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# UROPORPHYRINOGEN III SYNTHASE: STUDIES ON ITS MECHANISM OF ACTION, MOLECULAR BIOLOGY AND BIOCHEMISTRY

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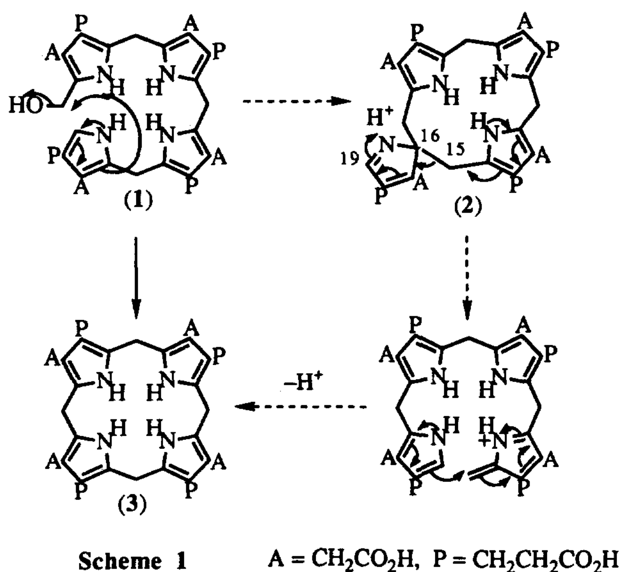
***Summary:*** A review is given of studies on the mechanism of action of uroporphyrinogen III synthase (cosynthetase, EC 4.2.1.75) based on synthesis of an inhibitory spiro-lactam. *HemD*, the *Escherichia coli* gene coding for uroporphyrinogen III synthase has been cloned and overexpressed at levels sixteen fold higher than in wild type *E. coli*. Evidence for *hemD* being part of an operon is reviewed. Uroporphyrinogen III synthase, which has been purified approx. 6000 fold, shows $M_r$ 28000 under denaturing conditions and has a pH optimum of $8.0 \pm 0.2$. The results from chemical modification of the enzyme point to the presence of arginine and lysine residues at or close to the active site.

## INTRODUCTION

Uroporphyrinogen III synthase (cosynthetase, EC 4.2.1.75) catalyses the cyclisation of the linear tetrapyrrole hydroxymethylbilane (1) to form uroporphyrinogen III (3), the macrocyclic precursor of haem, chlorophyll and vitamin $B_{12}$.[1] Uroporphyrinogen III synthase has been purified to homogeneity from human erythrocytes[2] and *Escherichia coli*,[3] and highly purified from *Euglena gracilis*[4] and rat liver.[5] In this paper we describe our studies on the molecular biology, biochemistry and chemistry of uroporphyrinogen III synthase.

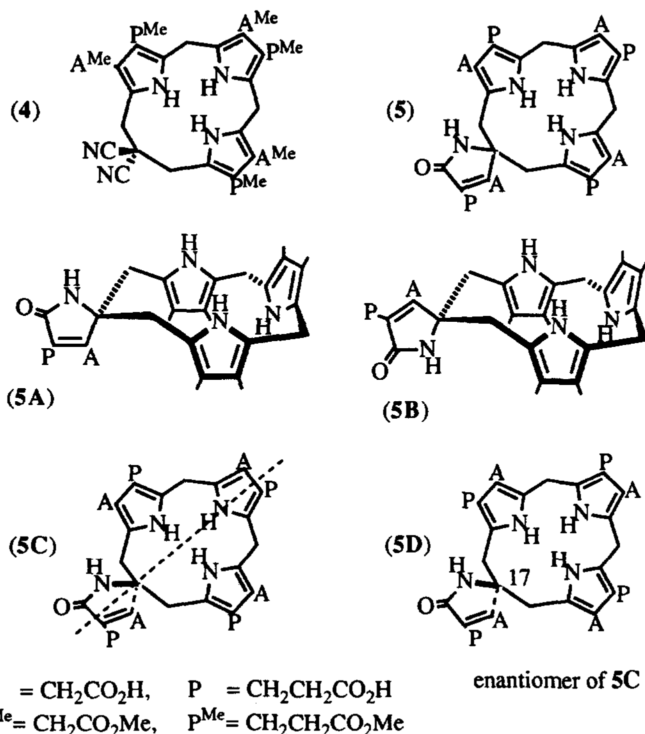



**Scheme 1** A = $CH_2CO_2H$, P = $CH_2CH_2CO_2H$

**The mechanism of uroporphyrinogen III synthase**

The mechanism for the enzymic cyclization of hydroxymethylbilane (**1**) to form uroporphyrinogen III (**3**) is believed to involve the initial formation of a bond between C-16 and the hydroxymethyl carbon, to generate a spiro-pyrrolenine (**2**). Cleavage of the C-15 to C-16 bond and formation of a bond between C-15 and C-19 produces a type III porphyrinogen, where the fourth ring is effectively turned around.[6,7] Strong support for this mechanism has been gained by a series of studies involving synthesis of molecules related to the spiro-pyrrolenine (**2**). The initial problem was to determine whether the tripyrrolic macrocycle of (**2**) can exist since it was initially thought to be too strained and it was suggested substantial *C*-protonation occurs to relieve this strain.[6] However, it was possible to synthesise the simplified macrocycle (**4**) carrying just two cyano groups at the quaternary centre[8] and it was both stable and crystalline. X-ray analysis showed the macrocycle to be markedly puckered with the NH groups of two pyrroles "pointing" in the opposite direction to the NH group of the third pyrrole. Space-filling models indicated that this is a locked conformation and if so, the two faces of the molecule are different.

When the synthesis was completed[9] of the spiro-lactam system (**5**), two isomers were formed, a major and a minor one, which were separable as their octa-esters. If the conformation of the macrocycle is locked, then there are two ways (**5A**) and (**5B**) in which the spiro-lactam ring can be fused to the larger ring; these are atropisomers and the existence of the minor isomer was interpreted on that basis.




If the spiro-pyrrolenine (**2**) is in fact the intermediate between the hydroxymethylbilane (**1**) and uroporphyrinogen III (**3**), then one of those two atropisomers should match it almost perfectly whereas the other will not. Since the spiro-lactam is a stable system which cannot undergo fragmentation-recombination,

the appropriate isomer should fit tightly into and block the active site of uroporphyrinogen III synthase so inhibiting its normal conversion of the bilane (**1**) into uroporphyrinogen III. The other isomer should not fit and so ought not to be an inhibitor. In the event, this is exactly what was found.[9] The inhibitory lactam showed a $K_I$ of *ca.* 1 μM whereas the other isomer had no detectable effect on uroporphyrinogen III synthase.

The foregoing synthetic spiro-lactams are both racemic and the stereochemical test was further refined by resolution of these materials.[10] This proved to be a major challenge because the difference is minimal between the pyrrole rings A and C attached to the chiral centre of (**5**) and inspection of the enantiomers of this structure reveals an interesting relationship between them. (**5D**) is the enantiomer of (**5C**) and can be obtained by reflection in the mirror plane passing through the spiro centre (C-17) and the centre of ring B (see dotted line). It can be seen that (**5C**) and (**5D**) differ only by having each acetate A on a pyrrolic ring replaced by a propionate P and each propionate P by an acetate A. It follows that though the active site which binds the putative spiro-pyrrolenine (**2**) should perfectly fit one enantiomer of the inhibitory spiro-lactam (**5**), when it accepts the other enantiomer it will still be presented with a set of six acidic side-chains in the correct locations but of the wrong size (A for P and P for A). Thus it was unlikely that there would be a "100% to 0%" relationship between the inhibitory effects of the two enantiomers, rather a "stronger to a weaker" relationship was to be expected.

The observed result was that one enantiomer of the inhibitory lactam was over 20 times more effective in blocking uroporphyrinogen III synthase than was the other enantiomer.[10] Moreover, the $K_I$ for the tightly binding enantiomer was more than an order of magnitude lower than the $K_m$ for the normal substrate, hydroxymethylbilane (**1**). If uroporphyrinogen III synthase has evolved to convert the bilane (**1**) initially into the spiro-pyrrolenine (**2**) then it would be expected to behave towards the correct enantiomer of the spiro-lactam in the way outlined above. Indeed, the sum of evidence piles up to give strong support to the intermediacy of the spiro-pyrrolenine (**2**) *en route* to uroporphyrinogen III (**3**). The pathway forward from the spiro-pyrrolenine (**2**) to uroporphyrinogen III (**3**) could then, in principle, either follow a fragmentation-recombination mechanism or go through a series of [1,5]-sigmatropic rearrangements. However, the known chemistry of closely related model pyrrolenines points to the former mechanism.[11,12]

## EXPERIMENTAL

### Chemicals

Bactotryptone and bactoyeast extract were obtained from Difco Laboratories. Geneclean® was obtained from Stratech Scientific. Electrophoresis grade agarose was obtained from Bethesda Research Laboratories. Isopropyl-ß-D-thiogalactopyranoside, *N,N,N',N'*-tetramethylethylenediamine, Bis-tris, chloramphenicol, ampicillin (sodium salt), ethidium bromide, Tris (Trizma grade), 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside, butane-2,3-dione, acrylamide, pepstatin, benzamidine hydrochloride, phenylmethylsulphonylfluoride, bis-acrylamide and dithiothreitol were all obtained from Sigma Chemical Co. L-[$^{35}$S]methionine was obtained from Amersham International. Formylbilane octamethyl ester and hydroxymethylbilane octamethyl ester were prepared and used immediately after basic hydrolysis.[13] Restriction endonucleases were obtained from Boehringer Mannheim GmbH. T4 DNA ligase was obtained from Amersham International, Amersham, Bucks., U.K. The cloned Klenow fragment of *E. coli* DNA polymerase was made available by Dr. R.T. Hunt (Department of Biochemistry, University of Cambridge). DEAE-cellulose, DE-52 ion-exchange medium was obtained from Whatman. Polybuffers® and Polybuffer exchanger®, and FPLC columns (MonoP, MonoQ and Superose 12) were obtained from Pharmacia. YM-10 ultrafiltration membranes and Centricon 10® concentrators were supplied by Amicon Ltd.

### Cloning and bacteria

*E. coli* TG1*recO* was obtained from Dr P. Oliver (Department of Genetics, Cambridge). The plasmid

pLC41-4 was obtained from B. Bachmann at the *E. coli* Genetic Stock Center (Department of Human Genetics, Yale University School of Medicine).[14] Plasmid pNC624 was prepared by inserting a 2.4 Kb fragment obtained from a *Bam* HI+*Hind* III digest of pLC41-4 into pIH223-3. Plasmid pPA327 was prepared by inserting a 1.9 Kb *Eco* RI, *Pst* I fragment from pLC41-4 into pPA101. pPA101 was made by replacing the *Pvu* II fragment of pUC18 (containing the multiple cloning site and regions of the *lacZ* and *lacI* genes) by a *Hae* III fragment of pKK223-3 (containing the *tac* promoter and multiple cloning site). All cloning steps were carried out following standard protocols.[15] DNA fragments excised from agarose gels were treated following the procedure supplied with the Geneclean® kit. Plasmids pNC624 and pPA327 was transformed into TG1*recO* cells using the method of Hanahan.[16]

*E. coli* strain TG1*recO*/PA327 was grown aerobically at 37°C in GA/TY medium (per litre; 1.6 g bactotryptone, 1 g bactoyeast extract, 0.5 g NaCl, 10 g glucose, 2 g $(NH_4)_2SO_4$, 0.8 g $MgSO_4$ and 10 g $KH_2PO_4$, 20 mg ampicillin, and 12.5 mg IPTG). The pH was maintained at 7.1. Cells were harvested using a Sharples continuous-flow centrifuge, resuspended in ice-cold buffer (0.1 M sodium phosphate, pH 8.0 containing 0.6 mM EDTA, pH 8.0, 0.1 mM DTT), centrifuged (8 000 rpm, 30 min, 4°C) and stored at -20°C.

**General procedures**

All water was deionised and glass distilled before use. All columns were equilibrated and run at 4 °C, unless stated otherwise. All buffers were prepared and adjusted to the required pH at room temperature regardless of the temperature at which they were subsequently used.

**Enzyme assays**

Uroporphyrinogen III synthase activity was detected using a lag assay.[4] A direct assay using synthetic hydroxymethylbilane (**1**)[13] was used for quantitative determinations.[4] One unit of enzyme activity is defined as the amount of enzyme necessary to produce 1 µmol of uroporphyrinogen per hour. HMBS was assayed using the procedure of Battersby.[17] Protein concentration was measured by the method of Bradford[18] using bovine serum albumin as the standard.

***In vitro* coupled transcription-translation**

The coupled transcription-translation of DNA was performed by the method of Zubay[19] using a cell-free system derived from *E. coli* strain PR7[20] with 1-2 µg of DNA.

**Polyacrylamide gel electrophoresis and molecular weight determination**

Polyacrylamide gel electrophoresis under denaturing conditions was carried out using SDS gels, incorporating 17.5 % polyacrylamide, according to the method of Laemmli.[21] Molecular weight ($M_r$) determination was carried out by comparing protein mobility using SDS-7 molecular weight markers as standards (Sigma Chemical Co.) Protein and enzyme activity was recovered from SDS PAGE gels using the method of Hager & Burgess.[22] Molecular weight determination under non-denaturing conditions was carried out by gel filtration on a Superose 12 HR 10/30 FPLC column, equilibrated with 15 mM TrisHCl, pH 8.5 (containing 0.6 mM EDTA, 1 mM DTT, 0.2 M NaCl) and run at 0.5 ml/min at room temperature. The $M_r$ standards used were bovine serum albumin (67000), ovalbumin (43000), chymotrysinogen A (25000), and ribonuclease A (13700).

**Isoelectric focusing**

Analytical isoelectric focusing on ultra-thin polyacrylamide gels, employing a pI gradient of 6.5 - 4.0, was performed on LKB Ampholine® PAG plates (LKB-Pharmacia).

**Protein sequencing**

Prior to sequence analysis, proteins were electroblotted from SDS/polyacrylamide gels onto Polyvinylidene Difluoride (PVDF) membranes[23] from which they were extracted prior to analysis. The analysis of the *N*-terminal amino acid sequences of proteins was carried out by the Protein Sequencing Facility, University of Cambridge, and performed on an Applied Biosystems 477A Protein Sequencer.

**Modification of arginine residues using butane-2,3-dione**

Uroporphyrinogen III synthase was incubated with 30 mM butane-2,3-dione in 50 mM sodium borate

buffer, pH 9.0. Aliquots were removed at intervals and assayed for activity. The experiment was repeated with enzyme that had been pre-incubated with 1.35 mM formylbilane for three minutes.

**Modification of lysine residues with pyridoxal 5'-phosphate**

Uroporphyrinogen III synthase was incubated with pyridoxal 5'-phosphate (5-30 mM) in 0.1 M sodium phosphate buffer, pH 7.0, in the dark and at 25 °C. 50 µl samples were removed at intervals and assayed. When the reaction had reached equilibrium, $NaBH_4$ was added and these samples were also assayed for activity. $NaBH_4$ alone had no effect on the enzyme.

## RESULTS AND DISCUSSION

### CLONING EXPERIMENTS

The *hemC* and *hemD* loci in *E. coli*, coding for hydroxymethylbilane synthase and uroporphyrinogen III synthase respectively, both map to minute 85 on the *E. coli* K12 chromosome.[24,25] The plasmid pLC41-4 from the Clarke and Carbon *E. coli* K12 colony bank[14] carries *hemC* and at least three further open reading frames (ORFs) downstream of the *hemC* locus.[26] These were designated ORF W, ORF X, and ORF Y and have predicted translation products of molecular weight 27797, 42961, and 45243 respectively. ORF W overlaps the 3' end of *hemC*, with the 3'-terminal nucleotide of *hemC* also serving as the first nucleotide of the initiation codon in ORF W.

There was reason to assume ORF W coded for *hemD*, particularly because it was transcriptionally coupled to *hemC*. This might be expected given that expression of HMBS without uroporphyrinogen III synthase would lead to the formation of toxic type I porphyrins. Furthermore, uroporphyrinogen III synthases from *E. gracilis*[4] human erythrocytes[2] and rat liver[5] all have $M_r$ values in the range 28000 to 31000, close to the molecular weight of the translation product of ORF W (27 797). It was therefore decided to subclone ORF W and with the aim of identifying *hemD* by overexpression of uroporphyrinogen III synthase activity. A series of constructs were made, two of which are described below.

**Cloning *hemD* and *hemC***

The level of uroporphyrinogen III synthase activity in cells harbouring the plasmid pLC41-4 and in wild-type (TG1*recO*) cells is indistinguishable from the control assay of the non-enzymic ring closure of hydroxymethylbilane (HMB). It was therefore decided to clone ORF W together with *hemC*, and its putative promoter (within a region 60 bp upstream of the start codon). It was anticipated that expression of *hem*C would act as a control and also give a measure of the extent of expression. Accordingly a 2.4 Kb fragment obtained from *Bam* HI+*Hin*d III digested pLC41-4 was ligated into pIH223-3 to generate plasmid pNC624. pIH223-3 is a derivative of pKK223-3 (Pharmacia), containing the strong *tac* promoter and a multiple cloning site, but lacking the *Bam* HI site upstream of the *tac* promoter in the more commonly used pKK223-3. Plasmid pNC624 was used to transform TG1*recO* cells and the resultant strain tested for expression of uroporphyrinogen III synthase activity using synthetic hydroxymethylbilane[13] for the assay.[4] The extent of expression (determined as apparent specific activities, calculated from initial rates of reaction and $A_{280}$ values) was estimated to be four times that of the wild-type cells. A similar level of overexpression of HMBS activity was found. ORF W was thus identified as *hemD*, a conclusion drawn independently by other groups on the basis of complementation studies[27] and by expression and enzyme assay.[28,29]

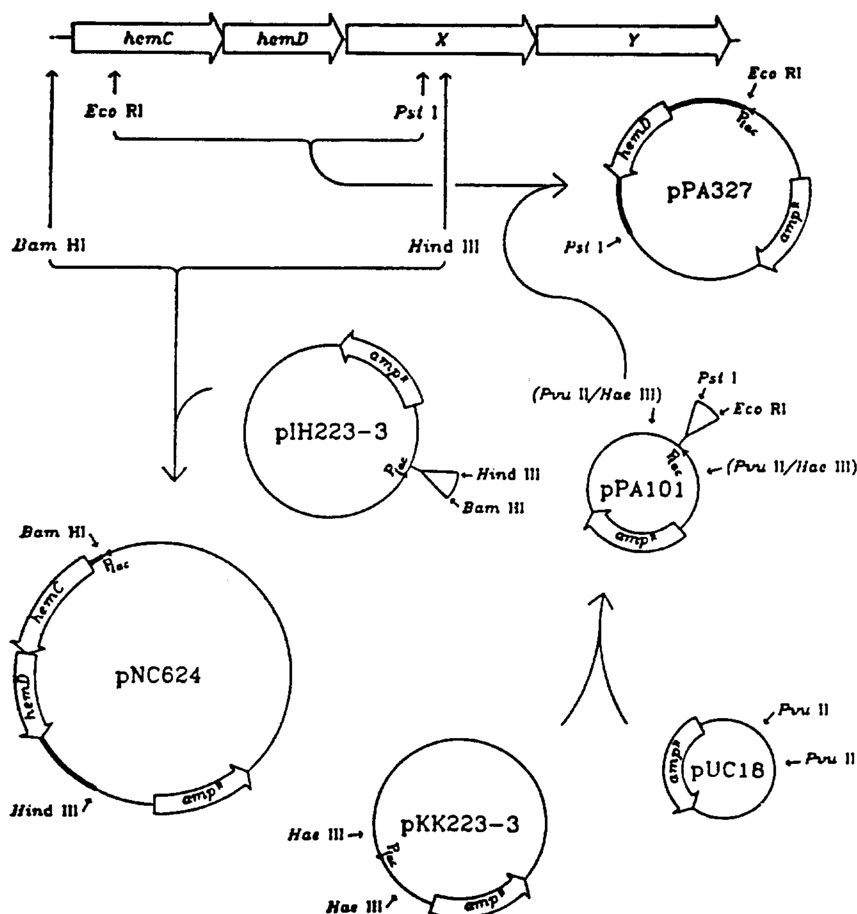
**Cloning of *hemD***

A 1.9 Kb *Eco* RI-*Pst* I fragment from pLC41-4 (containing the latter two-thirds of *hem*C, all of *hem*D and a portion of ORF X) was inserted into pPA101 to generate the plasmid pPA327. The high copy number plasmid pPA101, incorporating the *tac* promoter upstream of a multiple cloning site, was made by replacing the *Pvu* II fragment of pUC18 (containing the multiple cloning site and regions of the *lacZ* and *lacI* genes) by a *Hae* III fragment of pKK223-3 (containing the *tac* promoter and multiple cloning site). The plasmid pPA327 was transformed into TG1*recO* cells. Assays using hydroxymethylbilane on crude extracts of

TG1*recO*/PA327 showed sixteen-fold higher levels of uroporphyrinogen III synthase activity than wild-type (TG1*recO*) cells and four-fold greater than TG1*recO*/NC624 cells.

A thousand fold overexpression of uroporphyrinogen III synthase has been reported in a parallel study.[3] However, our attempts to improve the level of overexpression by using restriction sites nearer to the start codon of *hemD* were not successful. Likewise, the use of systems based on the T7 RNA polymerase promoter did not give levels of overexpression better than those obtained using *tac* promoter. (N. Crockett, C. Abell unpublished results). Therefore TG1recO/PA327 was used to overexpress protein for purification.

**Scheme II Construction of plasmids**




## Evidence for a uro operon

Analysis of a 4.2 Kb sequence of DNA within the plasmid pLC41-4 by the GCG program *Codon preference* (Genetics Computer Group, Version 1.2, University of Wisconsin, Biotechnology Center, Wisconsin, U.S.A.), shows four open reading frames with only two of them lying in the same frame. Furthermore, consideration of codon usage suggests the corresponding translation products will be expressed at low levels. These four open reading frames are all transcribed in the same direction opposite to the gene coding for adenylate cyclase (*cyaA*) which is also present in pLC41-4. The first two open reading frames are

*hemC* and *hemD*, respectively, and overlap by one base pair. Such overlap is common in polycistronic systems in *E. coli* (*e.g.* in the tryptophan operon).[30]

The observation that there is only a single consensus promoter sequence for the four ORFs, positioned within a region 60 bp upstream of *hemC*, and that no RNA polymerase termination signal has yet been found, suggest that these four ORFs may be part of an operon. This has been suggested previously[27,29] but not demonstrated. We therefore initiated a study to determine whether all four genes are transcribed into a single mRNA molecule by Northern blot analysis of total cellular RNA. A 1.3 Kb fragment containing *hemC* and the first 200 bp of *hemD* generated by a *Bam* HI+*Pvu* II digest of pNC624 was used to probe RNA isolated from TG1*recO* cells containing the plasmid pLC41-4 and run on a denaturing, glyoxal-agarose gel. Hybridisation experiments identified an intense band at approximately 6.5 Kb which was only seen in tracks containing TG1*recO*/LC41-4 RNA (N Crockett and C Abell, manuscript in preparation).

This Northern blot hybridisation experiment reveals a single mRNA molecule containing the transcript for *hemC*, presumably together with *hemD*, ORF X and ORF Y. These four genes would account for 4.2 Kb of the mRNA. The fact that mRNA of this size is not observed, and that there is no firm evidence for an RNA polymerase termination signal even beyond the 3' end of ORF Y[26] suggests there are other genes which may be in an operon. An open reading frame of at least 300 bp has been sequenced downstream of the ORF Y gene (P.R. Alefounder and C. Abell, preliminary results).

***In vitro* coupled transcription-translation**

The *in vitro* coupled transcription-translation system of Zubay[19] was used in an attempt to show that plasmids containing both *hemC* and *hemD* could express proteins of the predicted relative molecular weights of hydroxymethylbilane synthase (HMBS) and uroporphyrinogen III synthase. Using plasmid pNC624, two proteins were expressed with approximate $M_r$ of 34000 (corresponding to HMBS) and 28000. The latter band may correspond to uroporphyrinogen III synthase but is unfortunately indistinguishable from the band corresponding to ß-lactamase (seen for all plasmids harbouring the ampicillin-resistance gene). Using different constructs, it was similarly possible to express the protein products of ORF X and ORF Y as bands of 40000 and 45000 respectively.

Whilst physically and genetically mapping the *ilv-metE-udp* region of the *E. coli* chromosome, Aldea *et al.*[31] used an *in vitro* coupled transcription-translation system to express five proteins of $M_r$ 38000, 32500, 41000, 46000 and 70000 from a region of DNA proximal to the *cyaA* locus and on the same side of *cyaA* as *hemD*. Comparing this physical map[31] with that generated from the plasmid pLC41-4, it is clear that all five of these proteins are being expressed from genes lying within the *E. coli* chromosomal section contained within pLC41-4, the first four proteins being the translation products of *hemC*, *hemD*, ORF X and ORF Y.

The identity of the proteins corresponding to the open reading frames ORF X and ORF Y are unknown. There has been speculation that ORF X is *hemG*, coding for the penultimate enzyme on the pathway, protoporphyrinogen oxidase.[29] *HemG* has been mapped between the 85th and 86th minute on the *E. coli* chromosome,[25] between the *metE* and *rha* markers and very close to *chlB*.[32] It is possible that *hemG* might also lie within pLC41-4 and be part of the proposed operon. A two fold over-expression of protoporphyrinogen oxidase activity in strains harbouring the plasmid pLC41-4 has been reported.[29] Our experiments to confirm this have been inconclusive with similar low levels of expression sometimes being observed (N Crockett and C Abell, unpublished results).

## PURIFICATION OF UROPORPHYRINOGEN III SYNTHASE

### Preparation of a crude extract from TG1*recO*/PA327

Frozen cells of the *E. coli* overproducing strain, TG1*recO*/PA327 were thawed on ice in the extract buffer (supplemented with 6 mM EDTA, 1 mM DTT, 1 mM aprotinin, 1 mM pepstatin, 1 mM benzamidine

and 0.6 mM phenylmethylsulphonyl fluoride). The suspension was sonicated on ice using a Dawe Soniprobe (type 7530A) and the extract centrifuged (12 000 rpm, 30 min, 4°C).

**Ammonium sulphate fractionation**

Ammonium sulphate was added to the supernatant and the suspension stirred at 4°C before centrifugation at 6 000 rpm, for 10 min.. All the enzyme activity was found in the 25-40% fraction.

**Ion-exchange chromatography: DEAE-cellulose**

The dialysed ammonium sulphate 25-40% fraction was applied to a DEAE-cellulose (DE-52) column equilibrated with 15 mM Tris-HCl buffer, pH 7.5 (containing 6 mM EDTA and 1 mM DTT). The column was run at at 65 ml/hr with a step salt gradient by eluting with the buffer used for equilibration until the major protein peak had eluted then with the same buffer, containing 0.2 M NaCl, until all of the uroporphyrinogen III synthase activity eluted. Fractions containing enzyme activity were concentrated by ultrafiltration (Amicon 8200 with a YM-10 membrane, followed by Amicon Centricon-10, 2 ml micro-concentrators). Concentrated fractions could be stored at 4 °C for several days without appreciable loss in activity.

**Chromatofocusing on a Polybuffer Exchanger**

Concentrated fractions from the DE-52 column were dialysed against 15 mM Tris-HCl buffer, pH 7.5 (containing 6 mM EDTA and 1 mM DTT), at 4°C and applied to a column containing Polybuffer exchanger® (PBE 74, Pharmacia) equilibrated with 25 mM imidazole-HCl, pH 7.4. After a pre-gradient of equilibration buffer (5 ml), a pH gradient, pH 7.0-4.0, was set up using Polybuffer-HCl, pH 4.0 (Pharmacia; dilution of 12.5 % in $H_2O$). Uroporphyrinogen III synthase eluted at a pH of around pH 5.5. The active fractions were concentrated by ultrafiltration as above. SDS/polyacrylamide gel electrophoresis (17.5% acrylamide) of the active fraction showed four bands.

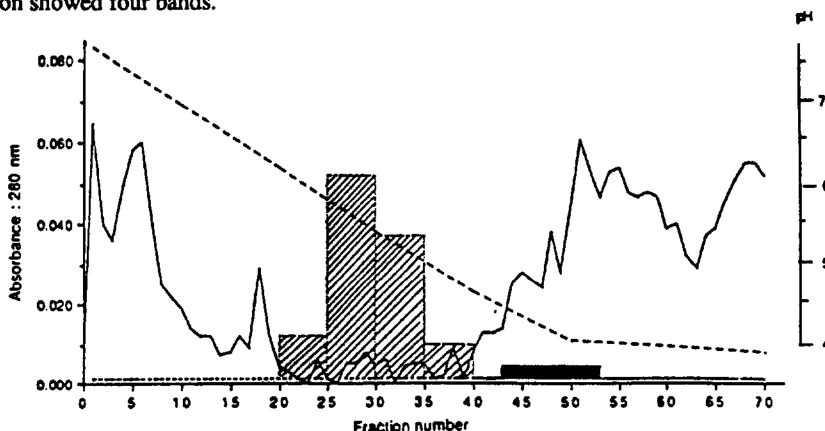



**Figure 1.** Chromatofocusing on Polybuffer exchanger® (PBE 74, Pharmacia) equilibrated with 25 mM imidazole-HCl, pH 7.4. After a pre-gradient of equilibration buffer (5 ml), a pH gradient, pH 7.0-4.0, was established using Polybuffer-HCl, pH 4.0 (Pharmacia; dilution of 12.5% in $H_2O$). The column was run at 75 ml/hr and 5 ml fractions were collected. (- - -) pH gradient; (////) uroporphyrinogen III synthase activity as determined by the lag assay; (▬) HMBS activity.

**Chromatofocusing on Mono P**

A second chromatofocusing step was carried out with the column at room temperature over a smaller pH interval using a Mono P HR 5/20 column (Pharmacia). The column was equilibrated with 15 mM Bis-tris-HCl, pH 6.4. The pH gradient of 6.0-5.0 was established using Polybuffer-HCl, pH 5.0 (30 ml, Pharmacia, 6% in $H_2O$) at a flow rate of 0.5 ml/min (Figure 1). The peak of uroporphyrinogen III synthase activity

coincided with an elution pH of 5.5. The active fractions collected were concentrated and stored at 4 °C. SDS/polyacrylamide gel electrophoresis revealed just two bands, of approximate relative molecular weights 70000 and 30000 .

**Gel filtration on Superose-12**

A Superose-12 HR 10/30 column (Pharmacia,) was equilibrated with 15 mM Tris-HCl buffer, pH 8.5 (containing 6 mM EDTA, 1 mM DTT and 0.2 M NaCl). Concentrated samples from the Mono P column were applied and the column eluted at 0.5 ml/min. Two major peaks were detected in the protein elution profile, of $M_r$ 66000 and 30000. All of the uroporphyrinogen synthase activity was associated with the smaller protein, which is a single band on SDS/PAGE..

**Recovery of uroporphyrinogen III synthase activity from SDS/PAGE.**

The enzyme preparation from the final Superose 12 column was subjected to SDS/polyacrylamide gel electrophoresis (17.5% acrylamide). The single band ($M_r$ of 28000) was excised and uroporphyrinogen III synthase activity (approximately 25 % of the applied activity) recovered, according to the method of Hager & Burgess.[22] This demonstrates that the enzyme does not require a reversibly bound cofactor.[33]

**Amino acid sequencing**

The single band from the isolated SDS/polyacrylamide gel was transblotted onto a PVDF membrane and the *N*-terminal amino acid sequence determined. Two sequences were found: *Ser-Ile-Leu-Val-Thr-Arg-Pro* which corresponds to the start of the uroporphyrinogen III synthase sequence derived from the nucleic acid sequence of the *hemD* gene[26,27,29] except that the predicted *N*-terminal methionine is absent in the mature protein. This sequence agrees with that previously reported,[3] and accounted for approximately 25% of the mixture. The major sequence was *His-Pro-Glu-Thr-Leu-Val-Lys* which is the sequence of an *E. coli* ß-lactamase protein from the 24th residue onwards. It is known that in *E. coli* the mature ß-lactamase enzyme has lost the first 23 amino acids predicted from the nucleic acid sequence.[34] The predicted $M_r$ of the mature ß-lactamase is 28906, very close to that derived for the purified, uroporphyrinogen III synthase preparation. This would explain why only one band was observed for the purified enzyme on SDS/polyacrylamide gel electrophoresis and only one peak on gel filtration.

The Table summarises the approximately 6000 fold purification of uroporphyrinogen III synthase. This preparation is an approximately 1:3 mixture with β-lactamase and has a specific activity of 190 units/mg. The specific activity of homogeneous uroporphyrinogen III synthase has been reported to be 1500 units/mg.[3]

**Table: Purification of uroporphyrinogen III synthase from *E. coli***

| Step | Volume (ml) | Protein (mg) | Total activity (units) | Specific activity (units/mg) | Yield (%) | Purif. (fold) |
|---|---|---|---|---|---|---|
| Crude extract | 170 | 12000 | 405 | 0.0338 | 100 | 1 |
| Ammonium sulphate fractionation (25-40%) | 18.7 | 2770 | 349 | 0.126 | 86 | 3.7 |
| Ion-exchange DEAE-cellulose (DE-52) | 37.6 | 94 | 209 | 2.22 | 52 | 66 |
| Chromatofocussing PBE 74, pH 7.4 - 4.0 | 2.3 | 2.54 | 123 | 48.4 | 30 | 1430 |
| Chromatofocussing Mono P, pH 6.4 - 5.0 | 0.3 | 0.79 | 75 | 94.9 | 18 | 2810 |
| Gel filtration Superose 12 | 0.3 | 0.36 | 68.7 | 191 | 17 | 5650 |

**Analytical isoelectric focusing**

The chromatofocussing experiments indicated an apparent pI for *E. coli* uroporphyrinogen III synthase of 5.5. This is very close to the reported pI values for *E. coli* ß-lactamase which has a major band corresponding to pI 5.4 and satellite bands close to pI 5.6.[35] It is therefore not surprising that ß-lactamase and uroporphyrinogen III synthase copurified on the chromatofocussing steps. Enzyme from the Superose 12 column reactivated after running on a SDS/polyacrylamide gel, was run on analytical isoelectric focusing gels with a pI gradient of 6.5-4.0 (on flat-bed, ultrathin PAG plates®, LKB-Pharmacia) and resolved into three bands with pI values 5.4, 5.5 and 5.6 (using LKB-Pharmacia pI markers as calibration standards).

Assuming the β-lactamase that copurifies with uroporphyrinogen III synthase is the product of the plasmid encoded ampicillin-resistance gene in pPA327, a simple substitution, at the DNA level, of the ampicillin-resistance gene for the kanamycin-resistance gene (kanamycin phosphotransferase, $M_r$ of 41758), should facilitate the final purification to homogeneity of uroporphyrinogen III synthase, using the highly reproducible procedure outlined in detail above.

## CHARACTERISATION OF UROPORPHYRINOGEN III SYNTHASE

**Molecular weight determination**

The relative molecular weight of uroporphyrinogen III synthase was found under non-denaturing conditions by gel filtration on a Superose 12 HR 10/30 FPLC column to be 32000 ± 2000. The $M_r$ value was found to be 28000 under denaturing conditions by SDS/polyacrylamide gel electrophoresis (17.5% polyacrylamide), using SDS-7 molecular weight markers as standards (Sigma Chemical Co.). This confirms that *E. coli* uroporphyrinogen III synthase is monomeric, as are the corresponding enzymes from *E. gracilis*,[4] rat liver,[5] and man.[2] The derived $M_r$ from the nucleic acid sequence of the *E. coli hemD* gene is 27766.

**Kinetic studies**

Steady-state kinetic experiments were carried out with purified uroporphyrinogen III synthase incubated in 0.2 M Tris-HCl buffer, pH 8.25 (containing 0.6 mM EDTA) at 25 °C using a freshly hydrolysed sample of hydroxymethylbilane octa-acid[13] as substrate in the assay. $K_m$ was determined from a Hanes plot to be 12 ± 4 μM, close to the value of 5 μM recently reported.[3] The $K_m$ for the *E. gracilis* enzyme (12-40 μM),[4] the rat enzyme (5 μM)[36] and the human enzyme (5-20 μM)[2] are all in the same range.

**Thermostability studies**

Uroporphyrinogen III synthase is particularly thermolabile, being denatured within minutes at 55 °C. At 25 °C approximately 20% of the activity is lost after one hour. Storage at 4 °C for 2 weeks resulted in 5% loss of activity.

**Effect of pH on uroporphyrinogen III synthase activity**

The pH-activity curves for purified uroporphyrinogen III synthase in 50 mM Tris-HCl buffer, 50 mM sodium phosphate buffer and 50 mM ethanolamine-AcOH show an optimum activity at pH 8.0 ± 0.2. Values between pH 7.4 and 8.0 have been determined for other uroporphyrinogen III synthases.[2,4]

**Modification of arginine residues**

Uroporphyrinogen III synthase was incubated with 30 mM butane-2,3-dione, a reagent known specifically to modify arginine residues.[37] Aliquots were removed at various time intervals and assayed for uroporphyrinogen III synthase activity (Figure 2). It was found that uroporphyrinogen III synthase is rapidly inactivated by butane-2,3-dione, giving a $t_{1/2}$ value of about 5 min. In the absence of butane-2,3-dione uroporphyrinogen III synthase retained 96% of its activity over the 40 min incubation. On pre-incubation of the enzyme with formylbilane (1.35 mM) (a substrate analogue available from the penultimate intermediate in the synthesis of hydroxymethylbilane)[13] there was total protection against inactivation by butane-2,3-dione. Similar protection from modification has been observed with the *E. gracilis* enzyme.[4]

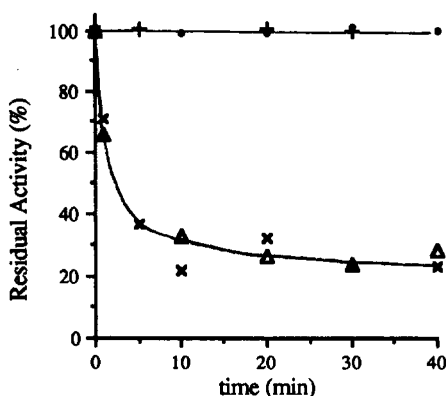



**Figure 2.** Residual enzyme activity after incubation of *E. coli* uroporphyrinogen III synthase with butane-2,3-dione. (+) enzyme with no butane-2,3-dione added; (•) enzyme incubated with formylbilane (1.35 mM) before addition of butane-2,3-dione; (x) and (Δ), incubations of enzyme with butane-2,3-dione (30 mM)

### Modification of lysine residues

Uroporphyrinogen III synthase was incubated with a range of concentrations of pyridoxal 5'-phosphate. This reagent has been used previously to modify lysine residues.[38] Uroporphyrinogen III synthase was inactivated to about 50% of its initial activity, with the inactivation reactions reaching equilibrium after 30 min (using concentrations of pyridoxal 5'-phosphate between 5 mM and 30 mM). Incubation of the enzyme with 10 mM pyridoxal 5'-phosphate for 30 minutes followed by the addition of $NaBH_4$ caused a further 10% decrease in residual activity. There was no reduction in enzyme activity when it was treated with $NaBH_4$ alone. These results indicate that there are lysine residues at or near the active site of uroporphyrinogen III synthase. Similar evidence for lysine residues close to the active site of uroporphyrinogen III synthase has also been presented for the rat[5] and *E. gracilis* enzymes.[4]

## CONCLUSIONS

Inhibition of uroporphyrinogen III synthase by resolved forms of the spiro-lactam inhibitor (**5**) highlight the specificity of the enzyme and support the intermediacy of spiro-pyrrolenine (**2**) in the enzymic mechanism. Cloning and overexpressing *hemD*, the *E. coli* gene coding for uroporphyrinogen III synthase, has allowed the approx. 6000 purification of the enzyme. However the very close similarity in physical properties of uroporphyrinogen III synthase and β-lactamase resulted in their copurification. *E. coli* uroporphyrinogen III synthase is similar to the enzyme from other sources in being thermolabile, having a pH optimum around 8.0, and being sensitive to lysine and arginine modifying reagents.

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