The *Escherichia coli* *cysG* gene encodes S-adenosylmethionine-dependent uroporphyrinogen III methylase

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The *Escherichia coli* *cysG* gene was successfully subcloned and over-expressed to produce a 52 kDa protein that was purified to homogeneity. This protein was shown to catalyse the S-adenosylmethionine-dependent methylation of uroporphyrinogen III to give a product identified as sirohydrochlorin on the basis of its absorption spectra, incorporation of ^{14}C label from S-adenosyl[$\text{Me-}^{14}\text{C}$]methionine and mass and ^1H -n.m.r. spectra of its octamethyl ester. Further confirmation of the structure was obtained from a ^{13}C -n.m.r. spectrum of the methyl ester produced by incubation of the methylase with uroporphyrinogen III, derived from [4,6- $^{13}\text{C}_2$]prophobilinogen, and S-adenosyl[$\text{Me-}^{13}\text{C}$]methionine.

INTRODUCTION

Cysteine synthesis in *Escherichia coli* requires the enzyme sulphite reductase to reduce sulphite to sulphide, which then reacts with O-acetylserine to form cysteine (for a review see Kredich, 1987). *E. coli* *cysG* mutants are unable to grow in the absence of cysteine, as they lack active sulphite reductase and also have no nitrite reductase activity (Cole *et al.*, 1980). Since both sulphite reductase and nitrite reductase contain a novel haem prosthetic group, termed sirohaem (Murphy *et al.*, 1973), it has been proposed that the *cysG* gene might encode for an enzyme that is responsible for sirohaem synthesis (Cole *et al.*, 1980). The *cysG* gene has also been shown to be required for cobalamin synthesis in *Salmonella typhimurium* (Jeter *et al.*, 1984), suggesting that the *cysG*-gene product synthesizes a precursor common to both sirohaem and cobalamin.

Sirohaem is synthesized from 5-aminolaevulinic acid along the same biosynthetic pathway as haem as far as the first cyclic tetrapyrrolic intermediate, uroporphyrinogen III (see Scheme 1). At this stage the pathway to sirohaem diverges from the haem pathway, as uroporphyrinogen III is methylated by a SAM-dependent methylase to produce sirohydrochlorin (also referred to as 'faktor II'; Deeg *et al.*, 1977). Sirohydrochlorin also serves as an intermediate in cobalamin biosynthesis, but further methylations and modifications are required *en route* to the final product, vitamin B-12. In sirohaem synthesis only chelation of iron is required to yield the fully formed prosthetic group from the isobacteriochlorin sirohydrochlorin.

Until now no biochemical evidence had been presented to show the function or activity of the *cysG*-gene product. In the present paper we demonstrate that the *cysG*-gene product is responsible for the SAM-dependent methylations of uroporphyrinogen III to form sirohydrochlorin.

MATERIALS AND METHODS

Chemicals

Prophobilinogen and [4,6- $^{13}\text{C}_2$]prophobilinogen were synthesized enzymically from 5-aminolaevulinic acid and

5-amino[3- ^{13}C]laevulinic acid by using purified amino-laevulinic acid dehydratase as previously described (Jordan & Berry, 1981). [$\text{Me-}^{14}\text{C}$]SAM was purchased from ICN Radiochemicals, Irvine, CA, U.S.A. [$\text{Me-}^{13}\text{C}$]SAM was prepared by the method of Hegazi *et al.* (1978). All other chemicals and reagents were purchased from Sigma Co. and were of highest grade obtainable.

Bacteria and plasmids

Plasmid pHM3 (MacDonald & Cole, 1985) was generously given by Dr. J. Cole (Department of Biochemistry, University of Birmingham, Birmingham, U.K.). *E. coli* strain CBK103 (*cysG*::Tn5) was generously given by Dr. M. Marinus (Department of Pharmacology, University of Massachusetts Medical School, Boston, MA, U.S.A.). All DNA manipulations were carried out by standard methods (Maniatis *et al.*, 1982). Bacterial strains CR204, CR211 and CR252, used for the isolation of the enzymes porphobilinogen deaminase, uroporphyrinogen III synthase and uroporphyrinogen III methylase respectively, were grown in LB broth containing ampicillin (100 mg/l) at 37 °C for 18 h.

Purification of enzymes

All procedures for the isolation of enzymes were carried out at 4 °C unless otherwise stated. The enzymes porphobilinogen deaminase and uroporphyrinogen III synthase (also referred to as co-synthase) were purified from recombinant *E. coli* strains harbouring plasmids containing the genes for these enzymes (Scott *et al.*, 1988; C. A. Roessner, unpublished work). Uroporphyrinogen III methylase was purified from 5 litres of *E. coli* strain CR252 as outlined below. The cells were harvested by centrifugation and resuspended in a minimal volume of 0.1 M-potassium phosphate buffer, pH 8.0, containing 2 mM-EDTA (PE buffer). The cells were lysed by sonication, and the cell extract was heated to 55 °C in a shaking water bath and maintained at this temperature for 15 min. The extract was cooled to 4 °C before centrifugation at 15000 rev./min (27000 g) for 15 min in a Sorvall SS-34 rotor. The supernatant was fractionated by differential saturation with $(\text{NH}_4)_2\text{SO}_4$. The precipitate obtained from 35–55 % saturation with

Abbreviation used: SAM, S-adenosyl-L-methionine.

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Scheme 1. Pathway to the biosynthesis of sirohydrochlorin

Sirohydrochlorin is synthesized from eight molecules of aminolaevulinic acid and two molecules of SAM. A = $\text{CH}_2\text{CO}_2\text{H}$; P = $\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$.

$(\text{NH}_4)_2\text{SO}_4$ was collected by centrifugation, resuspended in 10 ml of PE buffer and dialysed overnight against 2 litres of PE buffer. The extract was applied to a DEAE-Sephacel column (5 cm $\times$ 20 cm) that had previously been equilibrated in PE buffer. The column was washed with

0.5 litre of PE buffer, and the uroporphyrinogen III methylase was eluted from the column by passing through PE buffer containing 125 mM-NaCl. The fractions containing the methylase activity were pooled and concentrated in an Amicon ultrafiltration cell fitted with

a PM-10 membrane. The concentrate (20 ml) was applied to a Pharmacia Mono Q HR 10/10 column that had previously been equilibrated in 20 mM-Tris/HCl buffer, pH 7.5. The methylase was eluted from the column by application of a linear salt gradient (200–400 mM-NaCl, 100 ml total volume). SDS/PAGE was carried out by the method described by Laemmli & Favre (1973).

Assay of uroporphyrinogen III methylase activity

The methylase activity was determined by the conversion of uroporphyrinogen III into sirohydrochlorin. The uroporphyrinogen was generated from porphobilinogen by using a coupled assay system. The methylase activity was measured in a final volume of 0.5 ml containing 0.1 M-Tris/HCl buffer, pH 7.75, 2 mM-dithiothreitol, 250 μ M-SAM, 10 μ g of porphobilinogen deaminase, 25 μ g of uroporphyrinogen III synthase and between 0.2 and 1 mg of the methylase. The reaction was started by the addition of 40 nmol of porphobilinogen, the mixture was incubated at 37 °C for 30 min, and the reaction was stopped by the addition of 0.5 ml of 10% (w/v) trichloroacetic acid containing 0.5% (w/v) I_2 . After 5 min 5 μ l of saturated sodium bisulphite was added to decolorize the I_2 , and the precipitated protein was removed by centrifugation. The methylase activity was determined by comparing the absorption spectra of the supernatant in comparison with control experiments that lacked either the methylase or SAM. Methylase activity was associated with a reduced Soret band and the appearance of absorption maxima at 620 nm and 378 nm (Murphy *et al.*, 1973).

Preparation of tetrapyrrole methyl esters and their separation by h.p.l.c.

The tetrapyrroles in larger-scale incubations were isolated by adsorption on DEAE-Sephadex, which was then freeze-dried. After the freeze-drying the tetrapyrroles were esterified in methanol/ H_2SO_4 and extracted into chloroform as previously described (Scott *et al.*, 1978). The tetrapyrroles were separated by reverse-phase h.p.l.c. with an aq. 85% (v/v) methanol solvent system on a C_{18} silica column. The various tetrapyrroles were detected as they were eluted from the column by monitoring the absorbance at 378 and 405 nm with a Waters 990 photodiode array detector.

Spectral and radioactivity measurements

Absorption spectra were recorded on a Varian DMS90 u.v.–visible-absorption spectrophotometer.

Radioactivity determinations were made with a Packard model 3320 Tri-Carb scintillation spectrometer.

High-field Fourier-transform n.m.r. spectra were obtained with a Bruker WM-300 instrument equipped with a Bruker Aspect 2000 computer. All samples were dissolved in [2H_6]benzene (99.96 atom% 2H).

Fast-atom-bombardment mass-spectrometric determinations were made with a VG Analytical 70 S instrument.

RESULTS AND DISCUSSION

Expression of the *cysG* gene

The plasmid pHM3 (MacDonald & Cole, 1985), containing the *cysG* gene as well as portions of the *nir* operon on a 3.5 kb *EcoRI*–*PstI* fragment, was digested with *EcoRI* and *PstI* restriction enzymes. The 3.5 kb

Fig. 1. SDS/PAGE of purified *E. coli* uroporphyrinogen III methylase

Lane 1, molecular-mass standards; lane 2, purified *E. coli* uroporphyrinogen III methylase (10 μ g).

fragment was purified and ligated into the plasmid pUC19. The resultant plasmid was then digested with the restriction enzymes *HindIII* and *BstEII*, treated with the Klenow fragment of DNA polymerase I to fill in the single-stranded ends, incubated with ligase, and transformed into *E. coli* strain TB1. This treatment resulted in a plasmid (pCAR252) from which a large portion of the *nir* operon had been deleted and contained an insert of 1.88 kb in plasmid pUC19 in which *cysG* was the only complete gene. This plasmid, when transformed into *E. coli* strain CBK103, relieved the strain's requirement for cysteine.

Comparison by SDS/PAGE of a lysate of strain TB1 bearing plasmid pCAR252 (CR252) with a lysate of strain TB1 bearing plasmid pUC19 with no insert revealed a protein band with a molecular mass of about 52 kDa from strain TB1(pCAR252) that was not seen in strain TB1(pUC19).

This finding is evidence for the fact that the *cysG* gene had been successfully subcloned and over-expressed.

Purification of the over-expressed protein

The 52 kDa protein observed in strain CR252 extracts was purified to homogeneity by the procedure outlined in the Materials and methods section. When analysed by SDS/PAGE the purified protein ran as a single band with a molecular mass of 52 kDa (Fig. 1), which is comparable with the molecular mass of 49928 Da predicted from the DNA sequence of the *cysG* gene (Dr. Jeff Cole, personal communication). By gel-filtration chromatography on a Pharmacia Superose 12 column the protein was eluted with a molecular mass of 66 kDa, which strongly suggests that the protein product of the *cysG* gene exists as a monomeric species. From 4 litres of strain CR252 it was possible to obtain 50 mg of purified protein.

Fig. 2. Absorption spectra of the assay system described in the Materials and methods section with boiled enzyme (spectrum a) and with active methylase (spectrum b) and of authentic sirohydrochlorin (spectrum c)

Evidence that the *cysG* gene encodes uroporphyrinogen III methylase

By using the coupled assay system described in the Materials and methods section it was possible to demonstrate that the purified protein catalysed the SAM-dependent methylation of uroporphyrinogen III. The first line of evidence for this conclusion came from observing the enzyme incubations under u.v. light. Under u.v. only the tubes containing the 52 kDa protein and SAM appeared fluorescent, as a result of a change in the oxidation state of the uroporphyrinogen to that of uroporphyrin or sirohydrochlorin. Since the occurrence of the fluorescence was dependent on the presence of both the 52 kDa protein and SAM, it can be assumed that the change in oxidation level is caused by methylation to sirohydrochlorin and not by simple oxidation to form uroporphyrin.

Termination of incubations containing deaminase, co-synthase, methylase and SAM with I_2 /trichloroacetic acid and decolorization with sodium bisulphite produced a blue-green colour, whereas incubations containing only deaminase and co-synthase gave the normal red colour associated with uroporphyrin. Sirohydrochlorin (faktor II), isolated from *Propionibacterium shermanii* as previously described (Scott *et al.*, 1978), gives the same blue-green chromophore under acidic conditions. Comparison of the visible-absorption spectra of the various incubations with the sirohydrochlorin standard showed that only assay tubes with the 52 kDa protein and SAM gave a spectrum similar to that of sirohydrochlorin (Fig. 2). These spectra differed from that of uroporphyrin, which was formed in the control experiments, in having a much reduced Soret band and additional absorption maxima at 378 nm and 620 nm.

To prove that the methyl groups from SAM were being incorporated into the tetrapyrrole ring, [*Me*-

Fig. 3. H.p.l.c. profile of the esterified tetrapyrroles present after incubation of porphobilinogen with deaminase, co-synthase, methylase and SAM

Separation was carried out on a C_{18} reverse-phase column in aq. 85% (v/v) methanol. The absorbance was monitored at 378 nm.

^{14}C]SAM was added to the incubation mixture. At the end of the reaction the assay mix was passed down a small column of DEAE-Sephadex. The column was then washed with a 25-fold excess of water. The DEAE-Sephadex was then placed in a scintillation vial and the radioactivity determined. In control experiments with boiled enzyme only 514 c.p.m. was associated with the column material, but with active enzyme 47772 c.p.m. was recorded. This difference in radioactivity is explained by methylation of the uroporphyrinogen with the [^{14}C]methyl group derived from [*Me*- ^{14}C]SAM.

By increasing the scale of the methylase assay to 25 μ mol it was possible to isolate and purify larger amounts of product. After isolation by DEAE-Sephadex chromatography, esterification and extraction, the tetrapyrroles were separated by reverse-phase h.p.l.c. as described in the Materials and methods section. The esterified extract of an incubation mixture with the methylase and SAM gave a mixture showing an h.p.l.c. profile with one large peak and three minor peaks (Fig. 3). The major component was eluted at the same retention time (15 min) as sirohydrochlorin octamethyl ester, and two of the minor peaks were eluted at retention times corresponding to those of sirohydrochlorin monolactone heptamethyl ester and uroporphyrin octamethyl ester (11 min and 18.5 min respectively). The third minor peak, which had a retention time of 17 min, was probably the epimer of sirohydrochlorin octamethyl ester, although this was not verified. The 1H -n.m.r. spectrum of the compound that was eluted at 15 min exhibited chemical shifts identical with those of sirohydrochlorin octamethyl

ester [(8.86, 7.64, 7.54 and 6.78 p.p.m. (4 *meso*-protons), eight singlets between 3.15 and 3.42 p.p.m. (8 methyl esters) and 1.75 and 1.60 p.p.m. (2 methyl groups)] (Santander, 1987). The larger-scale incubation was repeated with [4,6-¹³C₂]porphobilinogen and [Me-¹³C]SAM as substrates. The ¹³C-n.m.r. spectrum of the purified esterified product showed four doublets corresponding to the chemical shifts of the two enriched methyl groups (19–21 p.p.m.) both coupled to ¹³C-enriched centres at positions 2 and 7 of the isobacteriochlorin (Santander, 1987). Analysis by fast-atom-bombardment m.s. gave a molecular ion (*m/z* 975) that corresponded to the [*M*+H]⁺ ion observed with authentic sirohydrochlorin octamethyl ester (Santander, 1987).

To summarize, we have subcloned and over-expressed the *cysG* gene. The product of this over-expression is a 52 kDa monomeric protein that catalyses the SAM-dependent methylation of uroporphyrinogen III to give sirohydrochlorin.

Since milligram quantities of this enzyme are now available, it should be possible to perform experiments to ascertain whether sirohydrochlorin is the true product of the reaction or an oxidation product derived (non-enzymically) from dihydrosirohydrochlorin, as has been postulated in vitamin B-12 biosynthesis (Scott *et al.*, 1978; Battersby *et al.*, 1982), and to identify which amino acid residues are involved in the catalytic process. Finally, the large amounts of protein produced by this over-expression will allow attempts at crystallization to be made.

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