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The *Escherichia coli cysG* gene encodes the multifunctional protein, siroheme synthase

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Previously, the *E. coli cysG* gene product had been shown to sequentially methylate uro'gen III to produce precorrin-2, hence it was given the trivial name uro'gen III methylase. We now report that in addition to methylase activity, the CysG protein catalyses both the NAD^+ dependent oxidation of precorrin-2 to sirohydrochlorin, but also the insertion of iron into this oxidized intermediate, thereby producing siroheme. Thus CysG is a multifunctional protein solely responsible for siroheme synthesis from uro'gen III in *E. coli*, and accordingly is renamed siroheme synthase.

Siroheme; Dehydrogenase; Sirohydrochlorin; Ferrochelatase; NAD^+

1. INTRODUCTION

Central to the biosynthesis of cysteine in bacteria, nature uses the 6 electron reduction of sulfite to sulfide to produce the latter nucleophilic form of sulfur necessary to react with *O*-acetyl serine. The complex reductase, M_r 670,000, has $\alpha_4\beta_8$ structure and utilizes a variety of cofactors and prosthetic groups to effect the transfer of electrons from NADPH to sulfite [1]. The final port of exit of the electrons to sulfite is through siroheme, a 'non-classical' heme with a dimethyl-isobacteriochlorin structure [2].

Remarkably, sirohydrochlorin (the metal free form of siroheme) also serves as a precursor of vitamin B_{12} in both anaerobic and aerobic B_{12}-producing organisms. Thus in *Propionibacterium shermanii* it has been shown that sirohydrochlorin (factor II) re-enters the biosynthetic pathway after undergoing enzymic reduction to the dipyrrocorphin, precorrin-2 [3,4].

Precorrin-2 therefore stands at the crossroads of both the B_{12} and siroheme biosynthetic pathways (Scheme 1) and is synthesized from uro'gen III and *S*-adenosylmethionine (SAM) in *Pseudomonas denitrificans* by uro'gen III methylase (SUMT) [5] and in *E. coli* by the *cysG* gene product [6,7]. Curiously, the M_r of SUMT (M_r 26K) is nearly half that of CysG (M_r 49K), and the sequence of the former shows high homology to the C-terminal terminus (a.a. 202–452) of CysG (Fig. 1), thus suggesting that the N-terminal section of CysG may contain additional, previously unearthed catalytic activities. The formation of siroheme from precorrin-2 involves oxidation of the latter to sirohydrochlorin (factor II) followed by chelation of Fe^{2+}. Precorrin-2 is readily oxidized (non-enzymically) in the presence of oxygen to factor II; however, to effect its oxidation in anaerobic organisms a different mechanism of oxidation is obviously at work. In this communication we report that NAD^+ fulfills the role of cofactor in the oxidation catalyzed by CysG. Furthermore CysG also serves as a ferrochelatase, inserting Fe^{2+} into sirohydrochlorin and, since it is a multifunctional protein capable of synthesizing siroheme from uro'gen III, should be named siroheme synthase.

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2. MATERIALS AND METHODS

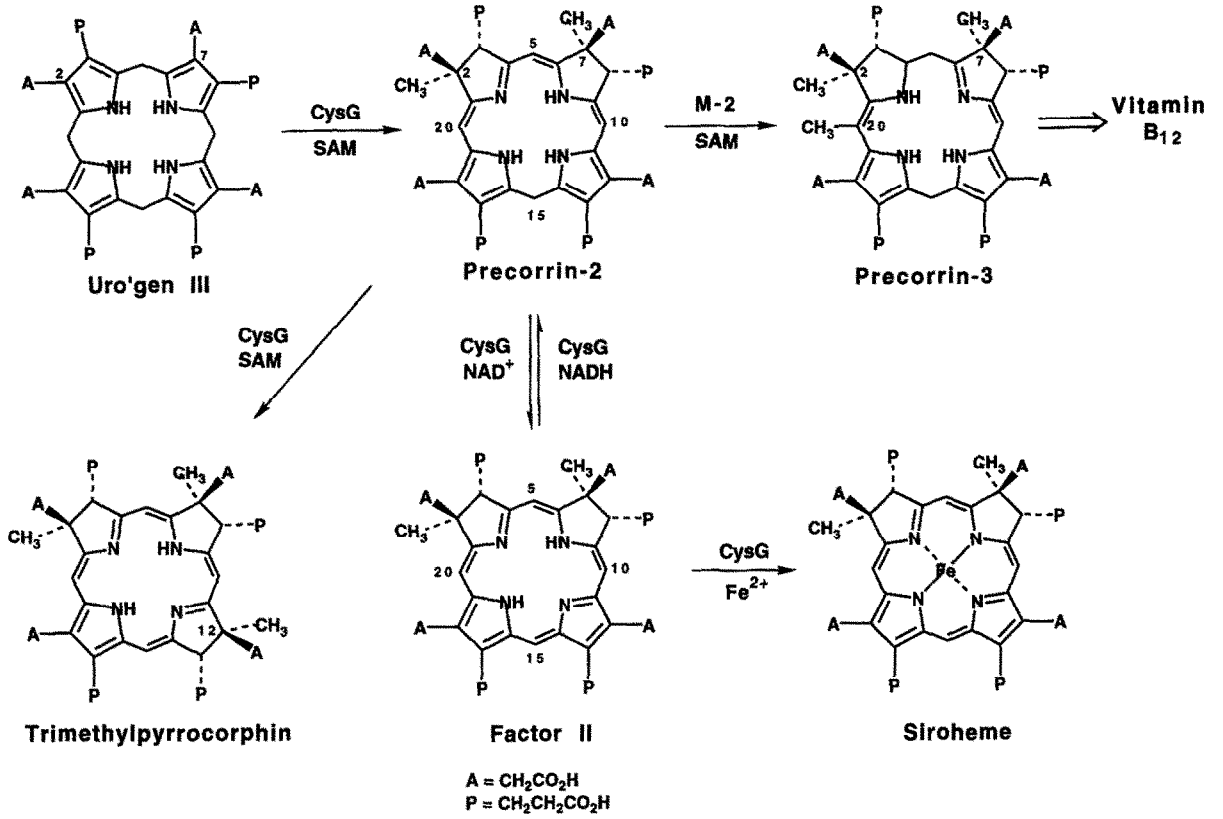
2.1. *Materials*

[2,11-$^{13}C_2$]PBG was synthesized enzymatically from [5-^{13}C]ALA using ALA dehydratase as previously described [8]. Precorrin-2 was prepared using the multi-enzyme synthesis as described earlier [8], omitting the homocysteine degrading system. *E. coli* uro'gen III methyltransferase (CysG) was overexpressed from the recombinant *E. coli* strain CR256 and purified as described previously [7] with the exception of employing a linear salt gradient (0–0.4 M KCl) during DEAE Sephacel purification. NAD^+, NADH and SAM were purchased from Sigma Chemical (St. Louis, MO).

2.2. *NAD^+-dependent oxidase of precorrin-2 by Siroheme synthase*

The ability of CysG to function as a dehydrogenase was assayed by incubating solutions of precorrin-2 (50 μM in 100 mM Tris, pH 8) alone or in the presence of either 0.5 mM NAD or NADP in a argon-purged globe box. The rate of oxidation was monitored spectrophotometrically by UV-VIS. For NMR samples, the incubation was scaled up to contain 5 mg precorrin-2, 15 mg CysG, and 0.5 mM NAD^+ in 100 ml buffer.

The reverse reaction, i.e. NADH dependent reduction of Factor II to precorrin-2, was performed in a similar manner, however using 0.5 mM NADH and incorporating methylase M2 [8] into the reaction, which converts precorrin-2 (but not Factor II) to precorrin-3. The resultant precorrin-3 was then converted to Factor III and determined by TLC and NMR methods.



Scheme 1.

2.3. *Metal insertion into sirohydrochlorin (Factor II) catalyzed by Siroheme synthase*

Solutions of Factor II were incubated in the presence of CysG and either 10 μM $FeSO_4$ or 10 μM $CoCl_2$. Metal concentrations higher than these usually resulted in inactivation of CysG. The rate of metal insertion was followed by fluorescent quenching of Factor II, and the incorporation verified by molecular weight determination by mass spectrometry.

2.4. *Analytical methods*

^{13}C-NMR spectra were obtained on a Bruker WM-300 spectrometer operating at a carbon frequency of 75.47 MHz. NMR samples were prepared under argon in 2 M KCl/20% D_2O and spectra were recorded at ambient temperature using bilevel low power WALTZ-16 proton decoupling to prevent sample heating as reported earlier [8]. Molecular weights were determined by laser desorption ionization FTICR mass spectrometry as described previously [9]. Samples for mass spectrometry were desalted by an additional absorbtion onto DEAE Sephadex, elution with 10% acetic acid, and lyophilization.

3. RESULTS AND DISCUSSION

CysG, when incubated anaerobically in the presence of SAM, sequentially methylates uro'gen III at C-2 and C-7 to form precorrin-2 (Scheme 1). Further incubation of CysG with precorrin-2 and SAM results in an additional methylation at C-12 to produce the nonphysiological product, 2,7,12-trimethylpyrrocorphin [10]. However, when NAD^+ is included in the incubation a new, pink chromophore accumulated and was shown to be indistinguishable from sirohydrochlorin (factor II), the oxidized equivalent of precorrin-2, by UV-VIS and ^{1}H NMR spectroscopy. Accordingly, when a sample of precorrin-2 was prepared from [2,11-$^{13}C_2$]PBG and incubated in the presence of CysG and 0.5 mM NAD^+, its conversion to factor II is quantitative as illustrated

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CysG   1 VDHLPIFCQLRDRDCLIVGGGDVAEPKARLLLDAGARLTVNALAFIPQFTAWADAGMLTLVEGPFDESLLDTCWLAIAATDDDALNQRVRQAAEARRIFC
CysG 101 NVVDAPKAASFIMPSIIDRSPLMVAVSSGGTSPVLARLLREKLESLLPLHLGQVAKYAGQLRGRVKQQFATMGERRRFWEKLFVNDRLAQSLANNDQKAI
CysG 201 TETTEQLINEPLDHRGEVVLVGAGPGDAGLLTLKGLQQIQQADVVVYDRLVSDDIMNLVRRDADRVFVGKRAGYHCVPQEEINQILLREAQKGKRVVRLK
SUMT   1 MIDDLFAGLPALEKGSVWLVGAGPGDPGLLTLHAANALRQADVIVHDALVNEDCLKLARPGAVLEFAGKRGGKPSPKQRDISLRLVELARAGNRVVLRLK

CysG 301 GGDPFIFGRGGEELETLCNAGIPFSVVPGITAASGCSAYSGIPLTHRDYAQSVRLITGHLKTG...GELDWENLAAEKQTLVFYMGLNQAATIQQKLIEH
SUMT 100 GGDPFVFGRGGEEALTLVEHQVPFRIVPGITAGIGGLAYAGIPVTHREVNHAVTFLTGHDSSGLVPDRINWQGIASGSPVIVMYMAMKHIGAITANLIAG

CysG 398 GMPGEMPVAIVENGTAVTQRVIDGTL..TQLGELAQQMNSPSLIIIGRVVGLRDKLNWFSN
SUMT 200 GRSPDEPVAFVCNAATPQQAVLETTLARAEADVAAAGLEPPAIVVVGEVVRLRAALDWIGA
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Fig. 1. Homology between *E. coli* CysG and *P. denitrificans* uro'gen III methyltransferase (SUMT). Percent similarity, 62.6; percent identity, 41.7. The predicted NAD(P)H binding site within the N-terminal of CysG is underlined.

in Fig. 2 by the complete conversion of the C15 meso carbon from sp^3 (δ 22, Fig. 2A) to sp^2 hybridization (δ 108, Fig. 2B). Moreover, no evidence of the formation of the over-methylated trimethylpyrrocorphin is observed, even after prolonged incubation. $NADP^+$ was also found to function as a cofactor for the oxidation though the rate of reaction was some 50 times slower than that with NAD^+. A comparison of the amino acid sequence of CysG to that of other NADH dependent dehydrogenases [11] reveals a potential NAD binding site located in the N-terminus of the protein (Fig. 1). To investigate this possibility experimentally, a truncated version of the CysG protein corresponding to the C-terminal homolog of SUMT (Fig. 1) was prepared and shown to be inactive as a dehydrogenase while still retaining its ability to methylate uro'gen III [12]. The full-length *cysG* gene product therefore is a multi-functional protein, with SAM dependent methylase activity lying entirely in the C-terminus, while the NAD^+ dependent dehydrogenase activity resides, at least partially, in the N-terminal domain.

Further experiments have also established that the

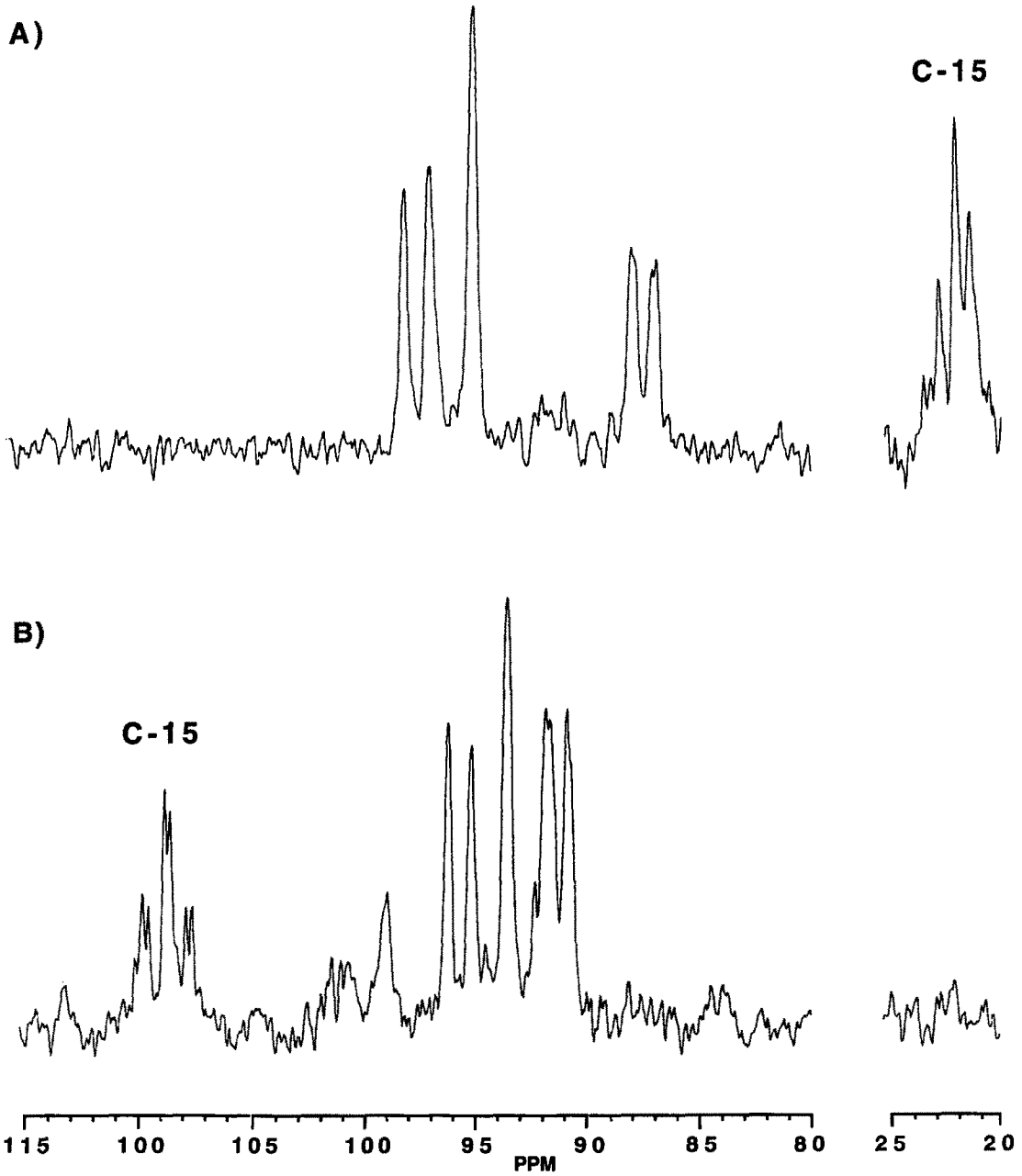

Fig. 2. Meso carbon regions of the ^{13}C-NMR spectra of (A) precorrin-2, derived from [2,11-$^{13}C_2$]PBG, and (B) precorrin-2 incubated anaerobically in the presence of CysG and NAD. In this pattern of ^{13}C enrichment, C-15 is coupled to both C-14 and C-16 (downfield and not shown) giving rise to the observed triplets centered at δ 22 (A) and δ 108 (B) in the reduced and oxidized versions, respectively. (The remaining signals shown arise from C-10, C-20 and C-5.)

oxidation reaction catalysed by CysG is reversible, precorrin-2 being formed from factor II in the presence of NADH. While the equilibrium of the reaction lies heavily in the favor of formation of factor II, this equilibrium could be effectively shifted by including the C-20 methyl transferase, M-2, in the incubation. This enzyme catalyzes the committed step in vitamin B_{12} biosynthesis by methylating C-20 of precorrin-2 (but not factor II) to form precorrin-3 [8] (Scheme 1). Thus in the presence of CysG, NADH and M-2, factor II is converted in good yield to precorrin-3. In *P. shermanii* it has been shown that factor II is reduced to precorrin 2 allowing re-entry into the B_{12} pathway, presumably by the same enzymatic sequence [3].

It is known that other oxidized B_{12} intermediates, namely factor III [13] and factor IV [14], may be reduced by an as yet uncharacterized dehydrogenase and become substrates for the respective B_{12} biosynthetic enzymes. To investigate the possibility that CysG catalyzes the former reaction, precorrin-3 was incubated with NAD^+ and CysG. Since no factor III was detected, the dehydrogenase activity of CysG is probably confined to the interconversion of precorrin-2 to factor II and yet another dehydrogenase must be responsible for the reduction of later B_{12} intermediates.

A third catalytic activity of CysG was uncovered when ferrous iron was added to its incubation with uro'gen III, SAM and NAD^+. A violet product was formed and identified as siroheme (Fig. 2). Subsequent incubations employing either precorrin-2 or factor II as substrates in the absence of NAD^+ proved that ferrous iron is inserted into factor II and not into precorrin-2. CysG is therefore a ferrochelatase and should be named siroheme synthase.

We have found that siroheme synthase is also capable of inserting Co^{2+} into factor II. It is therefore possible that siroheme synthase serves as the missing cobaltochelatase of the anaerobic B_{12} producing organisms (e.g. *Salmonella typhimurium* and *P. shermanii*)*. Additional evidence that all three catalytic functions of siroheme synthase are required for both siroheme and B_{12} pathways come from recent studies of *cysG* mutants in *S. typhimurium* [15]. Over 60 mutants in both the methylase domain of the siroheme synthase and the sequence encoding the dehydrogenase and ferrochelatase activities all prevent both siroheme and B_{12} biosynthesis. Siroheme synthase is therefore a multifunctional enzyme that, remarkably, plays a major role in two biosynthetic pathways.

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*Although *E. coli* does not produce B_{12}, CysG from this organism bears a very high homology to that of the CysG protein from *Salmonella* which does make B_{12} anaerobically.