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Enzymatic synthesis of dihydrosirohydrochlorin (precorrin-2) and of a novel pyrrocorphin by uroporphyrinogen III methylase

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Uroporphyrinogen III methylase was purified from a recombinant *hem*B⁻ strain of *E. coli* harbouring a plasmid containing the *cys*G gene. N-terminal analysis of this purified protein gave an amino acid sequence corresponding to that predicted from the genetic code. From the u.v./visible spectrum of the reaction catalysed by this SAM dependent methylase it was possible to observe the sequential appearance of the chromophores of a dipyrrocorphin and subsequently of a pyrrocorphin. Confirmation of this transformation was obtained from ^{13}C-NMR studies when it was demonstrated, for the first time directly, that uroporphyrinogen is initially converted into dihydrosirohydrochlorin (precorrin-2) and then, by further methylation, into a novel trimethylpyrrocorphin.

Uroporphyrinogen III methylase; Dihydrosirohydrochlorin; Precorrin-2; Dipyrrocorphin; Pyrrocorphin; NMR, ^{13}C-

1. INTRODUCTION

The structure proposed for precorrin-2 (**1**; R = H), an intermediate in vitamin B_{12} and sirohaem synthesis [1-4], is that of a dipyrrocorphin formed by C-methylation of uro'gen III at positions 2 and 7 by the action of a single SAM-dependent methyl transferase [5] as shown in scheme 1. Thus anaerobic isolation of the esterified dipyrrocorphin (**1**; R = Me) and the slightly enhanced cell free incorporation of the corresponding octa-acid, compared with that of the oxidised form, sirohydrochlorin (= factor II), into cobyrinic acid [3] provided strong, but indirect, evidence that (**1**; R = H) is the initial product of the action of uro'gen III methylase and that the dipyrrocorphin tautomer is not formed by prototropic rearrangement during the process of esterification, isolation and purification.

The enzyme responsible for the synthesis of precorrin-2, SAM-dependent uro'gen methylase, has recently been purified from two different sources as a direct result of genetic engineering. Blanche et al. [6] first described the purification of uro'gen III methylase (SUMT) from *Pseudomonas denitrificans* and, after oxidative esterification of the products of the enzymatic reaction, were able to isolate sirohydrochlorin octamethyl ester. However, since no spectral changes were observed during the course of the enzymic reaction (conducted under anaerobic conditions) is was assumed that the initial enzymatic product was the dipyrrocorphin, dihydrosirohydrochlorin (**1**).

Recently we reported the purification of uro'gen III methylase, the product of the *E. coli cys*G gene, and demonstrated that the enzyme catalysed C-methylation of uro'gen III at positions 2 and 7 to form sirohydrochlorin (factor II) after oxidation [7]. In this paper we provide direct evidence that the dipyrrocorphin tautomer (**1**) (precorrin-2) is not only synthesised by this enzyme but, furthermore, under the appropriate conditions, acts as a substrate for the enzyme and is transformed into a trimethylated pyrrocorphin by a further, anaerobic SAM-dependent C-methylation step.

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Abbreviations: uro'gen, uroporphyrinogen; SAM, *S*-adenosyl methionine; SUMT, *S*-adenosyl methionine uroporphyrinogen methyl transferase; NMR, nuclear magnetic resonance; HPLC, high pressure liquid chromatography; pbg, porphobilinogen

2. MATERIALS AND METHODS

Specimens of [2,11-$^{13}C_2$], [3,5-$^{13}C_2$] and [4,6-$^{13}C_2$]pbg were synthesised from the appropriately labelled 5-aminolaevulinic acid as previously described [7]. *S*-Adenosyl [^{13}C-methyl]methionine was prepared according to the method of Hegazi et al. [8]. Uro'gen III methylase was overexpressed and purified as described previously [7] from *E. coli* strain CR256 (RP523/pCAR252). RP523 is a *hem*B⁻, hemin permeable mutant [9] and, when grown on LB media supplemented with hemin, is unable to synthesise pbg and hence uro'gen III. The enzymes pbg deaminase and uro'gen III synthase (cosynthase) were also prepared from overproducing recombinant *E. coli* strains as previously described [7].

All enzymatic assays were carried out under strictly anaerobic conditions using buffer that had been degassed by freeze thawing under a vacuum of 10^{-3} Torr. Enzyme mixtures were lyophilised prior to

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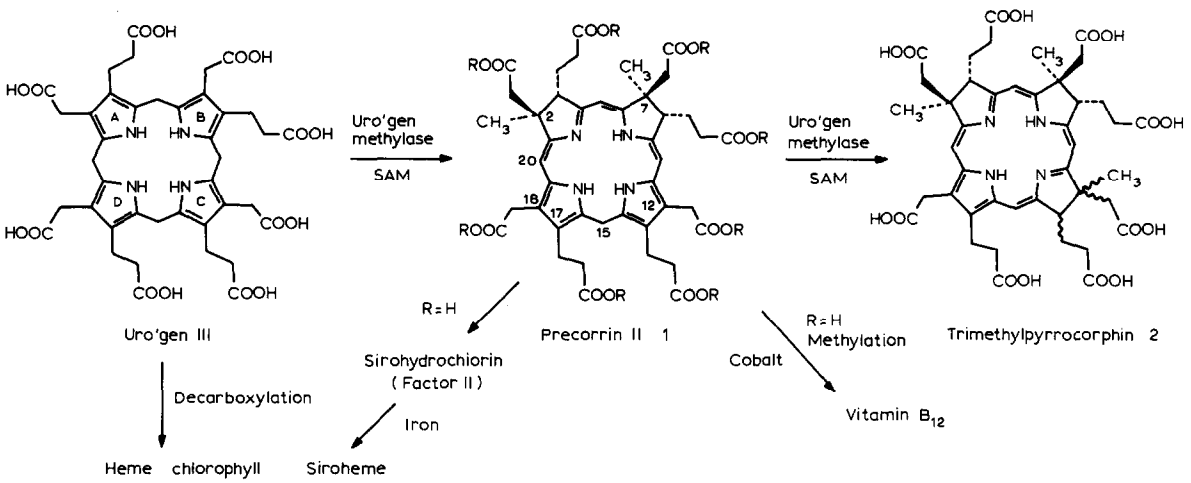

Scheme 1. Pathway to the biosynthesis of modified tetrapyrroles from uro'gen III. Methylation of uro'gen III at positions 2 and 7 by the action of uro'gen III methylase commits the pathway towards the biosynthesis of sirohaem and cobalamin via dihydrosirohydrochlorin (precorrin-2) (1; R = H). Further methylation of precorrin-2 by uro'gen methylase converts the dipyrrocorphin into a trimethyl pyrrocorphin (2). (The third methyl group is shown attached to C-12 but could also be attached to C-18.)

use. A typical reaction with uro'gen methylase was carried out as follows. In 5 ml of 0.1 M potassium phosphate buffer, pH 8.0, were dissolved 50 μg of pbg deaminase, 50 μg of uro'gen synthase, up to 1 mg of uro'gen methylase, 125 μg of pbg and 1 mg of SAM. The reaction was allowed to proceed at room temperature for 1–6 h under an atmosphere of argon in a glove box. The reaction was terminated by lyophilisation. Termination of larger scale reactions and isolation of the reaction products were achieved by the addition of DEAE Sephadex which was separated from the reaction mixture by filtration through a small sintered column. The column was washed with a large excess of buffer and the products were removed from the ion-exchange media with a 1 M NaCl solution. Esterification of the products, when necessary, was carried out as described previously [7].

Protein sequencing was carried out using an Applied Biosystems 470A pulsed liquid protein sequencer. HPLC analysis of the esterified reaction products was performed as described previously [7]. Uro'gen III concentration was determined after oxidation to uroporphyrin with I_2 by the HPLC method of Rideout et al. [10]. ^{13}C-NMR spectra were obtained at 75.47 MHz on a Bruker WM-300 spectrometer equipped with an Aspect 2000 computer. Spectra were recorded at ambient temperature (18–20°C) in a 10 mm ^{13}C selective probehead employing the following acquisition parameters. Repetition delay, 1.5 s; pulse width, 7.0 μs (60°); acquisiton time, 0.2 s. To avoid excessive sample heating, bi-level low power proton decoupling was accomplished using the WALTZ-16 sequence available in the Bruker-software. Exponential line broadening of 5 Hz was applied to the 16K data point FID prior to Fourier transformation.

3. RESULTS AND DISCUSSION

Uroporphyrinogen III methylase was purified to homogeneity from *E. coli* strain CR256 (RP523/pCAR252) by the same protocol as outlined previously [7]. CR256 is a recombinant *E. coli hem*B$^-$ strain harbouring a plasmid containing the *cys*G gene. The enzyme was purified from this strain because the strain used previously, CR252 (TB1/pCAR252), produced a large amount of endogenous factor II which was found to associate with the protein. By using a *hem*B$^-$ mutant strain this problem was overcome as the bacteria could no longer make the substrate for the methylating enzyme.

An N-terminal analysis of the protein gave the amino acid sequence shown in table 1. This sequence was found to be in complete agreement with that predicted by the DNA sequence (Dr Jeff Cole, personal communication) and has been used to confirm the translation start site on the DNA.

Table 1

Comparison of the predicted amino acid sequence, derived from the *cys*G gene, to the amino acid sequence obtained from the N-terminus sequencing of uro'gen methylase. The first amino acid predicted from the gene sequence (v) is encoded by the triplet GUG which, when it acts as the site of translation initiation, encodes for methionine

	Amino acid
DNA encoded sequence	V D H L P I F C Q L R D R D C L I V
Protein sequence	M D H L P I F ? Q L R D R D ? L I V

Table 2

Comparison of the amount of substrate remaining (measured as uroporphyrin) to the amount of sirohydrochlorin (Factor II) octamethyl ester (obtained by oxidative esterification of the reaction products) isolated at the end of a 4 h incubation with differing concentrations of uro'gen methylase

Reaction no.	Methylase concentration (mg/ml)	Amount of uro'gen remaining (nmol/ml)	Amount of factor II isolated (arbitrary units)
1	.000	14.0	.00
2	.008	12.1	.10
3	.020	10.3	.32
4	.040	6.9	.68
5	.100	2.9	.91
6	.200	0.0	.10

The effect of different enzyme concentrations on the methylation reaction was observed by recording the u.v./visible spectrum and by monitoring the disappearance of uro'gen and the production of sirohydrochlorin by HPLC analysis. As expected the amount of uro'gen decreased as the amount of methylase was increased (table 2). Concurrent with this decrease in substrate concentration was an increase in the amount of sirohydrochlorin isolated from the incubations with protein concentrations of up to 0.1 mg/ml (table 2). However, at 0.2 mg/ml the amount of sirohydrochlorin isolated decreased dramatically (table 2) and a new compound, with a longer retention time, was observed by HPLC analysis (data not shown). This result suggested that the enzyme was utilising its intended product as a substrate for further transformation. Additional evidence for this transformation was demonstrated by the dependence of the u.v./visible spectrum with enzyme concentration. As shown in fig.1, when the amount of enzyme was increased up to a concentration of 0.1 mg/ml the colour of the incubation increased to a dark yellow and a u.v./visible spectrum characteristic of a dipyrrocorphin developed [11,12]. At a protein concentration of 0.2 mg/ml the colour of the incubation changed from yellow to red and the u.v./visible spectrum changed from that of a dipyrrocorphin to that of a pyrrocorphin [11,12] (fig.1).

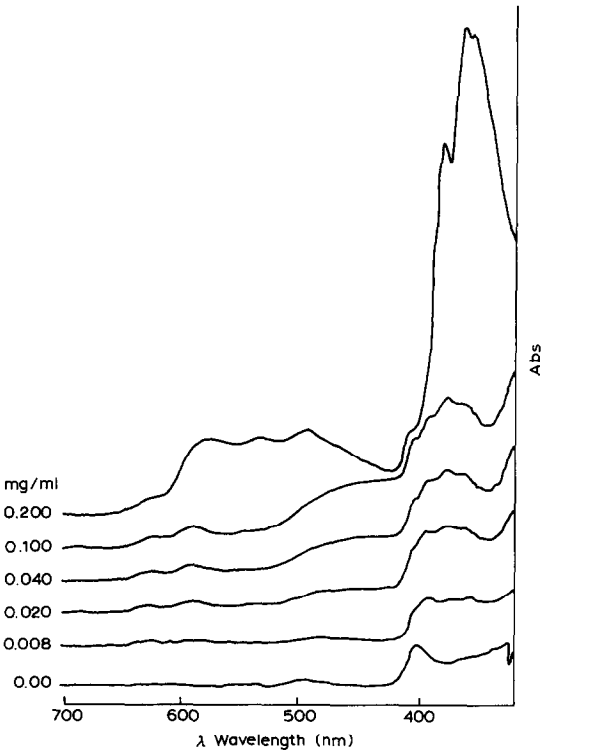

Fig.1. Ultraviolet/visible spectrum of the reaction catalysed by uro'gen methylase after incubation for 4 h. As the methylase concentration is increased so a spectrum of a dipyrrocorphin develops until, at a concentration of 0.2 mg/ml, the spectrum changes to that of a pyrrocorphin.

To confirm that uro'gen III was first being converted into a single tautomer of dihydrosirohydrochlorin the incubations were repeated on a larger scale using ^{13}C enriched substrates, stopped at the 'dark yellow' stage, and the products analysed by ^{13}C-NMR. Using uro'gen III derived from [4,6-$^{13}C_2$]pbg and [methyl-^{13}C]SAM a spectrum was obtained which indicated the presence of two (singlet) sp^2 enriched carbon atoms and two (doublet) sp^3 enriched centres coupled to SAM derived $^{13}CH_3$ at C-2 and C-7 (fig.2a). This spectrum, along with the corresponding NMR spectra obtained from incubations using [2,11-$^{13}C_2$] and [3,5-$^{13}C_2$]pbg derived substrate, which were all stopped at a similar stage in the reaction, provided the necessary proof that tautomer (**1**) was being produced (data not shown) thus allowing the unambiguous assignment of the dipyrrocorphin form (**1**) and confirmation that the anaerobic esterification sequence [3] had indeed preserved the dipyrrocorphin tautomer intact.

From the u.v./visible spectra presented in fig.1 it would appear that uro'gen methylase is also catalysing the transformation of the dipyrrocorphin (**1**), to a chromophore typical of a pyrrocorphin structure (as **2**). This process could occur either by tautomerization or by a further methylation. To differentiate between these two possibilities, incubations with the methylase were allowed to proceed to the 'red' stage using ^{13}C enriched substrates. The products were then again analysed by NMR. Using uro'gen III derived from [4,6-$^{13}C_2$]pbg the spectrum shown in fig.2b was obtained. The three signals in the sp^3 region and one in the sp^2 region are in agreement with a pyrrocorphin structure. Incubations with [2,11-$^{13}C_2$] and [3,5-$^{13}C_2$]pbg as substrate were also in accord with structure 2 (or isomers) (data not shown). To determine whether all sp^3 carbon atoms at the termini of the acetic acid side chains had undergone methylation, an incubation with [4,6-$^{13}C_2$]-pbg derived uro'gen III was conducted in the presence of [methyl-^{13}C] enriched SAM. The resultant spectrum showed that all three sp^3 enriched centres were coupled to enriched methyl carbons thus revealing that a third methyl group had indeed been attached to the macrocycle (at C-12 or C-18) (fig.2c). It was also possible, from pulse labelling experiments, to determine that these methyl groups were added in the sequence C-2 > C-7 > C-12 (or C-18).

There is no known physiological role for the pyrrocorphin described in this report, for in *E. coli* further methylation of precorrin-2 is not required for sirohaem synthesis (only oxidation and iron insertion are needed), and even in cobyrinic acid synthesis, which has not been demonstrated in *E. coli*, such a pyrrocorphin is unlikely to be an intermediate [13] since the third and fourth methyl groups are added to C-20 and C-17 respectively. The synthesis of this pyrrocorphin may therefore be artefactual due to the reaction being carried out under non-physiological conditions since, in

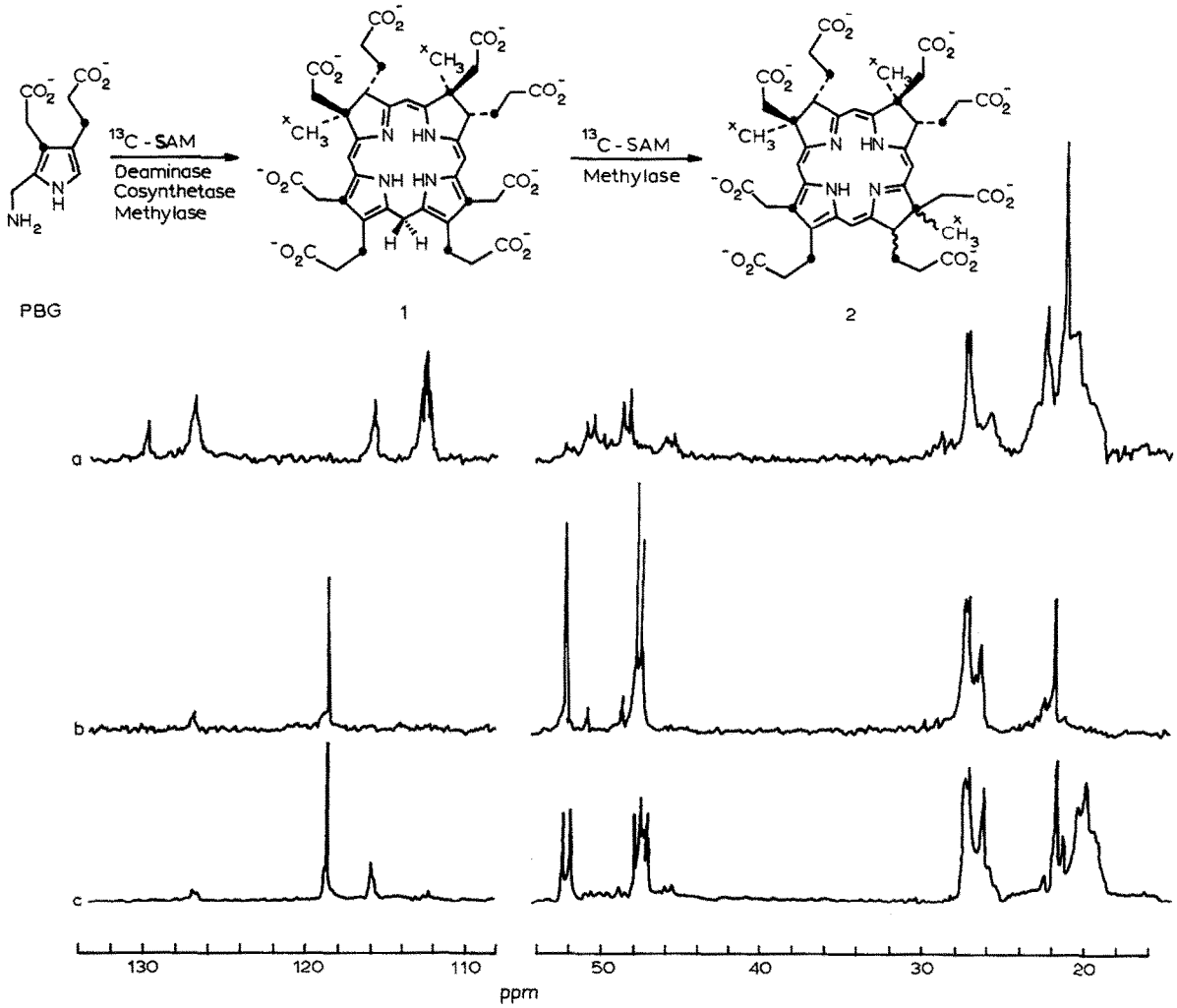

Fig.2. NMR spectra of the product of the reaction of uro'gen III, derived from [4,6-$^{13}C_2$]porphobilinogen, after: (a) incubation with uro'gen methylase and *S*-adenosyl[methyl-^{13}C]methionine. The reaction was stopped at the 'dark yellow' stage. The two signals (doublets) at 48 and 51 ppm correspond to the two sp^3 enriched carbons at positions 2 and 7 of precorrin-2 (**1**). The two signals (singlets) at 127 and 129 ppm are due to the two sp^2 enriched carbons at positions 12 and 17. The signals at about 27 ppm (two singlets) are from the enriched carbons in the propionic acid side chain of rings A and B whilst the broad signal at 22 ppm is due to the two enriched carbons in the propionic acid side chain of rings C and D (superimposed). The broad signals between 18 and 20 ppm arise from the enriched methyl groups derived from SAM. The other signals (116, 113 and 21 ppm) are due to unreacted porphobilinogen and uro'gen III. (b) incubation with uroporphyrinogen III methylase and SAM to the final 'red' stage. The three signals between 47 and 52 ppm correspond to the three sp^3 centres at positions 2, 7 and 12 (or 18) and the signal at 119 ppm arises from the sp^2 centre at position 18 (or 12) of the pyrrocorphin structure (**2**). The broad signals (three singlets) at around 27 ppm are due to the enriched carbons in the propionic acid side chains of the methylated rings A, B and C (or D) whereas the signal at 22 ppm is due to the enriched carbon atom of the propionic acid side chain of the remaining unmethylated ring D (or C). (c) as in (b) but using *S*-adenosyl-[methyl-^{13}C]methioninc. The spectrum corresponds to that observed in (b) except that the three singlets between 47 and 52 now appear as doublets and there is a broad signal between 18 and 20 ppm corresponding to the three SAM derived methyl groups.

the cell, the ratio of enzyme to substrate would be much lower and this in itself would probably prevent the formation of any pyrrocorphin. However, a uro'gen I derived tetramethylated metabolite, factor S_1, containing methyl groups at C-2, C-7, C-12 and C-18 has been described [14]. Perhaps the intrinsic structural and electronic factors reactions in these C-methylations [15] are connected with the change from a dipyrrocorphin to a pyrrocorphin chromophore with a consequent alteration in substrate specificity and a different methylase may insert the fourth methyl group in factor S_1 synthesis.

These results have revealed for the first time that dipyrrocorphin (**1**; R = H) is a direct product of uro'gen methylase, thereby confirming rigorously the structure proposed for precorrin-2. We have also biosynthesised the first uroporphyrinogen-derived pyrrocorphin and characterised it in its free acid form (**2**). This is of importance, since related pyrrocorphins are thought to be intermediates in vitamin B_{12} synthesis [2,4,13]. None of these intermediates have, as yet, been isolated, possibly due to the severe oxygen sensitivity of these compounds. We have found that, even after esterification, the pyrrocorphin (**2**) is very unstable when exposed to

air and suggest that the acquisition of the techniques necessary for handling this and related pyrrocorphins will lead to evolution of new strategies for isolation of the intermediates of the B_{12} pathway.

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