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Identification of HcgC as a SAM-Dependent Pyridinol Methyltransferase in [Fe]-Hydrogenase Cofactor Biosynthesis

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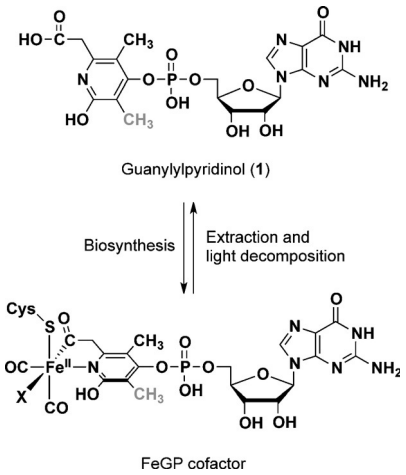
Abstract: *Previous retrosynthetic and isotope-labeling studies have indicated that biosynthesis of the iron guanylylpyridinol (FeGP) cofactor of [Fe]-hydrogenase requires a methyltransferase. This hypothetical enzyme covalently attaches the methyl group at the 3-position of the pyridinol ring. We describe the identification of HcgC, a gene product of the hcgA-G cluster responsible for FeGP cofactor biosynthesis. It acts as an S-adenosylmethionine (SAM)-dependent methyltransferase, based on the crystal structures of HcgC and the HcgC/SAM and HcgC/S-adenosylhomocysteine (SAH) complexes. The pyridinol substrate, 6-carboxymethyl-5-methyl-4-hydroxy-2-pyridinol, was predicted based on properties of the conserved binding pocket and substrate docking simulations. For verification, the assumed substrate was synthesized and used in a kinetic assay. Mass spectrometry and NMR analysis revealed 6-carboxymethyl-3,5-dimethyl-4-hydroxy-2-pyridinol as the reaction product, which confirmed the function of HcgC.*

Nature has developed three major types of hydrogenases, which use unique metal-containing cofactors to cleave and/or form H_2.[1] [Fe]-hydrogenases hydrogenate methenyltetrahydromethanopterin to methylenetetrahydromethanopterin within the methanogenic pathway of many methanogenic archaea.[2] This enzyme contains an iron guanylylpyridinol (FeGP) cofactor as a prosthetic group. The Fe center, which activates inert H_2, consists of a single iron atom coordinated by two CO molecules, a cysteine residue, one solvent molecule, a pyridinol nitrogen, and the acyl carbon of the 6-substituent of pyridinol (Scheme 1).[3] The solvent-binding site is postulated to be the H_2-binding site. Owing to several unique structural features, namely the two CO ligands, the acyl–iron bond, GMP–pyridinol conjugation, and the methyl substituents at the pyridinol ring, its biosynthesis is a chemically challenging process that is of great scientific and biotechnological interest.



Scheme 1. Structures of guanylylpyridinol **1** and the iron guanylylpyridinol (FeGP) cofactor of [Fe]-hydrogenase (X = solvent). Compound **1** is both the decomposition product of FeGP upon irradiation and most likely a genuine intermediate of the FeGP biosynthetic pathway. The 3-methyl group (shown in gray) of the pyridinol moiety is biosynthesized through a methyltransferase reaction.[8]

The FeGP cofactor biosynthesis enzymes are encoded by at least seven conserved genes (*hcgA-G*) that co-occur with *hmd* (the gene for [Fe]-hydrogenase).[2b] A previous analysis of the growth of *hcgA-G* knockout mutants of *Methanococcus maripaludis* indicated a relationship between these genes and biosynthesis of the FeGP cofactor.[4] Based on recent structure-to-function studies, HcgB was annotated as a guanylyltransferase that catalyzes the reaction between GTP and 2,4-dihydroxypyridines, most likely to afford guanylylpyridinol **1** (Scheme 1).[5] Interestingly, guanylylpyridinol **1**, an intermediate in the FeGP cofactor biosynthetic pathway, can be also obtained as a decomposition product of the FeGP

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cofactor upon irradiation with UV-A/blue light. Through this process, its acyl group is hydrolyzed to a carboxy group (Scheme 1).[3e,f] The activation of the carboxy group during FeGP cofactor biosynthesis is carried out by HcgE and HcgF. HcgE was identified as an adenylyltransferase that adenylates the 6-carboxy group of **1**. A subsequent transesterification reaction is catalyzed by HcgF by using an inherent cysteine that forms a thioester bond with **1**. The thioester is proposed to be a direct precursor of acyl–iron bond biosynthesis.[6] For HcgD, a function as an iron-trafficking protein was proposed.[7] The functions of HcgA, HcgC, and HcgG are unknown.

Stable-isotope labeling studies of the FeGP cofactor revealed that the 5-methyl group of the pyridinol moiety originates from C2 of acetate, and the 3-methyl group from the methyl group of methionine.[8] Therefore, we assume the 3-methylation to be catalyzed by an *S*-adenosylmethionine (SAM)-dependent methyltransferase. Several SAM-dependent methyltransferases have already been biochemically characterized and their participation in vital biological processes such as regulation and cofactor biosynthesis demonstrated.[9]

In this report, we predicted HcgC as the SAM-binding methyltransferase, primarily based on structural data. Subsequently, the HcgC-catalyzed reaction was verified using the predicted pyridinol substrate, which was chemically synthesized.

Targeted *hcgC* and *hcgB* knockout mutants in *M. maripaludis* were constructed (see the Supporting Information) to investigate the physiological effect of their gene products. No Hmd activity was detectable in strains lacking either *hcgB* or *hcgC* (Tables S1, S2, and S3, and Figure S1 in the Supporting Information), which demonstrates their participation in the biosynthesis of the FeGP cofactor and that they cannot be substituted by other genes.

On the basis of these results, we applied a structure-to-function strategy for annotating HcgC. We started the project with the determination of the crystal structure of HcgC (MJ0489) from *Methanocaldococcus jannaschii* at 2.7 Å resolution (Figure 1, Table S4, and Figure S2). HcgC was found in the crystalline state as a homotetramer, but was dominantly found as a homodimer in solution (data not shown). The monomers are composed of a Rossmann-like coenzyme-binding domain (31–190; 256–267), made of six parallel and one peripheral antiparallel strand, which is flanked by six α-helices and a smaller α/β domain (1–30; 191–255) termed the interface domain due to its extended contact with the partner monomer (Figure 1 a). According to the Dali server,[10] the coenzyme-binding domain is structurally most similar to those of NAD(P)H-dependent oxidoreductases[11] (e.g., shikimate dehydrogenase AroE; PDB ID:, 2HK9)[12] and SAM-dependent methyltransferases (e.g., 5-methyluridine methyltransferase RumA, PDB ID: 2BH2;[13] Figure S3). This relationship allows us to suggest HcgC as a binding protein for an adenosine moiety of either $NAD(P)^+$ or SAM; this prediction would be impossible to obtain on the basis of a primary structure comparison although HcgC revealed partial sequence identity to genes annotated as RNA methyltransferases. To obtain further information about its ligand-binding capabilities, HcgC was co-crystallized either with NAD^+, $NADP^+$, or SAM and subsequently structurally characterized. Extra electron density was only detectable for SAM, which could be reliably modeled at the expected position in front of the C-terminal loops of three parallel β-strands of the coenzyme-binding domain (Figure 1 a,b). SAM is embedded into a precisely tailored pocket and both the methionine and adenosine moieties are fixed by conserved residues, thus suggesting SAM as the natural substrate of HcgC. For example, the amino and carboxy groups of the methionine moiety are hydrogen-bonded to Lys34, Glu139, and Thr185. The two ribose-OH groups of the adenosine moiety interact with the invariant residue Asp77 (Figure 1 b and Figure S4). Furthermore, SAM binding induces specific conformational changes to encapsulate the active site. We

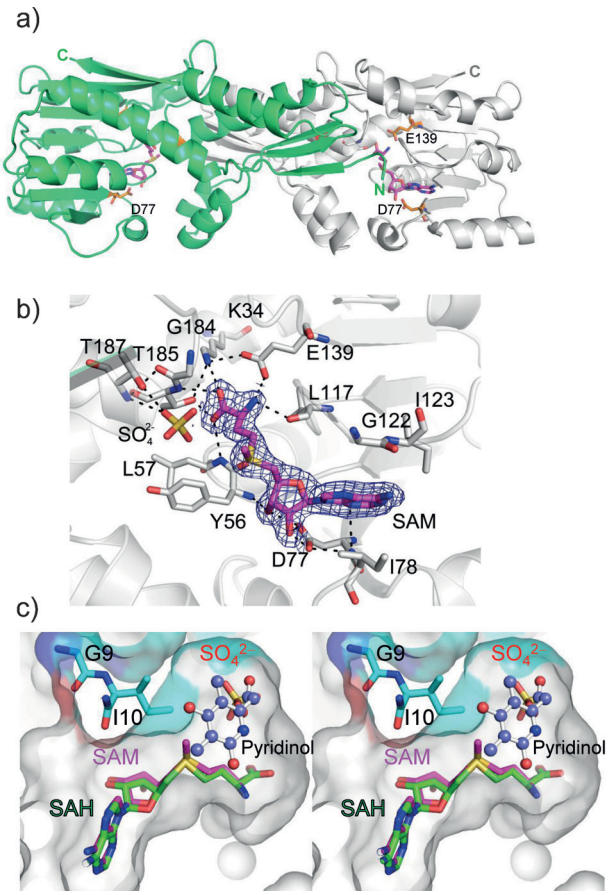


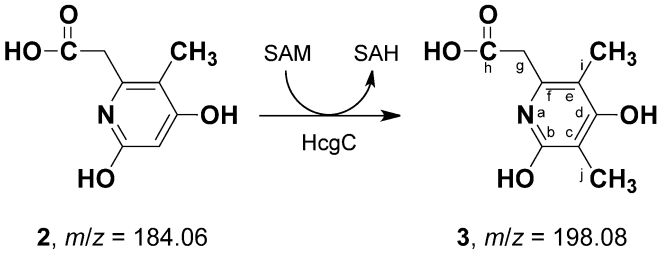
Figure 1. Structural information for HcgC. a) The overall fold of the HcgC homodimer. The monomers (green, gray) are composed of a larger coenzyme-binding domain and a smaller interface domain. Since the N-terminal residues of the partner monomers contribute to the methyl acceptor binding site, the dimer is considered as the functional unit. In the cocrystal structure, one *S*-adenosylmethionine (SAM) molecule (magenta stick model) is bound per monomer. The N and C termini and some important residues are indicated (carbon atoms shown in orange). b) The SAM-binding site. The $2F_o$-F_c electron density (blue mesh) for SAM was contoured at 1σ. Polar interactions are shown as dashed lines. c) Structure model of the HcgC/SAH/pyridinol **3** complex in stereoview. The orientation/position of pyridinol **3** (ball-and-stick model) was determined by docking calculations inside a properly sized/shaped pocket; SAH (green) and the superimposed SAM (magenta) are shown as stick models.

assume that the mentioned SAM–polypeptide interactions fix segment 79–92, which was disordered in the empty HcgC structure but is clearly visible partly as an α-helix in structure of the HcgC/SAM complex. More precisely, the flexible two-helix extension of the interface domain moves towards segment 79–92, thereby resulting in mutual stabilization by multiple mostly hydrophobic interactions and in closure of the cleft between them. As a result, SAM becomes partially shielded (Figure S2). Moreover, the absence of the cysteine-rich CX3CX2C motif that is characteristic of radical SAM enzymes that bind a [4Fe-4S] cluster[14] rules out HcgC as a radical-SAM enzyme, which might be required in the FeGP biosynthetic pathway for formation of the CO ligand[15] or heterocyclic ring formation.[16] These findings strongly argue for HcgC as a SAM-dependent methyltransferase.

Methyl transfer normally proceeds through an S_N2-type mechanism, which implies a binding site for the methyl-accepting atom in van der Waals contact with the methyl group of SAM. Indeed, a deep but solvent-exposed pocket occupied by a putative sulfate ion and several firmly bound water molecules was identified directly beside the SAM-binding site (Figure 1b). To gain information about the nature of the methyl acceptor and thus the function of the SAM-dependent methyltransferase, we also determined a crystal structure of HcgC soaked with SAH and the artificial guanylylpyridinol at 1.6 Å resolution (Figure S5 and Table S4). SAH binds equivalently into the same SAM-binding site of HcgC, and triggers the same induced-fit movement (Figure 1c and Figure S5), which supports HcgC as SAM-dependent methyltransferase. Unprecedentedly, the conserved residues 9–11 of the partner subunit, which are disordered in the previous structures, become rigid. The empty binding site for the methyl acceptor is thereby shrunk to a size which contains space for pyridinol but not for the larger guanylylpyridinol. Notably, the artificial guanylylpyridinol was partly visible at the interdimer interface of the HcgC tetramer, but this binding site is presumably not functionally relevant (Figure S5).

Docking simulations[17] of the possible 3-methylated product **3** (Scheme 2) into the decreased methyl acceptor binding site of SAH-bound HcgC resulted in a reasonable geometry between the 3-methyl group of pyridinol **3** and the SAH sulfur atom. Their distance of 3.8 Å is in the range to realize methyl transfer between **2** and SAM by HcgC (Figure 1c). Moreover, the carboxy group of pyridinol **3** is placed onto the putative sulfate-binding site of the superimposed HcgC/SAM complex structure, thus supporting the validity of the calculation. In conclusion, the size and hydrophobic profile of the postulated binding pocket in HcgC suggests that the 3-methyl group is introduced at the pyridinol stage. Although co-crystallization experiments with SAH and **3** have so far been unsuccessful, the results of the structure-to-function approach confidently suggest HcgC as a novel SAM-dependent methyltransferase, and all of the data are consistent with pyridinol **2** as the methyl acceptor (Scheme 2).

To confirm the predicted HcgC reaction, we synthesized pyridinol **2** ($m/z = 184.06$) through carboxylation of 5,6-dimethylpyridine-2,4-diol (for details, see the Supporting Information and Figure S6). Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF-MS) analysis of the HcgC enzyme assay indicated conversion of substrate **2** into a 14 m/z larger species (**3**; Figure 2a). Species **3** was not formed in the absence of HcgC. The attachment of one methyl group to **2** through a catalytic reaction is thus definitively confirmed. The HcgC reaction product contained three protons that are exchangeable with deuterons according to MALDI-TOF-MS data (Figure S7), which is in agreement with the chemical structure of **3**. The HcgC product **3** was also indirectly verified by its further reaction to give guanylylpyridinol **1** in the presence of GTP and HcgB, the enzyme that catalyzes the subsequent step in the FeGP cofactor biosynthetic pathway (Figure S8). The ^{1}H-^{13}C HMBC NMR spectrum of the HcgC product showed additional cross-peaks from the methyl protons at 1.9 ppm to C(b) (162 ppm), C(c) (105 ppm) and C(d) (163 ppm; Figure S9), which specified



2, m/z = 184.06 **3**, m/z = 198.08

Scheme 2. The proposed SAM-dependent methylation catalyzed by HcgC. Pyridinol **3** is subsequently conjugated to the GMP moiety in a reaction catalyzed by HcgB. The pyridinols appear to be present as pyridone and pyridinol tautomeric forms in dimethyl sulfoxide and water, respectively.

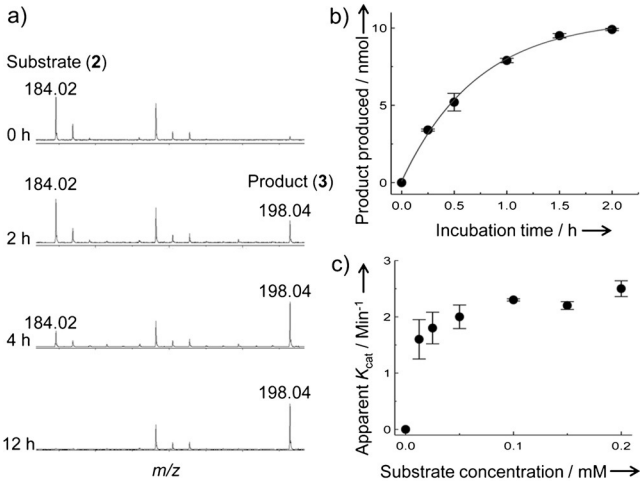


Figure 2. Kinetic analysis of the HcgC reaction. a) Conversion of the substrate (**2**, $m/z = 184$) into the methylated product (**3**, $m/z = 198$) by HcgC. The reaction mixture was incubated at 37 °C for defined periods in the presence of 0.4 mM **2** and then analyzed by MALDI-TOF-MS. Peaks from the matrix observed at the $m/z = 190$ region are not changed during the reaction. b) The time dependency of the production of **3** in the presence of 0.1 mM **2**, which was determined by HPLC. c) The dependency of the HcgC-catalyzed reaction on the substrate concentration. The error bar indicates standard deviation for duplicate measurements.

methylation in the 3-position [C(c)] of pyridinol **2**. The HcgC reaction was kinetically characterized through determination of the product concentration by HPLC (Figure 2b,c and Figure S10). The apparent k_{cat} was 2.5 min^{-1}.

In conclusion, this powerful structure-to-function approach provided us with sound information to postulate HcgC as a SAM-dependent methyltransferase and pyridinol **2** as the most likely methyl acceptor. On this basis, the assumed substrate was chemically synthesized. The predicted methyl transfer to the 3-position of pyridinol **2** as catalyzed by HcgC was demonstrated by product analysis.

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