROgen III was obtained from Frontier Scientific (UK). Electrophoresis reagents were purchased from BioRad Labs (Herts, UK) and BDH (Poole, UK).

Heterologous expression, quantification and isolation of CYPs In the present study we employed E. coli TB1 \{F, ara\Delta(lac-proAB)rps \phi 80d lacZ\DeltaM15hsdR17 (r\delta^+ m\kappa^-)\} strain harbouring pLi-CYP105D1 encoding the secretory form of CYP105D1, pAA-CYT coding for the secretory form of mammalian cytochrome b5 (16,17), pIN-CYP105 expressing the cytosolic CYP105D1 and the control progenitor plasmid pLi-Q (15). Plasmid pIN-CYP105D1 is a derivative of pLi-CYP105D1 and lacks the signal sequence. The vector was constructed by an inverse PCR strategy using pLi-CYP105D1 as the template and the following primers:

5’ ATGACGGAATCCACGACGG
5’ TTTATTTTCTCCATGTACAAATAC

The amplified DNA duplex was circularised by T4 DNA ligase and introduced into E. coli TB1. For induction of recombinant proteins, a 2% (v/v) inoculum of Luria Bertani broth-grown, saturated culture was added to a phosphate-limited (0.1 mM) MOPS medium (16) containing 75 µg ampicillin/ml. The culture was batch-cultivated at 35°C with orbital agitation at 125 rpm for periods specified elsewhere in the text. Unless stated otherwise, 5-aminolevulinic acid (ALA) at 1 mM was routinely included in the MOPS growth media.

All media used for subcellular fractionation or isolation of URO contained 5 mM ascorbic acid in order to reduce adventitious auto-oxidative generation of porphyrin. Periplasm extracts
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were isolated as described previously (15). To verify that authentic isolation of the periplasm had occurred, marker enzymes known to be specific for each of the sub-cellular fraction were routinely assayed as described (16). CYP content in biological samples were estimated according to the procedure described by Omura and Sato (18) using an absorption coefficient of 91 mM$^{-1}$ cm$^{-1}$. The oxidised cytochrome b$_5$ was quantified from the Soret absorption peak at 413 nm using the absorption coefficient of 115 mM$^{-1}$ cm$^{-1}$ (19). Protein content was estimated employing the Bio-Rad protein assay (Bio-Rad, UK) using bovine serum albumin as the standard (20).

The CYP105D1 enzyme was purified from the periplasmic extract of *E. coli* CYP105D1 (1 Lit culture) cultivated for 24 h at 28°C with 1 mM ALA supplementation. Following two-fold dilution of the periplasmic extract with 10mM Tris-acetate (pH 8) buffer (TA) containing 1 mM EDTA, the sample was applied twice through a TA-pre-equilibrated column of DEAE CL-6B Sepharose (5 ml bed volume). The column was successively washed with 5 ml of (i) TA, (ii) 0.1 M NaCl in TA and (iii) 0.3 M NaCl in TA. The enzyme was eluted with 25 ml 0.5 M NaCl in TA. The solution was concentrated by pressure filtration to 2.5 ml and the CYP105D1 further purified by FPLC by passage through a Superose column (1.5 cm x 65 cm) with 50 mM Tris-HCl (pH 7.5) buffer. The flow rate was 1 ml/min and fractions of 2 ml were collected. CYP105D1-enriched fractions were pooled and concentrated using an Amicon Centricon 10 micro-concentrator (LKB-Pharmacia, UK).

**Isolation and estimation of URO** Periplasmic and cytoplasmic extracts were derived from *E. coli* cultured in 100 ml MOPS media supplemented with ALA and 75 µg ampicillin/ml. Each extract, finally recovered in 5 ml TA, was acidified with HCl to 0.2 M final concentration (pH < 2) and left on ice for 5 min. The deproteinised supernatant was recovered by
centrifugation at 13,000 g for 2 min. The URO in the supernatant fraction after appropriate dilution were estimated spectroscopically from absorbance at 406 nm using the absorption coefficient 541 mM⁻¹ cm⁻¹ (21). The values were standardized to nmol/L culture.

For estimation of URO in the ‘spent’ growth medium, 20 ml of the growth medium was applied onto a DEAE CL-6B Sepharose column (1 ml bed volume). After washing the column with 5 ml of 0.3 M NaCl in TA, the pigments were eluted with 2 ml 1 M NaCl in TA, acidified and spectroscopically quantified as described above.

For the analysis and estimation of URO by HPLC (22), the periplasmic fraction (5 ml volume recovered from 100 ml culture) was first de-proteinised with 0.2 M HCl as described above. The pH of the supernatant was adjusted to 7.5 by titration with Trizma base. Following five-fold dilution with distilled water, the solution was applied onto a DEAE Sepharose CL-6B column (1 ml bed volume). After washing the column with 5 ml TA, the pigments were eluted with 1 ml 1 M NaCl in TA. HPLC was performed on an ODS2 column (250 mm x 4.6 mm; 5 µm particle size) eluted at a flow rate of 1 ml/min with an acetonitrile-1 M ammonium acetate (pH 5.16) (14.3:85.7 (v/v)) as the mobile phase. The amounts of porphyrins in each peak were calculated by comparing with the standard curve derived from known amounts of URO I and III. Compounds were detected by measuring the absorbance at 406 nm in a continuous flow cell and the data processed by Thermochrom II software (LDC Analytical, UK).

**Heme solution** A 10 mM heme solution was prepared as follows. Bovine hemin, after dissolving in a 1/20 of the final volume of freshly-prepared 1 M NaOH, was sequentially mixed with an equal volume of 1 M Tris-HCl (pH 7) and 8/10 final volume of ethylene glycol. The pH was adjusted to 8.2 with 1 M HCl. The solution was made up to the final volume with distilled water,
filter-sterilized by passage through a 0.2 μm filter and stored at 4°C. To test the ability of *E. coli* TB1 strain to utilize exogenous heme, the stock heme solution was applied in the MOPS medium at five-fold increasing concentrations ranging from 1 to 50 μM.
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RESULTS

A fluorescent compound generated during periplasmic expression of CYP105D1 is a URO

We recently reported that an E. coli strain harbouring an engineered pLi-CYP105D1 plasmid expressed an exportable form of the Streptomyces griseus CYP105D1 under the transcriptional control of the tightly-regulated phoA promoter (23). The periplasmic localisation of CYP105D1 was facilitated by N-terminal appendage of a bacterial secretory signal that utilises the sec-dependent translocon export pathway (24). The pre-CYP105D1 was correctly processed, folded and heme-assembled to yield substantial quantities of the functional recombinant hemoprotein in the periplasm.

E. coli pLi-CYP105D1 cultured in the MOPS medium in the presence of 1 mM ALA for periods ranging from 12 h to 36 h displayed a strong brown hue in contrast to comparably cultured control cells containing the progenitor plasmid pLi-Q lacking cyp105D1. The color was partly attributed to the presence of significant quantities of the holo CYP105D1 that had accrued in the periplasm. Interestingly, UV-illumination of E. coli pLi-CYP105D1 but not E. coli pLi-Q cells exhibited an intense red fluorescence emission. Preliminary sub-fractionation studies indicated that the fluorescent agent(s) was particularly concentrated in the periplasmic extract.

In order to isolate and characterise the fluorescent compound(s), the periplasmic extract from E. coli pLi-CYP105D1 was subjected to anion exchange separation by DEAE Sepharose CL-6B chromatography. Following sequential washes with 0.1 M, 0.2 M and 0.3 M NaCl in TA an intense dark brown layer remained firmly bound to the column. A further wash with 0.5 M NaCl in TA eluted a light brown-colored fraction containing the CYP105D1 protein together with a pigment which showed predominant absorption peaks between 486-492 nm. A final wash with 1 M NaCl in TA displaced a rose-colored fraction which gave an intense red fluorescence
upon UV light illumination. Spectrofluorometric analysis of the isolated compound(s) upon excitation at 398 nm displayed a major emission peak at 615 nm together with smaller peaks 642 nm and 678 nm (Fig. 1). Absorption spectroscopy in the visible region gave a major peak at 398 nm (Fig. 2A) with four smaller peaks at 502 nm, 538 nm, 560 nm and 612 nm (Fig. 2B); the spectrum matched well with that of authentic URO I (25). The etio-nature of the UV-visible spectrum suggested that the potential URO was in a metal-free state (26). Acidification of the solution with HCl (0.2 M final concentration) shifted the Soret absorption peak to 406 nm (Fig. 3A) and yielded two additional minor peaks at 550 nm and 592 nm (Fig. 3B). Further analysis of the pigment(s) by electrospray ionisation spectrometry revealed a major, stable predominant species with a relative molecular mass of 831 (Fig. 4) that was not detectable in the E. coli pLi-Q extract. The spectral characteristics and the mass of the bacterially-produced fluorescent compound were identical to a commercially-obtained authentic URO I (Figs. 2-4). This could be indicative of URO I but notably URO III, the counterpart physiological isomer, also has identical spectral characteristics. However, further comparison of the unknown pigment with the two authentic pigments by HPLC analysis (22) identified type I isomer as the major species constituting about 85%. Identification was further confirmed by thin layer chromatography (27) whereby the relative mobility of the bacterial pigment matched with the authentic URO I. Under the present conditions of analysis other intermediates of the pathway such as hepta-, hexa-, penta and tetra-carboxylporphyrins were not detected.
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URO accumulation is confined to the periplasm and concomitantly coupled with the exported holo CYP105D1

The quantities of URO in the periplasm and cytoplasm compartments and in the growth media of *E. coli* strains harbouring pLi-Q or pLi-CYP105D1 cultured in the presence of 1 mM ALA were monitored as a function of time (Table 1). In both strains, the periplasmic URO accrued with the duration of culturing. However, the control pLi-Q strain showed only a five-fold increase ranging from 2 nmol/Lit culture at 12 h to about 10 nmol URO/Lit culture at 36 h compared to an approximately 100-fold increase in URO in the recombinant strain. The latter produced 800 nmol URO/L by 36 h. In the pLi-Q strain, URO profile followed a similar pattern to that seen in its periplasm. In contrast, the cytoplasmic level of URO in pLi-CYP105D1 increased substantially by nearly ten-fold, reaching 25 nmol URO/Lit culture following 36 h of culturing. Given the high accumulation of URO in the periplasm, we cannot discount the likelihood that URO in the cytoplasmic fraction could be, at least partly, derived from contamination during cellular fractionation or *in vivo* leakage from the periplasm. In contrast, the URO found in the growth media in the control and pLi-CYP105D1-expressing strains were negligible (Table 1).

We considered the possibility that over-expression and export of the apo CYP105D1 may have induced synthesis and periplasmic export of heme. This possibility was explored by measuring the periplasmic and cytoplasmic pools of URO in an *E. coli* TB1 strain pAA-CYT that efficiently exports a mammalian recombinant cytochrome b₅ under comparable expression conditions to CYP105D1 (16). Interestingly, although cytochrome b₅ is produced at about a 10-fold molar (24 h) excess over CYP105 (Table 2), the periplasmic, cytoplasmic and medium URO
levels in this strain are similar to those in the control pLi-Q strain throughout the duration of culturing (Table 1).

To investigate whether URO production was an event specifically coupled with expression of the periplasmic form of CYP105D1, we constructed a cell line that expressed a non-secreted, cytoplasmic CYP105D1. The cytoplasmic CYP105D1 was expressed more efficiently than the secretory form (Table 2) and supported URO formation, though to a significantly lesser extent than that catalysed by the periplasmic catalyst (Table 1). Identities of the URO accumulated in the periplasmic fractions of the control and recombinant strain was confirmed by subjecting de-proteinated extracts to HPLC analysis. Using the standard conditions described under Experimental Procedures, URO (I and III) were clearly resolved and URO I was the dominant species. The overall pattern and yields of URO detected by HPLC were similar to that monitored spectroscopically in the periplasmic extracts.

URO production requires exogenous ALA and co-expression of holo CYP105D1

In view of the long periods (> 12 h) required to generate significant URO production, it was of interest to explore whether this phenomenon was related to synthesis and/or assembly of CYP105D1 in the periplasm. Hence, this necessitated an investigation on ALA dependency on production of holo and apo CYP105D1 (Fig. 5). The periplasmically-recovered soluble CYP105D1 was separated by non-denaturing gel electrophoresis. The apo and holo forms were detected and distinguished by a combination of heme-staining and Western blotting using anti-CYP105D1. Apo but not holo cyp105D1 was detectable in the E. coli cultured for 12 h without ALA. By 24 h of culturing in the absence of ALA apo CYP105D1 almost doubled, of which approx 6% was converted to holo form. With supplementation of increasing ALA concentrations
in the medium, conversion of apo form to holo CYP105D1 accrued hyperbolically and were saturated at 2 mM ALA. Substitution of ALA with heme in the medium did not enhance the production of holo CYP105D1. Moreover, significant synthesis of URO in the periplasm of *E. coli* pLi-CYP105D1 occurred only in the presence of exogenously-supplemented ALA (Fig. 6). Peak production of URO was reached at 0.25 mM ALA and at higher doses inhibition was imposed. Interestingly, supplementation of the cultures with additional ALA at 12 h slightly enhanced the productivity of URO but beyond 0.2 mM ALA a more drastic inhibition of URO was imposed (Fig. 6). In contrast, formation of holo CYP105D1 was more directly proportional to ALA concentration up to 0.8 mM such that at the highest tested concentration of 2 mM ALA, a 20-fold increase in holo hemoprotein was reached (Figs 5 and 6). These findings suggest that formation of both holo CYP105D1 and URO production were clearly dependent upon ALA supplemented in the medium except that in the latter case a higher concentration of ALA caused a decrease in URO accumulation for reasons which are not clear.

**Selective fungicides inhibit URO production**

We investigated the effects of the fungal P450 inhibitors, clotrimazole, miconazole and triadimefon, which interfere with the sterol biosynthesis (28) on the productivity of URO. These compounds are well known to exert their inhibitory effects by interacting with the heme iron of CYPs (29). Standard batch cultures were incubated with the inhibitors at concentrations ranging from 0 to 2.5 mM, and the periplasmic URO monitored after 24 h of culturing (Fig. 7). Clotrimazole at 0.5 mM produced the most pronounced decline (65%) in URO accumulation. However, all of the inhibitors suppressed URO production in a dose-dependent manner and yielded a similar inhibition of about 60% at 2.5 mMazole. These inhibitors did not affect the growth of *E. coli*. Moreover, clotrimazole that most potently inhibited URO formation in the
Periplasmic uroporphyrin formation catalysed by an exported CYP recombinant cell line, did not significantly affect the production of URO in the control cell line. β-diethylaminoethyl diphenylpropyl acetate (proadifen, SKF525A), a non-imidazole CYP inhibitor that is structurally and mechanistically unrelated to azoles (30), had no significant effect on URO production in E. coli pLi-CYP105D1.
DISCUSSION

In the present study we have investigated the formation of a fluorescent pigment in *E. coli* TB1 strain in ALA-treated cells expressing a secreted or periplasmic-localized CYP105D1. The pigment has been firmly identified by absorption and fluorescence spectroscopy, electron-spray ionisation and HPLC. Several pieces of evidence strongly point to catalytic involvement of CYP105D1 in the transformation of UROgen I to URO I. Over a hundred-fold higher level of URO was found in *E. coli* pLi-CYP105D1 than in the comparable control harbouring pLi-Q or pAA-CYT (Table 1). The azole compounds, known to be potent inhibitors of CYPs, considerably reduced the *in vivo* productivity of URO in the recombinant but not in the non-recombinant cell lines (Fig. 7). In contrast to the control strain pLi-Q and another over-expressing, secreted hemoprotein, cytochrome b₅, production of URO I was most effective in *E. coli* harbouring pLi-CYP105D1 expressing the periplasmically-localised CYP105D1. Nevertheless, a small but significantly elevated production of URO was also observed in the cytosolic-expressing CYP105D1 cells (Table 1). Thus, we conclude that periplasmic targeting of the catalyst was a pre-requisite for generating a uroporphyrin-like state. This raises interesting issues regarding the temporal nature of periplasmic CYP-catalysed conversion of UROgen I to URO I in *E. coli*. The important points to address are: (i) the derivation of the substrate from the heme biosynthetic pathway in the cytosol (ii) the preferential generation of URO I rather than URO III isomer (iii) the likely mechanism(s) by which UROgen I reaches the periplasm for the CYP105D1-catalysed conversion to URO I, and (iv) the nature of electrons source and the likely mechanism of the transformation by CYP105D1.

ALA is the first committed precursor of tetrapyrrole synthesis and its intracellular concentration limits the rate of porphyrin synthesis in *E. coli* (31). In prokaryotes, ALA synthase
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is a regulatory enzyme whose activity can be repressed by heme feed-back inhibition. However, exogenously-supplied ALA can cause an overproduction of porphyrins (32,33) and heme as shown in this study. However, it seems unlikely that uroporphyrin accumulation merely resulted from increased hemoprotein synthesis since over-expression at ten-fold molar excess over the exported CYP105D1 of the periplasmically-exported hemoprotein, the globular core of cytochrome b\textsubscript{5}, failed to promote porphyrin production. Expression of the substantial amounts periplasmic apo CYP105D1 and its conversion to holo form demands a higher than normal cellular heme supply which is derived from exogenously-supplied ALA in the growth medium (Figs. 5 and 6). In the absence of ALA only a small proportion (< 10%) of the expressed CYP105D1 was converted to the holo form (Fig. 5). Exogenous heme supplementation in the culture medium did not improve the generation of holo CYP105D1 suggesting that the prosthetic group was not made available in the periplasm of \textit{E. coli} TB1 cells as previously reported for the secreted form of cytochrome b\textsubscript{5} (34) and that the outer membrane of \textit{E. coli} is normally impermeable to heme (35). Nevertheless, the alleviation of this heme deficiency by ALA supplemented in the culture medium (Figs. 5 and 6) indicated that heme, during the assembly of CYP in \textit{E. coli}, was derived metabolically via the \textit{de novo} pathway (36). This suggests that the limiting step in heme synthesis during overproduction of CYP105D1 occurs at or prior to ALA synthesis (37). Thus, excess ALA is required to produce maximal holo CYP which when expressed periplasmically produces massive URO accumulation.

The second issue relates to the question of preferential periplasmic accumulation of URO I rather than the III isomer with co-expression of periplasmic CYP105D1. Indeed both URO I and URO III can accumulate either in the presence or absence of ALA under some circumstances in a variety of cells. Moreover, the UROgens, which are the true intermediates, also need to be
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oxidized to generate the porphyrins. The production of all heme intermediates would be expected to occur exclusively in the cytosol where the complete hem-encoded pathway is localised. Hydroxymethylbilane synthase (porphobilinogen deaminase) condenses four porphobilinogen molecules to form the first linear tetrapyrrole, hydroxymethylbilane (38). Hydroxymethylbilane, being unstable in solution can non-enzymatically cyclize to form UROgen I which is a physiologically redundant end product. However, hydroxymethylbilane is normally rapidly converted to UROgen III by the action of UROgen III synthase (39). Both I and III forms of UROgens have the potential to be oxidised to their respective porphyrin forms in the cytosol, although this apparently does not occur to a large extent under the physiological conditions studied here. UROgen III would be expected to be removed by the action of UROgen decarboxylase and its auto-oxidative conversion to URO III may be partly suppressed due to a higher reducing condition within the cytoplasm. Whilst UROgen I may also accumulate in the cytosol and/or periplasm, UROgen III can be removed by the action of UROgen III decarboxylase, to be eventually converted to heme. Although the accumulated UROgen I can also be decarboxylated by UROgen decarboxylase to give coproporphyrinogen I, we are unable to explain why the oxidized intermediate, coproporphyrin I was not observed here. Nevertheless, the findings imply that UROgen III decarboxylase is absent in the periplasm. A plausible model of the likely metabolic events is depicted in Fig. 8.

UROgen I must accumulate in the cytoplasm but then how does it resist auto-oxidation within the cytoplasm and become available in the periplasm? Perhaps under the more reducing condition within the cytoplasm, endogenous auto-oxidation of UROgen may be partially suppressed. Another possibility is that the overproduced heme intermediate may be translocated into the periplasm (40) where its conversion to URO I can be accounted for by catalytically-
enhanced oxidation by CYP105D1 in the more amenable oxidising environment of the periplasm (Fig.8).

The periplasmic reaction involving production of URO I would be expected to require reducing equivalent(s) and molecular oxygen for the P450 catalysis and a yet unidentified reductase system to transfer the reducing equivalents to CYP105D1. Reducing equivalents such as NADH or NADPH are unlikely to be present in periplasmic space but they could be scavenged from the electron transport chain or other periplasmic oxido-reductant reactions. However, electron transfer to the heterologous CYP105D1 must require a reductase, most likely a ferredoxin reduction type system. Genomic analysis of E. coli suggests presence of a periplasmic ferrodoxin-type reductase which uses a TAT-dependent export pathway (41). The removal of six hydrogen atoms from UROgen I, two from the pyrrolic nitrogens and four from the methene carbons would result in the formation of URO I. As yet, the mechanism for the mediation of UROgen oxidation by CYP remains unknown.

Clearly, ALA-dependent URO I production can be induced in a recombinant E. coli expressing periplasmic CYP105D1 and we have made similar observations for a secreted form of human CYP17. Further examples are likely and will extend our understanding of such metabolic effects on the ontogenesis of porphyria. Exposure of eukaryotic cells with exogenous ALA also increases URO due to insufficiency of decarboxylase (42) which can be potentiated by pre-exposure to xenobiotics such as chlorinated biphenyls (43). The essential role of a methycholantherene-inducible (44) and other CYPs (45-47) in the oxidation of UROgen suggests that the CYP enzyme(s) catalytically participate in the conversion of the heme intermediate UROgen to its oxidised form, URO, thus implicating it as a major potential factor in the initiation of URO overload. The present findings are consistent with the more recent
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observations of CYP knock-out mice which fail to accumulate URO upon exposure to the classic inducers of CYP1A2 and ALA (48). Nevertheless, this is the first study providing clear evidence that the highest levels of uroporphyrin accumulation are remarkably correlated with expression of a broad substrate-range CYP105D1 in *E. coli*.

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Table 1: URO amounts in isolated periplasmic and cytoplasmic fractions and in the growth medium of *E. coli* strains containing derivatives of pLiQ expressing secreted CYP105D1 (pLi-CYP105D1), secreted cytochrome b$_5$ (pAA-CYT) and cytoplasmic-resident CYP105D1 (pIN-CYP105D1)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Incubation period</th>
<th>E. coli pLi-Q</th>
<th>E. coli pLi-CYP105D1</th>
<th>E. coli pAA-CYT</th>
<th>E. coli pIN-CYP105D1</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>12h</td>
<td>24h</td>
<td>36h</td>
<td>12h</td>
<td>24h</td>
</tr>
<tr>
<td>URO in periplasm</td>
<td>1.9 ± 0.4</td>
<td>2.01 ± 0.5</td>
<td>10.3 ± 1.5</td>
<td>2.8 ± 0.6</td>
<td>511 ± 51</td>
</tr>
<tr>
<td></td>
<td>(95 ± 3)</td>
<td>(87 ± 5)</td>
<td>(416 ± 30)</td>
<td>(89 ± 4)</td>
<td>(12,270 ± 610)</td>
</tr>
<tr>
<td>URO in cytosol</td>
<td>2.2 ± 0.4</td>
<td>4.3 ± 0.5</td>
<td>9.6 ± 1.1</td>
<td>2.6 ± 0.08</td>
<td>17.9 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>(9 ± 0.5)</td>
<td>(14 ± 1.1)</td>
<td>(29 ± 3.5)</td>
<td>(10 ± 0.6)</td>
<td>(68 ± 5)</td>
</tr>
<tr>
<td>URO in growth medium</td>
<td>0.06 ± 0.01</td>
<td>0.05 ± 0.02</td>
<td>0.05 ± 0.01</td>
<td>0.07 ± 0.03</td>
<td>0.05 ± 0.01</td>
</tr>
</tbody>
</table>

The values as nmol URO /Llt culture or pmol URO /mg protein (in brackets) are presented as means ± SD of triplicate measurements. URO were monitored in deproteinised, HCl-acidified (pH < 2) samples as described under Experimental Procedures. Protein was not detectable in the growth medium. P, *PhoA* promoter; SD, Shine-Dalgarno sequence; SS, signal sequence of alkaline phosphatase; b$_5$, globular core of cytochrome b$_5$. 
Table 2: Holo forms of CYP105D1 and cytochrome b5 in *E. coli* extracts as a function of culture duration

<table>
<thead>
<tr>
<th></th>
<th>Holo hemoprotein content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>E. coli</em> pCYP105D1</td>
</tr>
<tr>
<td></td>
<td>(periplasm))</td>
</tr>
<tr>
<td>12h</td>
<td>24h</td>
</tr>
<tr>
<td>nmol hemoprotein /L culture</td>
<td>12 ± 1.7</td>
</tr>
<tr>
<td>nmol hemoprotein /mg protein</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>24h</td>
<td>87 ± 10</td>
</tr>
<tr>
<td>20 ± 6</td>
<td>29 ± 7</td>
</tr>
<tr>
<td>36h</td>
<td>77 ± 11</td>
</tr>
</tbody>
</table>

The estimations of hemoproteins were performed in the isolated periplasmic extracts as described in the Experimental Procedures. Data are presented as means ± SD of triplicate measurements.
Figure 1. The fluorescence scans of the pigment eluted from a DEAE CL-6B Sepharose column following washing with 1 M NaCl in TA buffer (pH 8). The spectra were recorded across a 1 cm light path and at one nm wavelength intervals following excitation at 398 nm. The emission spectra were performed using a Shimadzu RF-5301PC spectrofluorophotometer.
Figure 2. Absorption spectra of the putative porphyrin pigment isolated from *E. coli* periplasm compared to authentic URO I. The spectra were recorded in TA buffer (pH 8). The rose-colored pigment displayed a major Soret absorption peak at 398nm (A) together with four minor peaks at 502nm, 538nm, 560nm and 612nm, shown more clearly in the inset (B).
Figure 3. Absorption spectra of the HCl-acidified putative porphyrinic pigment (pH < 2) isolated from *E. coli* periplasm compared to authentic URO I.
Figure 4. Electrospray ionisation spectroscopy of the isolated pigment. A 70 μl portion of the 1 M NaCl TA buffer (pH 8)-eluted-pigment from DEAE-Sepharose CL-6B was 100-fold pre-diluted with acetonitrile:water (50:50 (v/v)) containing 0.1% (w/v) formic acid, and applied continuously through a Harvard syringe pump at a flow rate of 5 μl/ min through a silicon tubing (0.7 μm I.D x 1 m) into an LCT-ES spectroscope (Micromass, UK). Each scan was integrated over a 30sec period and the data deconvoluted using Mass Lynx software (Ver 3.2).
Periplasmic uroporphyrin formation catalysed by an exported CYP

**Figure 5.** Expression of CYP105D1 detected in the in periplasm extracts of *E. coli* pLi-CYP105D1 cultured without or with varying doses of ALA supplemented in the growth media. CYP105D1 visualised either by (A) heme-activity staining (49) or (B) immuno-detection. Band I, apo CYP105D1; band II, holo CYP105D1. Periplasmic proteins were separated in a non-denaturing polyacrylamide gel (6 % (w/v), pH 6.5 (50) containing glycine and 0.2 M urea. For Western-blot analysis, the gel was first gently agitated with the denaturing buffer (0.2 % (w/v) sodium dodecyl sulphate, 50mM Tris-HCl (pH 8.2) for 2 h and the proteins transferred onto a nitrocellulose transfer membrane essentially as described (51). The membrane was probed with goat anti-CYP105D1 antiserum (1:400) followed by affinity-purified guinea pig anti-goat IgG-coupled to alkaline phosphatase. The antigen-antibody interaction was detected by alkaline phosphatase activity after incubating the blot with 0.5 mg/ml α-naphthyl pyrophosphate and 0.5 mg/ml 4-chloro-o-toluidine diazonium in 30 mM Tris-HCl (pH 9). The densitometrically scanned (Epson GT-12000) profiles were quantified (C) using Phoretix 1D Advanced software (Ver 3.01) operating under MS Windows XPTM.
Figure 6. Effects of supplementing ALA in the growth medium on the periplasmic formations of URO I (A) and expression of CYP105D1 (B). 

_E. coli_ pLi-CYP105D1 were cultured for 24 h and ALA was supplemented either at the start (0 h, pre-treated) and both at the start and again at 12 h (pre- and post-treated) of cultivation.
Figure 7. *In vivo* inhibition of URO I production in *E. coli* pLiCYP105D1 and *E. coli* pLiQ by azoles. The MOPS media containing the dissolved azoles were filter-sterilised. The *E. coli* strains were cultured for 24 h as described in Experimental procedures and the URO estimated in the isolated periplasmic fractions. The values are average determinations of three independent culture measurements.
Figure 8. Scheme showing UROgen I production from heme biosynthetic pathway, translocation of UROgen I to E. coli periplasm, CYP105D1-catalysed conversion of UROgen I to URO I. HelABCD, heme translocator; PPT, pre-protein translocase; COPROgen, coproporphyrinogen; pre CYP105D1, precursor of CYP105D1; PBG, porphobilinogen; HMB, hydroxymethylbilane; IM, inner membrane; OM, outer membrane. The thickness of the arrowed lines indicates the extent of metabolite flow.
Export of a heterologous cytochrome P450 (CYP105D1) in Escherichia coli is associated with periplasmic accumulation of uroporphyrin

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