

Identification of the HcgB Enzyme in [Fe]-Hydrogenase-Cofactor Biosynthesis**

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The [Fe]-hydrogenase from methanogenic archaea, one of three types of hydrogenases,^[1] reversibly catalyzes the cleavage of H₂ and the subsequent transfer of a hydride ion to methenyltetrahydromethanopterin.^[2] For the activation and production of H₂,^[3] [Fe]-hydrogenase contains an iron guanylylpyridinol (FeGP) cofactor, which is composed of guanylylpyridinol and an iron center with two CO ligands; the pyridinol ring is ligated to the iron center through its nitrogen atom and an acyl carbon atom (Figure 1, top).^[4] Upon irradiation with UV-A/blue light, the extracted FeGP cofactor photochemically decomposes into Fe, CO, and the iron-free FeGP cofactor (abbreviated as GP); the acyl group of the cofactor is hydrolyzed to give a carboxy group (Figure 1, bottom).^[5] Because of its unique structural and functional features, the biosynthesis of the FeGP cofactor is a major challenge and therefore of great interest in chemistry and biology. Based on retrosynthetic analysis and stable-isotope labeling data, we have proposed a pathway for the biosynthesis of the FeGP cofactor that involves various complicated reactions for pyridinol formation, pyridinol methylation, and formation of the iron center.^[6] Whereas the guanosine monophosphate (GMP) moiety of the FeGP cofactor was

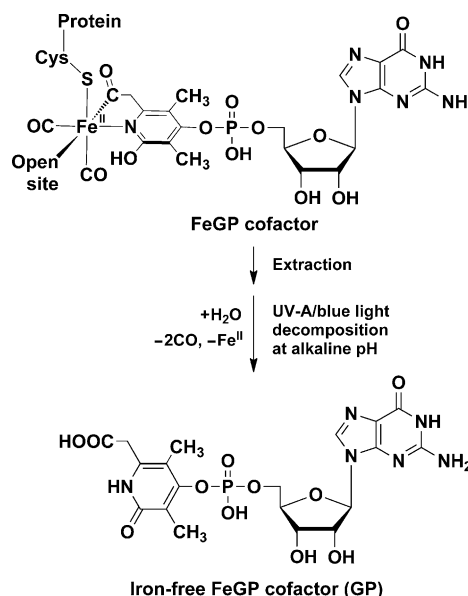


Figure 1. Formation of GP from the FeGP cofactor extracted from [Fe]-hydrogenase in the presence of 2-mercaptoethanol or acetate.^[5b] In the crystal structure of the native enzyme, a solvent molecule is bound at the “open” site. Upon irradiation with UV-A/blue light, the iron complex decomposes, and the acyl group is hydrolyzed to a carboxy group.

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formed through a canonical pathway,^[6] the reactions postulated for the synthesis of natural pyridinol were rather hypothetical (for possible pyridinol precursors, see the Supporting Information, Figure S1). None of the pyridinol precursors were experimentally confirmed, the order in which they are synthesized in the cell is unclear, and the enzymes involved have not been characterized. Nevertheless, it was expected that the biosynthesis of the FeGP cofactor includes a guanylyltransferase reaction through which GMP and a structurally unknown pyridinol precursor are conjugated.

Herein, the function of HcgB as a guanylyltransferase was predicted by structural genomics^[7] by detecting structural similarities between HcgB and nucleoside triphosphatases (NTPases) that cleave off pyrophosphates from nucleoside triphosphates (Figure 2a). To confirm this proposal, we successfully imitated the guanylyltransferase reaction using artificial pyridinol derivatives and guanosine triphosphate (GTP) as the substrates. Crystal structure analysis of complexes of HcgB with the product of the model reaction or with GP provided not only an explanation for product–substrate binding and the mechanism of the catalytic process on a molecular level, but also a proposal for a possible reaction

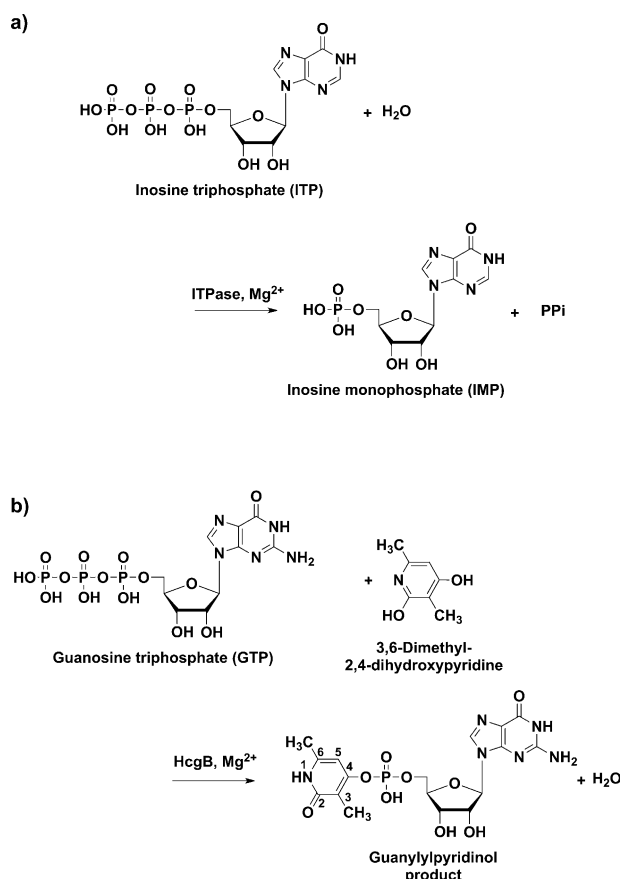


Figure 2. a) A reaction catalyzed by inosine triphosphatase (ITPase).
b) A model reaction catalyzed by HcgB.

sequence of the biosynthesis of the FeGP cofactor. This study is a notable example for the functional characterization of an enzyme that was guided by a structural genomics approach and verified by various analytical methods.

We initially analyzed seven conserved genes (*hcgA–G* genes) that are located adjacent to the [Fe]-hydrogenase-encoding gene^[2b,8] with respect to the potential activity of their corresponding enzymes in the biosynthesis of the FeGP cofactor. The structure of HcgB obtained from *Methanothermobacter thermautotrophicus* has already been solved by a structural genomics project (PDB code: 3BRC). Comparative studies revealed structural similarities between HcgB and NTPase, which could not be deduced on the sequence level (for the sequences of HcgB, see Figure S2). Superimposed structures of HcgB and NTPase with inosine triphosphate and Ca²⁺ (PDB code: 2Q16)^[9] indicated that the NTP binding cleft of NTPases is similar to that of HcgB (Figure S3). In comparison with NTPases, a crucial aspartate, the base catalyst for hydrolysis of nucleoside triphosphates, is exchanged for a glycine in HcgB. Therefore, HcgB might be able to bind and activate nucleoside triphosphates, but lacks activity as an NTPase. These findings made us wonder whether HcgB might catalyze a guanylyltransferase reaction for the conjugation of the pyridinol ring to GMP (Figure 2b).

To confirm this hypothesis, biosynthetic model reactions with GTP and pyridinol derivatives as the substrates were

performed in the presence of Mg²⁺ and HcgB from *Methanocaldococcus jannaschii* to form the corresponding guanylyl-pyridinol products. Experiments with some of the most promising pyridinol substrates, including the pyridinol derivative of GP, 6-carboxymethyl-2,4-dihydroxy-3,5-dimethylpyridine, could not be performed because the compounds are not commercially available and difficult to obtain by synthetic means. However, one of these substrates is probably the natural substrate of the HcgB reaction (see below). Therefore, commercially available pyridine derivatives with 2-hydroxy and/or 4-hydroxy substituents were applied as model substrates to imitate the possible precursor of the pyridinol moiety of the cofactor (Figure S4; for the possible natural substrates, see Figure S1). The products were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). For 2,4-dihydroxypyridine derivatives (3,6-dimethyl-2,4-dihydroxypyridine, 2,4-dihydroxy-6-methylpyridine, and 2,4-dihydroxypyridine), but not for mono-hydroxypyridine compounds, mass peaks that correspond to the GMP-pyridinol conjugates were observed (Figure S4 and S5). This finding indicated that both of the 2- and 4-hydroxy groups of the pyridinol substrates are crucial for HcgB catalysis. We selected 3,6-dimethyl-2,4-dihydroxypyridine as a pyridinol substrate for further studies, because its structure is most closely related to that of the pyridinol in the FeGP cofactor. UV/Vis spectroscopy of the corresponding product supports the hypothesis that conjugation of the pyridinol to GMP has occurred (Figure 3b). For the ¹H and ¹³C NMR spectra, all resonances could be assigned to the GMP and pyridinol moieties (Figure S6a–d). Peak splitting in the ¹³C NMR spectrum was caused by long-range coupling between ¹³C and ³¹P (Figure 3c) and indicated that only one regioisomer of the product had been formed (Figure 2b); its configuration was in agreement with that of the FeGP cofactor. Finally, we identified 3,6-dimethyl-2,4-dihydroxypyridine–GMP in the crystal structure of HcgB from *M. jannaschii* co-crystallized with this model product (produced by the HcgB reaction; Figure 4a). 3,6-Dimethyl-2,4-dihydroxypyridine–GMP was located inside the predicted substrate-binding cleft. The pyridinol moiety was accommodated into a well-designed pocket and interacts with Arg 104 and Arg 20 through its pyridinol 2-hydroxy substituent and with Asp 23 through its pyridinol nitrogen atom (Figure 4a). The structure obtained by X-ray analysis is in agreement with the regioselectivity of the HcgB-catalyzed reaction that was determined by NMR spectroscopy.

The catalytic reaction of HcgB with the model substrate 3,6-dimethyl-2,4-dihydroxypyridine was further explored by kinetic studies. The product formation rate was dependent on the amount of HcgB added (Figure 3a) and obeys Michaelis–Menten kinetics; the rate constant (*k*_{cat}) and the Michaelis constant (*K*_M) for GTP were 1.4 ± 0.3 min^{−1} and 410 ± 50 μM, respectively. A plausible mechanism for the guanylyltransferase reaction was postulated on the basis of the structure of HcgB complexed with 3,6-dimethyl-2,4-dihydroxypyridine–GMP (Figure S7). Nucleophilic substitution of the β,γ-pyrophosphate group of GTP is accomplished by the 4-hydroxy group (not the 2-hydroxy substituent) of pyridinol, which is activated by the conserved active-site residues Arg 20, Asp 23,

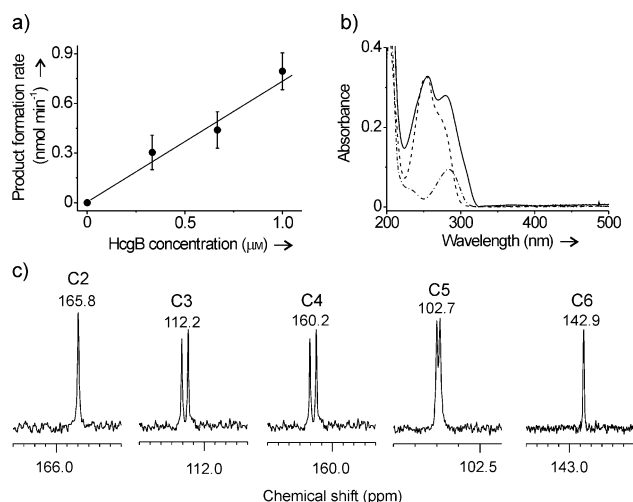


Figure 3. Enzymatic synthesis of guanylylpyridinol from GTP and 3,6-dimethyl-2,4-dihydropyridine catalyzed by HcgB. a) Dependency of the product formation rate on the concentration of HcgB in the assays. b) UV/Vis spectra of purified guanylylpyridinol produced by the HcgB-catalyzed reaction (—), GMP (---), and 3,6-dimethyl-2,4-dihydropyridine (—•—). c) Peak splittings in the ¹³C NMR spectrum of the resonances of the C3, C4, and C5 carbon atoms of the pyridinol moiety of the product, which are due to long-range ¹³C–³¹P coupling, and lack of splitting of the peaks corresponding to the C2 and C6 carbon atoms; thus, the C4 carbon atom is connected to the phosphate group.

and Arg104. Thus, the active site is well adapted to catalyze the guanylyltransferase reaction; this finding corroborates the function proposed for HcgB.

Thus far, it has been shown that model pyridinol compounds react with GTP to form a pyridinol–GMP product. However, the structure of the natural pyridinol substrate remains elusive. The structure of the complex of HcgB and the model product revealed space for an additional methyl group at the 5 position and a carboxylate group adjacent to the methyl substituent at the 6 position; both functional groups are present in the pyridinol of GP. In the complex of HcgB with the product of the model reaction, the potential binding site for the carboxylate is occupied by one water molecule. Thus, the pyridinol moiety of GP (6-carboxymethyl-2,4-dihydroxy-3,5-dimethylpyridine) might form additional interactions to its binding site, and therefore, GP might be the preferred substrate for HcgB. To substantiate this hypothesis, GP was prepared by photochemical decomposition of the FeGP cofactor extracted from [Fe]-hydrogenase (Figure 1)^[5] and cocrystallized with HcgB. The crystal structure of GP-bound HcgB (Figure 4b) strongly supports the idea that GP is a natural intermediate of the biosynthetic pathway for the synthesis of the FeGP cofactor (Figure 5). The carboxymethyl group at the 6 position of GP perfectly fits into a small pocket and forms a hydrogen bond to Ser132. Although the HcgB reaction could not be tested with the pyridinol part of GP as the substrate because of its unavailability, we speculate that the reaction efficiency is higher for natural pyridinol than for the pyridinol analogues used for the model reactions, owing to an increased number of polypeptide–substrate interactions.

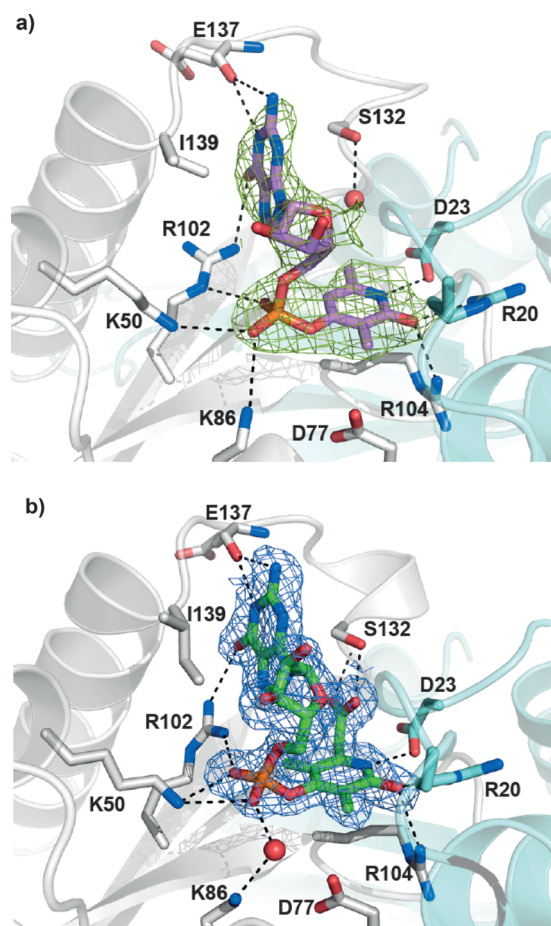


Figure 4. Crystal structures of the complex between HcgB and guanylylpyridinol. a) HcgB in a complex with 3,6-dimethyl-2,4-dihydropyridine–GMP, purified by HPLC. b) HcgB with GP prepared by irradiation with UV-A/blue light of the FeGP cofactor extracted from [Fe]-hydrogenase. Ligands and contacting residues are shown by stick models. Water molecules are represented by red spheres. The 2F_o–F_c electron densities (contoured at 1σ) of 3,6-dimethyl-2,4-dihydropyridine–GMP and GP are shown as green and blue meshes, respectively.

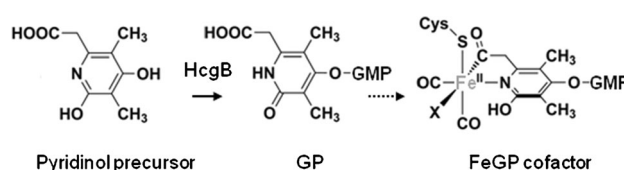


Figure 5. Proposed biosynthetic pathway of the FeGP cofactor. All substituents of the pyridinol precursor are synthesized prior to the HcgB-catalyzed GTP-dependent conjugation reaction. Then, the iron center of the FeGP cofactor is formed by other enzymes using GP, Fe²⁺, and CO as the substrates. The carboxymethyl group is presumably converted to the acyl ligand of the FeGP cofactor during iron-center formation.

The biochemical and structural data of HcgB presented herein provide valuable suggestions for the reaction sequence in FeGP cofactor biosynthesis, and complement the results of the retrosynthetic analysis.^[6] First, the 2,4-hydroxy groups are essential for the HcgB reaction and must therefore be added to the pyridine ring before this reaction occurs. Second, the

pyridinol nitrogen atom and the adjacent 2-hydroxy group are bound to the active site of HcgB by specific interactions, so that no space for an iron atom or an iron-complex exists nearby (Figure 4). This structural feature suggests that the iron center is formed after guanylylpyridinol biosynthesis is completed (Figure 5) and consequently that HcgB catalyzes the final reaction of the guanylylpyridinol biosynthesis.

Experimental Section

The model reactions were performed in MOPS/KOH (10 mM; pH 7.0) containing MgCl_2 (1 mM), GTP (0–1 mM), the substrate (1 mM; 3,6-dimethyl-2,4-dihydroxypyridine), and HcgB (1 μM) from *M. jannaschii* at 80°C. Crystals were obtained by sitting-drop vapor diffusion at room temperature. The FeGP cofactor was prepared from *M. marburgensis* [Fe]-hydrogenase.^[5a] GP was prepared by irradiation of the extracted FeGP cofactor with UV-A/blue light and purified by HPLC as reported previously.^[5] The resulting structures were established at 2.4 Å (sulfate-bound HcgB; PDB 3WB1), 2.4 Å (3,6-dimethyl-2,4-dihydroxypyridine-GMP-bound HcgB; PDB 3WB2), and 1.9 Å (GP-bound HcgB; PDB 3WB0). Data collection and refinement statistics are summarized in Table S1.

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