Evidence for a covalent intermediate in the S-adenosyl-L-methionine-dependent transmethylation reaction catalysed by sirohaem synthase

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INTRODUCTION

The cysG gene encodes a protein that is involved in the final stages of sirohaem biosynthesis in both Escherichia coli and Salmonella typhimurium [1,2] and has recently been shown to be a multifunctional enzyme (EC 2.1.1.107) [3–5]. The enzyme performs two S-adenosyl-L-methionine (AdoMet)-dependent transmethylation steps at positions 2 and 7 of uroporphyrinogen III [6]. These methyl transfers produce precorrin-2 (dihydrosirohydrochlorin) (Scheme 1), a known intermediate in both the cobalamin and sirohaem biosynthetic pathways. An NAD\(^+\)-dependent dehydrogenation of precorrin-2 yields the iso-bacteriochlorin sirohydrochlorin, and a subsequent ferrochelation yields sirohaem (Scheme 1) [3–5]. Sirohaem is required by both sulphite and nitrite reductases and mutants associated with the former enzyme are unable to make the amino acid cysteine [1]; hence the prefix cys is found affiliated both with genes that encode for the subunits of the enzyme and genes that are required for the biosynthesis of the prosthetic group. CysG is also known to be required for cobalamin biosynthesis in S. typhimurium where precorrin-2 acts as the substrate for corrin ring formation [7].

In E. coli, sirohaem synthase (CysG) performs all the aforementioned reactions concerning with the transformation of uroporphyrinogen III into sirohaem [3,4]. The protein contains 457 amino acids [8] and the polypeptide chain can be thought of as folded into at least two functional domains (Figure 1) [4]. The N-terminal domain, amino acids 1–201, contains the NAD\(^+\) binding site (14–41) as well as the catalytic machinery to perform the ferrochelation reaction. The C-terminal domain, amino acids 202–457, contains the AdoMet binding site (218–234) and performs the methyl transfer reaction [3,4,9]. Gene dissection has permitted the expression of the C-terminal domain of the CysG protein as a truncated protein and it has been shown that this truncated protein retains the ability to methylate uroporphyrinogen III in the same way as CysG but is unable to catalyse the macrocyclic oxidation step or the ferrochelation reaction [4]. In this respect the truncated protein is similar to CobA, the uroporphyrinogen methylase involved in corrin biosynthesis from Pseudomonas denitrificans [10] and Bacillus megaterium [11] and for this reason the domain has been termed CysG\(^\text{t}'\) in contrast with the N-terminal domain, which has been termed CysG\(^\text{t}\) [4]. In the absence of NAD\(^+\), both CysG and CysG\(^\text{t}'\) perform an ‘ overmethylation’ whereby an extra methyl group is transferred to position 12, producing a trimethylpyrrocorphin with no known physiological function [12,13]. In S. typhimurium, CysG is also required for cobalamin biosynthesis as precorrin-2 can be directed along the corrin pathway by methylation at position 20 [7,14]. As S. typhimurium belongs to the corrin-producing class of bacteria that make cobalamins anaerobically and insert cobalt at an early stage in the corrin pathway [15,16], CysG may also be responsible for cobalt chelation of precorrin-2 (E. Raux, A. Lanois, F. Levillayer, M. J. Warren, E. Brody, A. Rambach and C. Thermes, unpublished work). The full summary of reactions catalysed by CysG is shown in Scheme 1.

The AdoMet-dependent C-methylation reactions performed by CysG are similar to the other C-methylations carried out during the biosynthesis of vitamin B\(_{12}\) [18–22]. Corrin biosynthesis requires a total of eight peripheral methylations, at C-2, C-7, C-20, C-17, C-12, C-1, C-5 and C-15, whose addition is mediated by six methyltransferases, CysG (or CobA), CobI, CobJ, CobM, CobF and CobL. The discrepancy between the number of methylations (eight) and the number of enzymes (six) is explained by the fact that two of the methyltransferases are bifunctional, CysG (CobA) and CobL [21,22]. The amino acid sequences of the methyltransferases involved in corrin biosynthesis reveal a certain similarity in terms of their primary structure, and evolutionary genetic analysis suggests that they are all derived from a common ancestor [21]. Such structural similarity would also be indicative of a common mechanism. The
Scheme 1 Role played by CysG in tetrapyrrole modification

Reactions catalysed by the multifunctional CysG. The enzyme performs two AdoMet-dependent methyl transfers, initially generating precorrin-1 and subsequently precorrin-2. Sirohaem is generated from precorrin-2 by an NAD\(^+\)-dependent dehydrogenation and a ferrochelation event. In the absence of NAD\(^+\) and at high enzyme concentrations, CysG will 'overmethylate' precorrin-2 to yield a trimethylpyrrocorphin with no known physiological role. CysG may also chelate cobalt at the level of precorrin-2 to give cobalt–precorrin-2 and thus direct the intermediate towards cobalamin biosynthesis.

Figure 1 Domain structure of CysG

CysG can be envisaged as being divided into two functional domains. The C-terminal domain is responsible for the transmethylation reactions and is termed CysG\(A\), and the N-terminal domain catalyses the NAD\(^+\)-dependent dehydrogenation and ferrochelation reactions and is termed CysG\(B\). The relative positions of the putative NAD and AdoMet binding sites are shown.

methyltransferases in corrin biosynthesis have been assigned not only on their sequence similarity but also on their ability to bind AdoMet [23]. An AdoMet binding assay, in which \(S\)-adenosyl[methyl-\(^3\)H]methionine is mixed with the protein and then passed down a small gel-filtration column, has been used to confirm the function of several of the corrin methylases. Coelution of AdoMet with the protein during gel filtration identifies the protein as a methylase, whereas AdoMet was found to separate from the protein and to be eluted later in the small-molecules fraction in control experiments with BSA [23]. Such tight binding as that observed between the methylases and AdoMet was assumed to be due to a low dissociation constant \(K_d\). In this paper we provide evidence that the binding is due to a covalent attachment between the AdoMet and the protein.

MATERIALS AND METHODS

Chemicals

Most chemicals, reagents and antibiotics were purchased from the Sigma Chemical Corporation. Sephacryl S-300 and DEAE-Sephacel were obtained from Pharmacia Fine Chemicals. Tryptone, yeast extract and agar were purchased from Difco Laboratories. Mimetic Green-I affinity chromatography resin
was purchased from Affinity Chromatography Ltd, Cambridge, U.K. 
S-Adenosyl-L-[methyl-3H]methionine (80 Ci/mmol), S-
adenosyl-L-[carboxyl-14C]methionine (50 mCi/mmol) and 
Hyperfilm-MP were purchased from Amersham International.
Bio-Rad protein assay was obtained from Bio-Rad. AutoFlor was 
purchased from National Diagnostics. Porphobilinogen (PBG) 
was synthesized from 5-aminolaevulinic acid (5-ALA) from 
purified 5-ALA dehydratase as previously described [24].

Bacterial strains

E. coli strain TB1 transformed with pCR2552, pMW1 and 
pMW2 was used for the overexpression of CysG, CbiF and CbiH 
respectively [6,25]. Strains were grown from 5% inocula for 18 
h at 37 °C in LB medium supplemented with ampicillin (50 mg/l).

Enzyme purification

CysG was purified by the method described by Warren et al. [5].
Porphobilinogen deaminase and uroporphyrinogen III synthase 
were isolated from recombinant E. coli strains harbouring 
plasmids containing the hemC and hemD genes respectively, as 
previously described [26]. CbiH and CbiF were purified by the 
method described by Roessner et al. [25]. Proteins were stored 
at −20 °C in 50 mM Tris buffer, pH 7.8, with 10 mM 2-
mercaptoethanol.

Rapid AdoMet binding assay

Purified protein (1 mg) was incubated with either 0.5 µCi of 
[methyl-3H]AdoMet or [carboxyl-14C]AdoMet in a final volume 
of 200 µl of 50 mM Tris buffer, pH 7.8, and 1 mM 2-
mercaptoethanol at 37 °C for 15 min. The specific activity of 
the AdoMet was adjusted to approx. 50 µCi/mmol with un-
labelled AdoMet before use. The reaction mixture was applied to 
Sephadex G25 column (1 cm × 15 cm) and fractions (1 ml) were 
collected. Protein-containing fractions were identified by the 
Bio-Rad protein assay and the radioactivity of the individual 
fractions was determined with a Pharmacia Wallac 1410 scint-
tillation counter.

Analysis of AdoMet binding to CysG by fluorography

Labelled protein (20 µg) was boiled for 2 min in sample disruption 
buffer and then loaded on to an SDS/12% polyacrylamide gel. 
After electrophoresis at 40 mA for 30 min the gel was stained 
with Coomassie Blue and destained in 7% (v/v) acetic acid. The 
gel was soaked in AutoFlor for 1–2 h before drying to enhance 
the detection of the labelled protein. The gel was exposed to X-
ray film at −80 °C.

Generation of uroporphyrinogen III

Uroporphyrinogen III was generated in situ by using purified 
PBG deaminase and uroporphyrinogen III synthase. Generation 
of 25 µM uroporphyrinogen III was achieved by incubating 
0.1 mM PBG with 10 µg/ml PBG deaminase and 1 µg/ml 
uroorphyrinogen III synthase at 37 °C for 10 min in 50 mM 
Tris buffer, pH 8.0. For fluorography experiments, methylated 
CysG (2 mg/ml) was mixed with the appropriate amounts of 
deaminase and uroporphyrinogen III synthase. The sample was 
divided into two and uroporphyrinogen III was generated in one 
of the samples by the addition of PBG. The two samples were

subsequently boiled in disruption buffer and subjected to 
SDS/PAGE in 12% gels in adjoining lanes.

CD spectra

CD spectra were obtained at the National Chirotical Spectroscopy Centre (EPSRC) in the Chemistry Department, 
Birkbeck College, London, U.K. CD spectra were recorded at 
temperature over the range 180–260 nm on a Jasco J720 
spectropolarimeter with a cell pathlength of 0.02 cm.

RESULTS AND DISCUSSION

Previous work has demonstrated that some of the Ps. denitrificans 
AdoMet-dependent methyltransferases involved in the peripheral 
me-thylation of the corrin ring structure of vitamin B12 bind 
AdoMet very tightly [23]. This was demonstrated by the 
association of radioactivity derived from S-adenosyl-L-[methyl-
3H]methionine with the methyltransferase after separation of an 
AdoMet/enzyme mixture by gel filtration chromatography. The 
rapid AdoMet binding assay is a useful technique not only for 
ascriming the function of ‘unknown’ proteins in the cobalamin 
biosynthetic operons but also for detection of the methyl-
transferases during purification. Although this result was inter-
preted as being due to a high affinity of the enzyme for AdoMet 
(a low Keq), it could also be due to the covalent attachment of the 
AdoMet to the protein or methylation of the protein itself. These 
possibilities were not examined.

The CysG protein was also analysed with the rapid binding 
assay to see if it demonstrated the same tight binding to AdoMet. 
The addition of [methyl-3H]AdoMet (0.5 µCi, 10 nmol) to a 
CysG solution, 200 µl of a 5 µg/ml solution, followed by gel 
filtration chromatography yielded two radioactive peaks (Figure 
2a). These peaks corresponded to AdoMet-associated protein 
and unbound AdoMet. When the experiment was performed with 
BSA as a control, only one radioactive peak was observed, 
corresponding to unbound AdoMet (Figure 2b). The radio-
activity associated with fractions 5–7 contained 95% of 
the applied protein, whereas the radioactivity that was eluted in 
fractions 11–14 contained no protein. CysG is therefore able to 
bind AdoMet tightly, in agreement with its role as an AdoMet-
dependent methyltransferase. This result is similar to the ob-
servations made by Crouzet et al. [23], who used this test with the 
CobA and CobF methyltransferases from Ps. denitrificans. A 
detailed kinetic analysis of CysG has shown that it has a 
Kd for AdoMet in the micromolar range, which is similar to the 
Km for NAD+, another substrate required in the oxidation of precorri-
2 into sirohydrochlorin (S. C. Woodcock and M. J. Warren, 
unpublished work). However, the latter dinucleotide does not 
associate with the enzyme on the gel filtration column, indicating 
that NAD+ does not bind as tightly as AdoMet (results not 
shown). For tight binding, as is apparently observed with 
AdoMet, a Keq in the nanomolar range would be required. The 
discrepancy between the Keq and the apparent Keq led us to 
investigate further the association of AdoMet with CysG.

To test whether the labelled moiety that associated with the 
CysG fraction on gel filtration was covalently bound to the 
enzyme, the following two experiments were performed. First, 
the enzyme was mixed and incubated with [methyl-3H]AdoMet at 
37 °C for 10 min and was subsequently treated with 4 M urea. 
Incubation with urea above a concentration of 4 M with CysG 
leads to denaturation of the protein, a conclusion deduced from 
a study of the circular dichroism (CD) spectrum. The native 
protein shows a CD spectrum consistent with a mixture of helical 
(8% ) and β-strand (36.5%) conformations, whereas there is a
Figure 2 Rapid AdoMet binding assay with BSA and CysG

Graphs showing the elution profile of radioactivity from the rapid AdoMet binding assay performed on a Sephadex G-25 column. Protein was eluted in fractions 5–7 whereas AdoMet was eluted in fractions 11–14. (a) Elution profile observed when a CysG/[methyl-3H]AdoMet mixture was analysed; (b) elution profile of a BSA/[methyl-3H]AdoMet mixture; (c) elution profile of CysG after mixing with [carboxyl-14C]AdoMet; (d) comparison of the elution profile of CysG denatured with urea before the addition of [methyl-3H]AdoMet (C) and CysG denatured with urea after it had been mixed with [methyl-3H]AdoMet (O). Only the elution profile surrounding fractions 1–9 is shown in this instance to demonstrate the marked difference between them.

Figure 3 CD spectrum of CysG

Comparison of the CD spectra of native CysG (thick line) with that of CysG in 4 M urea (thin line). The native CysG spectrum is characteristic of a protein containing both helical and extended conformations. Addition of 4 M urea leads to a protein with a greatly reduced amount of defined secondary structure and increased random coil conformation. Data acquisition was not possible below 205 nm owing to interference from the urea.

large change in the spectrum when the protein is treated with 4 M urea, associated with the abolition of the major secondary-structure elements (Figure 3). A urea-treated enzyme/[methyl-3H]AdoMet mixture was chromatographed on Sephadex G-25. The same elution pattern of two radioactive peaks was observed as previously shown, in that a high proportion of the radioactivity was eluted with the protein-containing fractions 5–7 (Figure 2d). Importantly, if the protein was first denatured with urea before the addition of [methyl-3H]AdoMet, none of the label was eluted with the protein-containing fractions but was all accounted for in the unbound fractions 11–14 (Figure 2d). These experiments prove (i) that the tertiary structure of the protein is important for AdoMet binding, and (ii) that the labelling of the protein is due to a specific reaction between CysG and AdoMet. Moreover, the fact that the label remains bound to the protein, even after denaturation, indicates that the association is probably of a covalent nature. Similar AdoMet binding results are also obtained when CysG is treated with 1% (w/v) SDS, although the protein is not denatured to the same extent as by treatment with urea; the CysG/SDS CD spectrum is only slightly altered from the native protein spectrum (results not shown).

The stoichiometry of AdoMet binding was also investigated by increasing the concentration of [methyl-3H]AdoMet to CysG until a saturating incorporation of label was observed. This allowed a crude estimate of the binding ratio of AdoMet to protein to be determined and this was found to be about 1 nmol of label per 10 nmol of protein under the conditions tested. Several reasons can be attributed for this apparent low binding ratio (10%) of AdoMet to CysG. In the absence of an accurate active-site titrant, it is not known whether all the protein is fully functional or, as a dimer, whether it demonstrates half-site reactivity; likewise it is also not known what proportion of the enzyme is already associated with unlabelled AdoMet. Taking these arguments into account the observed 10% incorporation of label into the protein must be highly significant.

The second experiment that demonstrates the covalent link between the AdoMet-derived methyl group and CysG comes from the association of the labelled methyl group with the
It was also found that if CysG was preincubated with an excess of unlabelled AdoMet before incubation with [methyl-3H]AdoMet, no incorporation of label was observed by either gel filtration or fluorography. Conversely if CysG, labelled with [methyl-3H]AdoMet, was incubated with an excess of unlabelled AdoMet, the protein was found to retain the label. These results suggest that, once attached to the protein, the AdoMet moiety is not readily exchanged. In fact, the labelled protein was found to be stable to both acidic (pH 2) and alkaline (pH 10) environments for at least 1 h and did not dissociate in 1 M hydroxylamine. However, high salt concentrations (up to 1 M NaCl) affected the association of the AdoMet with CysG (results not shown).

The covalent modification of AdoMet methyltransferases has been reported before but in a very different context. Previous modifications of AdoMet-dependent enzymes have been achieved with photolabelling techniques whereby the enzyme–AdoMet complex is irradiated with UV [27,28]. The ensuing free-radical chemistry gives rise to covalently bound adducts of AdoMet at the active site of the proteins. This type of labelling, as reported with the EcoRII and CheR methyltransferases, is substantially different from the experiments reported here because UV irradiation of CysG–AdoMet complexes leads to a decrease in the incorporation of label into the protein (results not shown). Another protein covalently inactivated by AdoMet is amino-cyclopropane carboxylate synthase [29]. This enzyme uses pyridoxal phosphate as a cofactor to generate amino-cyclopropane carboxylate from AdoMet. Under certain conditions the enzyme becomes inactivated by the AdoMet-derived aminobutyrate, which covalently modifies the active-site lysine residue. In all these examples the enzymes are irreversibly inactivated and the covalent modification of the protein is not part of the catalytic cycle of the enzymes.

To determine whether CysG is complexed with the complete AdoMet molecule or just the labile methyl group of AdoMet, the binding of [methyl-3H]AdoMet to CysG was compared with the binding of [carboxyl-14C]AdoMet. When [carboxyl-14C]AdoMet was used in the AdoMet column binding assay two radioactive peaks were again observed (Figure 2c). The [carboxyl-14C]AdoMet behaved in the same way as the [methyl-3H]AdoMet in that denaturation of the CysG–AdoMet complex with urea did not release the bound label. To prevent the [carboxyl-14C]AdoMet from binding to CysG the protein had to be denatured first with urea (results not shown). These results prove that it is not the transfer of the methyl group from the AdoMet to CysG that is giving rise to the labelled protein. The experiments suggest that the protein is modified with the whole AdoMet molecule, although assays with labelled adenine AdoMet need to be performed to confirm this unambiguously.

The final question raised by this intriguing binding observation is whether the bound AdoMet subsequently transfers its methyl group to the other reaction substrate, uroporphyrinogen III. To answer this question two further experiments were performed. Thus the [methyl-3H]AdoMet–CysG complex, isolated after gel filtration chromatography, was incubated with uroporphyrinogen III. After incubation, the uroporphyrinogen III was separated from the enzyme by anion-exchange chromatography. Most of the protein was eluted from the anion-exchange resin by 0.5 M NaCl whereas the majority of the tetrapyrrolic material was eluted by 2 M NaCl. The protein fraction contained 33% of the radioactivity, whereas the uroporphyrinogen III fraction contained 67%. Most of the label had therefore been transferred to the uroporphyrinogen III. The remainder of the label associated with the protein fraction probably represents unseparated uroporphyrinogen III, as it is difficult to achieve full separation of the protein from the porphyrinogen. Further
convincing evidence that uroporphyrinogen III was able to remove the methyl group from the labelled \textit{[methyl-}$^{3}$\textit{H]}AdoMet–protein was obtained from SDS/PAGE and fluorography. Two identical samples containing \textit{[methyl-}$^{3}$\textit{H]}AdoMet–CysG as well as small amounts of the enzymes PBG deaminase and uroporphyrinogen III synthase were prepared. Addition of PBG to one of the samples generated uroporphyrinogen III \textit{in situ}. After 10 min incubation at 37 °C the samples were boiled in disruption buffer and subjected to SDS/PAGE; the resulting gel was analysed by fluorography. Visualization of the fluorogram clearly showed that the protein sample to which uroporphyrinogen III had been added had lost the majority of the label (Figure 4b, lane 2), whereas the label was retained in the untreated sample (Figure 4b, lane 1). Together these two experiments demonstrate that uroporphyrinogen III is able to remove the methyl group from the protein and that uroporphyrinogen III is itself the final recipient of the methyl group.

The work of Crouzet et al. [23] has also shown that the \textit{P. denitrificans} CobA and CobF transmethylases of corrin biosynthesis bind AdoMet very tightly, probably in the same way as described in this paper. To ascertain whether other cobalamin biosynthetic AdoMet-dependent methyltransferases also bind AdoMet covalently, the \textit{S. typhimurium} CbiF and CbiH, which are homologues of the \textit{Ps. denitrificans} CobM and CobJ, were purified from the appropriate recombinant strains [25] and analysed in the rapid AdoMet binding assay. CbiF is the methylase responsible for methylation at C-11, whereas CbiH is responsible for methylation at C-17 and ring contraction. The CbiH from AdoMet with CysG would be consistent with this result as long as the methyl group were not transferred first to the enzyme. Interaction with the next substrate, uroporphyrinogen III, produces precorrin-1 and \textit{S}-adenosyl-L-homocysteine by transmethylisation of the methyl group from the AdoMet–protein complex to the tetrapyrrole. The enzyme undergoes another round of AdoMet modification which, in turn, catalyses the transformation of precorrin-1 into precorrin-2 (Scheme 2).

The steric course of AdoMet-dependent transmethylation reactions has been studied by using chiral methyl AdoMet and this has shown that the reactions go by overall inversion [30], in agreement with a direct \textit{S}_2\text{N}_2\text{E}\text{E}\text{E}\text{E} displacement reaction between AdoMet and uroporphyrinogen III. After rearrangement the process is repeated at position 7 to give precorrin-2. (b) Methylation cycle for CysG consistent with the observations made in this study.

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The results presented in this paper provide strong evidence that CysG catalyses the AdoMet-dependent methyltransferase reaction to uroporphyrinogen III in a multistep process. The protein is first modified by the attachment of AdoMet to form an enzyme intermediate. Interaction with the next substrate, uroporphyrinogen III, produces precorrin-1 and \textit{S}-adenosyl-L-homocysteine by transmethylisation of the methyl group from the AdoMet–protein complex to the tetrapyrrole. The enzyme undergoes another round of AdoMet modification which, in turn, catalyses the transformation of precorrin-1 into precorrin-2 (Scheme 2).

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and to where is the covalent bond made on the AdoMet? Furthermore why is such a tight association required? There thus remain several important mechanistic questions to be addressed in this very interesting family of related AdoMet-dependent methyltransferases.

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