Two Distinct Roles for Two Functional Cobaltochelatases (CbiK) in *Desulfovibrio vulgaris* Hildenborough[†]

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ABSTRACT: The sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough possesses a large number of porphyrin-containing proteins whose biosynthesis is poorly characterized. In this work, we have studied two putative CbiK cobaltochelatases present in the genome of *D. vulgaris*. The assays revealed that both enzymes insert cobalt and iron into sirohydrochlorin, with specific activities with iron lower than that measured with cobalt. Nevertheless, the two *D. vulgaris* chelatases complement an *E. coli cysG* mutant strain showing that, *in vivo*, they are able to load iron into sirohydrochlorin. The results showed that the functional cobaltochelatases have distinct roles with one, $CbiK^C$, likely to be the enzyme associated with cytoplasmic cobalamin biosynthesis, while the other, $CbiK^P$, is periplasmic located and possibly associated with an iron transport system. Finally, the ability of *D. vulgaris* to produce vitamin B_{12} was also demonstrated in this work.

Modified tetrapyrroles such as hemes, siroheme, and cobalamin (vitamin B_{12}) are characterized by a large molecular ring structure with a centrally chelated metal ion. This family of compounds share a common pathway until the formation of the first macrocyclic intermediate, uroporphyrinogen III, at which point the pathway branches. Each branch contains a unique chelatase that performs the insertion of a specific metal ion into the modified tetrapyrrole ring (*1, 2*). In heme biosynthesis, for example, ferrous iron is inserted into the protoheme precursor protoporphyrin IX by the protoporphyrin IX ferrochelatase (*3*). In the case of vitamin B_{12} biosynthesis, cobalt is inserted into the macrocycle by a cobaltochelatase, which is either specific to the aerobic (oxygen-dependent) or anaerobic pathway for cobalamin biosynthesis (*2*). Along the aerobic pathway, precorrin-2 undergoes several enzymatic modifications that result in the formation of hydrogenobyrinic acid *a*,*c*-diamide where cobalt is inserted by the class I ATP-dependent cobaltochelatase CobN-S-T (*4, 5*). Along the anaerobic pathway, cobalt insertion occurs at an early stage, at the level of sirohydrochlorin (*6*), by the class II ATP-independent cobaltochelatases CbiX or CbiK (*1, 5, 7*). The CbiX protein was first identified as a 320 amino acid enzyme, the so-called long form ($CbiX^L$), whereas the archaeal orthologue is constituted by only around 110−145 amino acids, the short form ($CbiX^S$). The N-terminal and C-terminal domains of the long form of $CbiX^L$ share a high degree of amino acid sequence similarity. CbiKs are formed by ∼300 amino acid residues and share a low level of similarity with $CbiX^L$. Analysis of circular dichroism (CD) spectra suggested that the cobaltochelatases CbiK, $CbiX^L$, and $CbiX^S$ may have a similar overall topology (*5*). The X-ray structure determination of the CbiK from *Salmonella enterica* revealed also that the enzyme is highly similar to the protoporphyrin IX ferrochelatase from *Bacillus subtilis* (*8, 9*). More recent structural work on $CbiX^s$ has confirmed that this protein shares the same basic protein architecture as that found in CbiK (*10*).

Sulfate-reducing bacteria (SRB[1]), belonging to the genus *Desulfovibrio,* are considered to be early organisms on the evolutionary scale and contain a very large number of proteins with modified porphyrins, some of them unusual. Examples of this include the rubredoxin oxygen oxidoreductase from *D. gigas* that contains iron uroporphyrin I (*11*) and bacterioferritin of *D. desulfuricans* ATCC 27774, which has an iron-coproporphyrin III cofactor (*12*). Proteins containing cobalt−porphyrin were also reported for *D. gigas* and *D. desulfuricans* (Norway) (*13, 14*), and the nature of the cobalt−porphyrin macrocycle was identified as Co^{III}-syrohydrochlorin (*15*). Studies in *D. vulgaris* suggested that in these organisms an alternative pathway for the biosynthesis of heme may be operative since coproporphyrinogen III is

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[1] Abreviations: SRB, sulfate-reducing bacteria; LB, Luria broth medium IPTG, isopropyl-β-D-thiogalactopyranoside; ALA, 5-aminolevulinic acid; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; SAM, *S*-adenosyl-L-methionine; NAD^+, nicotinamide adenine dinucleotide; MM, minimal medium.

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formed not directly from uroporphyrinogen III but from the vitamin B_{12} precursor, precorrin-2 (*16*). However, the biosynthesis of porphyrins in *Desulfovibrio* sp. remains poorly understood.

The genome of *D. vulgaris* Hildenborough encodes two putative cobaltochelatases, DVU0650 and DVU1365, that share a significant degree of amino acid sequence identity with *S. typhimurium* CbiK. In order to clarify the role of these proteins and their involvement in modified tetrapyrrole biosynthesis in a *Desulfovibrio* sp., the genes were cloned, the proteins produced recombinantly and the isolated enzymes characterized.

EXPERIMENTAL PROCEDURES

Cloning and Expression of Recombinant Cobaltochelatases. The putative cobaltochelatases encoded by the genes DVU0650 and DVU1365 have 894 bp and 849 bp, respectively. Amplification of the two genes was achieved in PCR reactions using genomic DNA of *Desulfovibrio vulgaris* Hildenborough and appropriated oligonucleotides specifically designed for each case. A second DNA fragment of the DVU0650 gene was also amplified in order to construct a truncated protein that starts at amino acid 29 (Δ28DVU0650). The various DNA fragments were cloned into pET-28a(+) (Novagen) giving pET-28a(+)-*DVU0650*, pET-28a(+)-Δ*28DVU0650*, and pET-28a(+)-*DVU1365* allowing the encoded proteins, DVU0650, Δ28DVU0650 and DVU1365, respectively, to be produced with an 6x-His-tag in the N-terminal region. Sequencing of the PCR products guaranteed the integrity of all gene sequences. To produce DVU0650 and Δ28DVU0650 proteins, the recombinant plasmids were transformed in *Escherichia coli* BL21Gold(DE3) (Stratagene) and the cells were grown, at 30 °C, in Luria−Bertani (LB) medium containing kanamycin (30 μg/mL) until an $OD_{600} = 0.3$. At this point, 200 μM of IPTG (isopropyl-β-D-thiogalactopyranoside) was added and the medium supplemented with 50 μM 5-aminolevulinic acid (ALA) and 100 μM $FeSO_4$. The culture was then grown overnight, at 15 °C. The overexpression of DVU1365 was achieved by transforming the recombinant plasmid in the BL21Gold(DE3) and growing the cells, at 37 °C, in LB medium containing 30 μg/mL kanamycin until an OD_{600} of 0.8, followed by the addition of 200 μM of IPTG and 100 μM $FeSO_4$. The cells were then grown at 37 °C for 4 h.

Protein Purification and Characterization. Cells expressing DVU0650, Δ28DVU0650, and DVU1365 proteins were harvested, resuspended in 20 mM Tris-HCl buffer at pH 7.5 (buffer A) with 20 μg/mL DNase, and disrupted in a French Press. The soluble fraction was separated from the membranes by ultra centrifugation for 2 h at 160,000 × *g*. All purification steps were performed at 4 °C, either in aerobic or anaerobic conditions.

DVU0650 protein was purified under aerobic and anaerobic conditions. The fraction isolated from the aerobic purification was used for the biochemical and spectroscopic characterization. For the aerobic purification of DVU0650 protein, the soluble fraction from *E. coli* cells expressing DVU0650 was applied onto a Chelating Sepharose fast flow column (130 mL) (GE, Healthcare), previously charged with $NiCl_2$ and equilibrated with buffer A containing 400 mM NaCl. Eleven volumes of a linear gradient up to 250 mM imidazole were applied to the column, and the recombinant protein was eluted at 250 mM imidazole. After dialysis against buffer A, the protein fraction was loaded onto a Q-Sepharose High-Performance column (20 mL) (GE, Healthcare), previously equilibrated with buffer A. Ten volumes of a linear gradient up to 400 mM NaCl followed by four volumes of a linear gradient from 400 mM up to 1 M NaCl was applied, and the protein was eluted with ~200 mM NaCl.

DVU0650, Δ28DVU0650, and DVU1365 proteins were purified under anaerobic conditions. The anaerobic purification steps were performed in a Coy model A-2463 anaerobic chamber filled with a gas mixture of 95% argon plus 5% hydrogen. The soluble fraction from the cells expressing DVU0650, Δ28DVU0650, and DVU1365 was applied onto a HiTrap Chelating HP column (GE, Healthcare). The recombinant DVU0650 and Δ28DVU0650 proteins were eluted with 5 volumes of buffer A containing 0.5 M NaCl and 200 mM imidazole, while the DVU1365 protein was eluted with 5 volumes of buffer A plus 250 mM imidazole. The protein fractions containing DVU0650 or Δ28DVU0650 were passed through a PD10 desalting column (GE, Healthcare) in order to exchange the elution buffer to 50 mM Tris-HCl buffer at pH 8 with 100 mM NaCl, while the DVU1365 protein fraction was dialyzed overnight against 50 mM Tris-HCl buffer at pH 8 with 8% glycerol.

The purity of the proteins was analyzed by SDS−PAGE gel (*17*) and the protein concentration was determined by the bicinchoninic acid method (*18*) using protein standards from Sigma. Heme content was assayed by the hemochromopyridine method (*19*), and heme extraction was performed according to the method described by Lubben et al. (*20*). The protein molecular mass was determined by gel filtration in a Superdex 200 column according to the instructions of the manufacturer (GE, Healthcare), using commercially available standards from GE Healthcare.

Comparisons of the primary amino acid sequences were done using ClustalX (*21*), and the prediction of sorting signals was achieved using the SOSUI signal program (*22, 23*).

UV−visible spectra were recorded at room temperature in a Shimadzu UV-1700 spectrophotometer with 4.5 μM of protein, and sodium dithionite was used as a reductant. The redox titration was performed at 25 °C, under continuous agitation and argon atmosphere using 2.8 μM of purified protein and 12 μM of mediators, diluted in 50 mM Tris-HCl at pH 7.5. The redox mediators used allowed to cover the interval range between +340 mV and −225 mV: *N*,*N*-dimethyl-*p*-phenylene-diamine ($E_o' = 340$ mV), *p*-benzoquinone ($E_o' = 240$ mV), 1,2-naphtoquinone-4-sulfonic acid ($E_o' = 215$ mV), 1,2-naphtoquinone ($E_o' = 180$ mV), trimethylhydroquinone ($E_o' = 115$ mV), phenazine methosulfate ($E_o' = 80$ mV), 1,4-naptoquinone ($E_o' = 60$ mV), duraquinone ($E_o' = 5$ mV), menadione ($E_o' = 0$ mV), plumbagin ($E_o' = -40$ mV), phenazine ($E_o' = -125$ mV), 2-hydroxy-1,4-naphtoquinone ($E_o' = -152$ mV), and anthraquinone sulfonate ($E_o' = -225$ mV). A silver/silver chloride electrode, previous calibrated in a saturated quinhydrone solution was used. The titration was performed from the oxidized to the reduced state, and an experimental curve was obtained from the potential measured at 560 nm. The experimental data was analyzed using MATLAB (Mathworks, South Natick, MA) for Windows.

Concentrated fractions of DVU0650 (95 μM) were prepared for EPR spectroscopy, and the spectra were acquired at 15 K, on a Bruker ESP 308 spectrometer equipped with an Oxford Instruments continuous flow helium cryostat.

The NMR sample was prepared in D_2O, and the pH and ionic strength were set using 10 mM phosphate buffer at pH 6.9. 1D ^{1}H NMR spectra (1k scans) were acquired, at 298 K, in a Bruker Avance 500 spectrometer using a QXI probe. The residual water signal was saturated using a selective pulse of 500 ms. The sample was reduced by adding the appropriated volume of a saturated solution of sodium dithionite.

Activity Assays. The chelatase activity assays were performed with proteins purified under either aerobic/anaerobic conditions, and the activity was measured anaerobically following the formation of cobalt- sirohydrochlorin or siroheme, by the decrease of absorbance at 376 nm and using the extinction coefficient 2.4 $\times$ 10^5 M^{-1} cm^{-1} (*24*). The plasmid pETcoco-2ABCDC (*25*) harboring the genes encoding for uroporphyrinogen III methyltransferase (CobA) of *Methanosarcina barkeri*, porphobilinogen synthase (HemB) and precorrin-2 dehydrogenase (SirC) of *Methanothermobacter thermautotrophicus*, and porphobilinogen (PBG) deaminase (HemC) and uroporphyrinogen III synthase (HemD) of *Bacillus megaterium* was introduced in *E. coli* BL21(DE3)pLysS (Novagen), and the proteins were overexpressed in order to produce sirohydrochlorin. To this end, the cells were grown overnight in LB medium containing ampicillin (50 μg/mL), chloramphenicol (34 μg/mL), and 0.2% (w/v) glucose. The cells were then inoculated in LB medium with the same antibiotics described and grown at 37 °C, until an OD600 ~0.5, followed by the addition of 0.02% (w/v) of L-arabinose. After 2 h, 400 μM of IPTG was added, and the cells were grown overnight at 24 °C. After breaking the cells in a French Press, the lysate was transferred to the anaerobic chamber where 2 mL of lysate was incubated with 2 mg of SAM (*S*-adenosyl-L-methionine), 1 mg of ALA, and 1 mg of NAD^+, in a total volume of 6 mL in 50 mM Tris-HCl buffer at pH 8.0 containing 100 mM NaCl. The pH of the reaction mixture was adjusted to 8 with 2 M KOH. After overnight incubation, at room temperature, the reaction mixture was filtered (0.22 μm filter) and passed through a DEAE-resin (Sigma), and sirohydrochlorin was eluted with 50 mM Tris-HCl buffer at pH 8.0 containing 1 M NaCl. The activity was measured in triplicate using a Shimadzu UV- 1203 spectrophotometer. The assays were performed in 1 mL reaction in 50 mM Tris-HCl buffer at pH 8.0 with 100 mM NaCl, with 4.2 μM of sirohydrochlorin, 20 μM of Co^{2+} or Fe^{2+}, and with different amounts of enzyme.

Complementation of E. coli cysG Mutant Strain with D. vulgaris Cobaltochelatases. The genes from pET-28a(+)*DVU0650*, pET-28a(+)Δ*28DVU0650*, and pET-28a(+)*DVU1365* were subcloned in pETac, a plasmid under the control of the *tac* promoter. The resulting plasmids, pETac-*DVU0650*, pETac-Δ*28DVU0650*, and pETac-*DVU1365*, were transformed into *E. coli* 302Δa, a strain deleted in the *cysG* and harboring plasmid pCIQ-*sirCcobA* for expression of the *M. thermoautotrophicus sirC* and *Pseudomonas denitrificans cobA* genes. The *E. coli cysG* mutant strain containing the recombinant plasmids was selected on LB plates supplemented with 100 μg/mL ampicillin and 35 μg/mL chloramphenicol. Strain *E. coli* 302Δ*a* was also transformed with the plasmid pKK223.2-*cysG*, which expresses the *E. coli* CysG, or with an empty pETac plasmid and grown on MM plates in the absence and in the presence of cysteine, in order to provide the positive and negative controls of the experiment, respectively.

Bioassay for Detection of Vitamin B_{12} in D. vulgaris Cells. *Desulfovibrio vulgaris* Hildenborough was grown anaerobically at 37 °C for 48 h in sulfate/lactate growth medium supplemented with a trace element solution (*26*). The cells were collected and the bioassay was performed according to the technique previously described (*27*).

RESULTS

The analysis of the *D. vulgaris* Hildenborough genome revealed the presence of two gene loci, DVU0650 and DVU1365, encoding putative cobaltochelatases that share ~30% amino acid sequence identity with CbiK from *S. enterica* and *Porphyrimonas gingivalis* (*28, 29*) (Figure 1). In order to study the physiological and biochemical role of these two genes, they were amplified, cloned, and the enzymes produced, purified, and characterized.

D. vulgaris Cobaltochelatase DVU0650 Contains a Heme b. After overproduction in *E. coli* and isolation, the soluble fraction containing the purified DVU0650 exhibited an intense pink−orange color, which was shown to be due to the presence of a heme cofactor (1 mol/dimer). For convenience, DVU0650 was termed $CbiK^P$ (periplasmic form of CbiK).

As-isolated $CbiK^P$ migrated in SDS−PAGE with an apparent molecular mass of 28 kDa (Figure 2A), which could correspond to a protein in which a cleavage of 28 residues had occurred. In fact, the removal of a signal peptide was confirmed by N-terminal sequencing, which showed that the mature protein starts at residue 29. Gel exclusion chromatography of purified $CbiK^P$ suggests that the protein exists in solution as a homotetramer (data not shown).

A UV−visible spectrum of the oxidized form of the protein exhibited a Soret band at 414 nm and a broadband between 515 and 580 nm; upon reduction with sodium dithionite, the Soret band shifted to 424 nm with concomitant appearance of two defined bands at 530 nm and at 560 nm, suggesting the presence of a heme *b* prosthetic group (Figure 2B). To elucidate further the type of heme present, pyridine hemochrome analysis was performed in the total protein and in its isolated cofactor. In both cases, the redox spectrum presented a β band with an absorption maxima of 555 nm confirming the presence of a heme *b* type (*19*). To characterize the heme *b* present in DVU0650, a redox titration was performed, and a midpoint redox potential of −130 mV was determined (Figure 2C). NMR spectroscopy was used to infer the nature of the heme ligands through the analysis of the reduced form of the protein. The sample presented a spectrum typical of low-spin paramagnetic heme protein, and upon reduction, the paramagnetic signals in the high frequency region disappeared. The low frequency region of the spectrum did not show the fingerprint for a diamagnetic heme protein with a histidine-methionine coordination (*30, 31*), i.e., the signal of the methionine ε methyl group usually observed at approximately −3 ppm was absent, suggesting that the heme has a bis-histidinyl coordination (data not shown). EPR analysis of the as-isolated DVU0650 exhibited

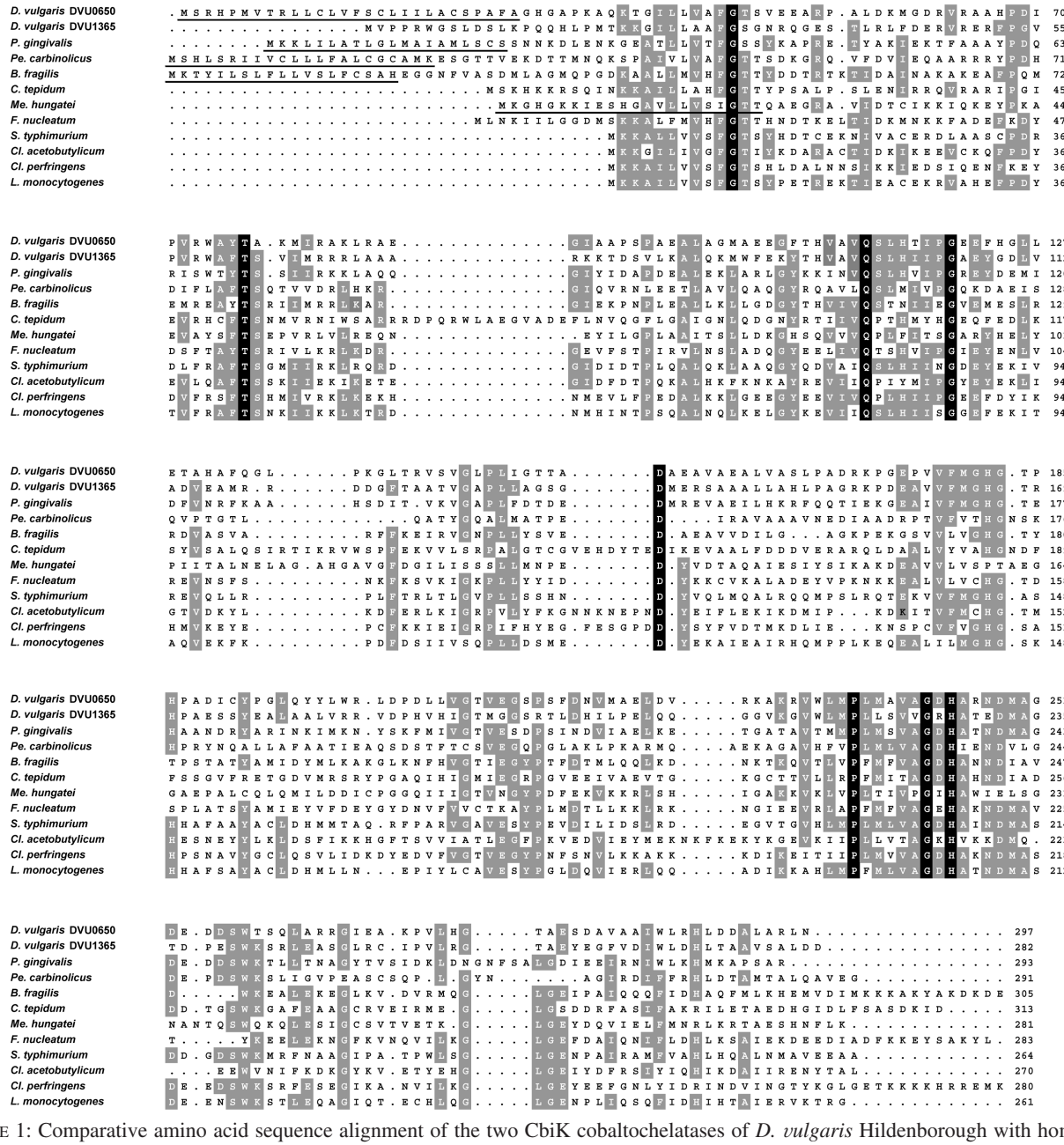

FIGURE 1: Comparative amino acid sequence alignment of the two CbiK cobaltochelatases of *D. vulgaris* Hildenborough with homologue sequences from other prokaryotes. The alignment was performed in ClustalW and viewed in GeneDoc. The strictly conserved residues are represented in black boxes, and the conserved residues are highlighted in gray boxes. Organism and NCBI accession number: *D. vulgaris* DVU0650 (YP_009872) and DVU1365 (YP_010584); *Porphyromonas* (*P.*) *gingivalis* (NP_904946); *Pelobacter* (*Pe.*) *carbinolicus* (YP_355908); *Bacteroides* (*B.*) *fragilis* (YP_099794); *Chlorobium* (*C.*) *tepidum* (NP_661293); *Methanospirillum* (*Me.*) *hungatei* (YP_501756); *Fusobacterium* (*F.*) *nucleates* (NP_604160); *Salmonella* (*S.*) *typhimurium* (NP_460970); *Clostridium* (*Cl.*) *acetobutylicum* (NP_347221); *Clostridium* (*Cl.*) *perfringens* (YP_695881); *Listeria* (*L.*) *monocytogenes* (NP_464727). The leader sequences predicted by the SOSUIsignal program are underlined.

g-values of 2.94, 2.26, and 1.53, indicative of the presence of a low spin heme group (Figure 2D).

CbiK^P (DVU0650) Is a Sirohydrochlorin Cobaltochelatase. The DVU0650 enzyme purified under aerobic conditions had activity values very similar to those obtained with the protein purified anaerobically. A truncated protein that lacks the signal peptide was also produced (see Experimental Procedures). This protein, named Δ28CbiK^P, exhibited the same molecular mass profile observed for CbiK^P on both SDS-PAGE gel and gel exclusion chromatography (data not shown). However, no heme content was determined in the Δ28CbiK^P protein. The chelatase activity could be then assessed for two forms of the enzyme, one containing the heme cofactor and another lacking the chromophore, allowing us to infer the influence of the heme in the enzymatic activity of CbiK^P.

The chelatase activity assays revealed that both CbiK^P and Δ28CbiK^P were able to insert cobalt and iron into sirohydrochlorin and that the activities were not affected by the presence of the heme since both proteins had very similar specific activities. These results also showed that the two forms of the enzymes have a slightly higher activity with cobalt than with iron (Table 1).

DVU1365 Is a Second Sirohydrochlorin Cobaltochelatase. As mentioned above, *D. vulgaris* DVU1365 encodes a second putative CbiK cobaltochelatase that shares 39% amino

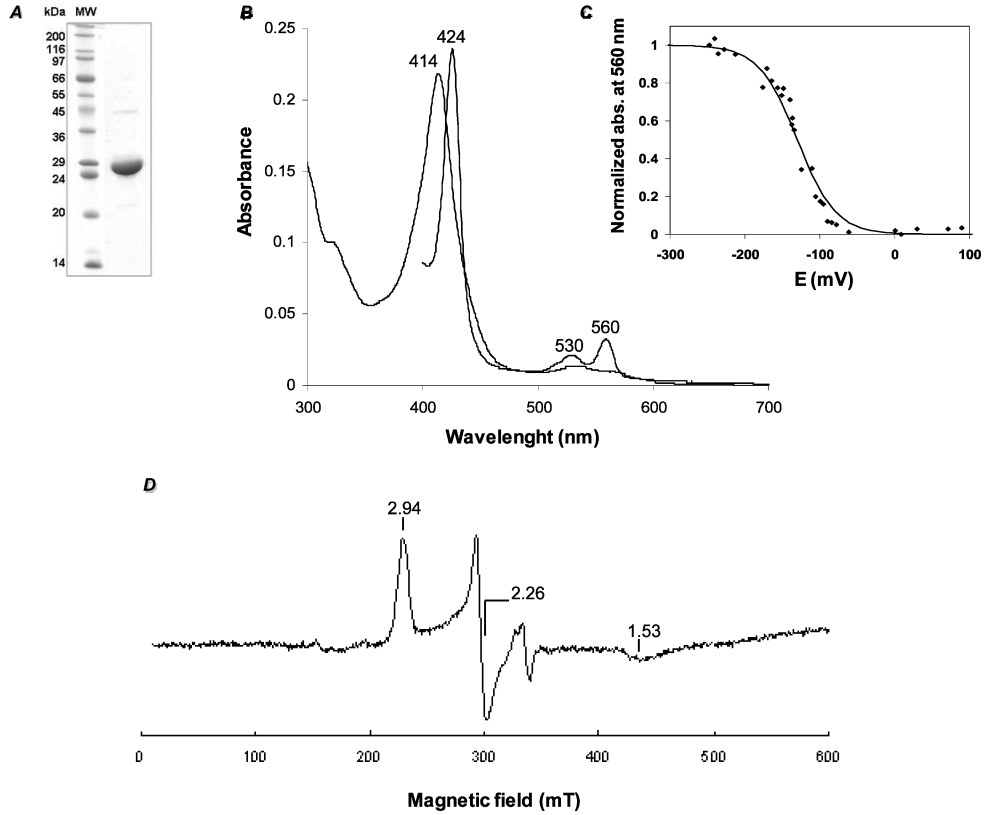

FIGURE 2: Biochemical features of the DVU0650 CbiK^P cobaltochelatase of *D. vulgaris*. Panel A: SDS−PAGE gel of the purified CbiK^P protein and molecular weight marker (MW). Panel B: UV−visible spectra of oxidized (black line) and reduced (gray line) CbiK^P enzyme indicating the presence of a heme *b*. Panel C: Redox titration of the heme *b* of *D. vulgaris* CbiK^P with a redox midpoint potential of −130 mV. Panel D: EPR spectrum of CbiK^P protein with *g* values typical for the presence of low spin heme.

Table 1: Sirohydrochlorin Cobalt and Iron Chelatase Specific Activity Measured for DVU0650, Δ28DVU0650, and DVU1365 Enzymes[a]

	activity[a] (nmol·min^{-1}·mg^{-1} of protein)	
protein	Co^{2+}	Fe^{2+}
DVU0650	22 ± 3	13 ± 3
Δ28DVU0650	25 ± 2	18 ± 2
DVU1365	4 ± 0.1	1.5 ± 0.1

[a] The activity values are presented with the standard deviation.

acid sequence identity with CbiK^P (DVU0650) (Figure 1) but lacks an N-terminal transit peptide. Consequently, this form of CbiK was termed CbiK^C (for cytosolic). The gene was cloned, and the enzyme was overproduced in *E. coli*. In contrast to CbiK^P, no cofactor was detected in the purified CbiK^C. The purified CbiK^C migrates on a denaturating gel with a molecular mass of approximately 31 kDa. The chelatase activity was assayed with both cobalt and iron in the same conditions described before. CbiK^C has an approximate order of magnitude lower activity than CbiK^P with a specific activity of 4 nmol·min^{-1}·mg^{-1} with cobalt and 1.5 nmol·min^{-1}·mg^{-1} with iron (Table 1).

Δ28CbiK^P and CbiK^c Complement an E. coli Sirohydrochlorin Ferrochelatase Mutant Strain. Previously, it has been shown that cobalatochelatases can complement a sirohydrochlorin ferrochelatase deficient strain of *E. coli*. This is due to the lack of metal ion specificity displayed by these chelatases and their ability to insert either ferrous or cobalt ions into sirohydrochlorin (*10, 29*). In order to investigate whether the *D. vulgaris* CbiK^P and CbiK^C were able to complement the sirohydrochlorin ferrochelatase deficiency of an *E. coli* strain, the genes were cloned into a pETac plasmid and introduced in *E. coli* 302Δ*a* that harbored pCIQ*sirCcobA* (i.e., a strain that is able to make sirohydrochlorin but not siroheme). To overcome the potential problem of CbiK^P being exported to the periplasm in *E. coli*, the complementation was performed with the truncated version. Both CbiK^C and Δ28CbiK^P cobaltochelatases were found to complement the mutant strain as observed by growth of the transformed strains on minimal medium in the absence of cysteine. Thus, both enzymes are able to act as sirohydrochlorin ferrochelatases in the biosynthesis of siroheme.

D. vulgaris Produces Vitamin B_{12}. D. vulgaris cells were grown on a defined medium in the absence of any exogenous cobalamin. After growth, the cells were harvested and analyzed for the level of cobalamin that was produced. By bioassay, the strain was found to produce cobalamin to a level of about 10 nmol/L of culture.

DISCUSSION

In this work, we characterized two putative CbiK cobaltochelatases present in the genome of *D. vulgaris* encoded in the loci DVU0650 and DVU1365. Production of the recombinant DVU0650 (CbiK^P) revealed that the protein was processed to a form that lacks the first 28 amino acid residues in accordance with the predicted prokaryotic leader sequence. A leader sequence in cobaltochelatases has so far only been reported for the *P. gingivalis* enzyme (*28*); however, a search in the protein database revealed that other prokaryotic CbiKs are also predicted to contain signal peptide sequences (Figure 1).

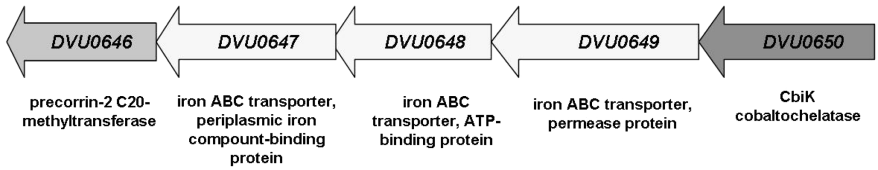

FIGURE 3: *D. vulgaris* genomic organization of the predicted operon encoding DVU0650 CbiK^P cobaltochelatase, DVU0646 CbiL precorrin-2 methyltransferase, and three genes for proteins involved in the putative iron ABC transport system (DVU0647, DVU0648, and DVU0649).

The presence of redox centers, whose role remains unclear, has been previously reported in some prokaryotic chelatases. A recent study revealed that a [4Fe-4S]$^{+2/+1}$ center exists in the CbiX^L cobaltochelatases from *Bacillus megaterium* and *Synechocystis* PCC6803 (*6*) whereas several bacterial protoporphyrin ferrochelatases contain [2Fe-2S]$^{+2/+1}$ clusters (*32, 33*). However, the presence of a heme in a chelatase enzyme is so far unique. Our study showed that in *D. vulgaris* CbiK^P cobaltochelatase the heme is not required for the fully enzyme activity since CbiK^P and Δ28CbiK^P were able to insert cobalt and iron into sirohydrochlorin and the absence of the heme did not affect the activity of metal insertion.

Insertion of iron into sirohydrochlorin, yielding siroheme, has been reported for other cobaltochelatases but in a lower ratio compared to that of cobalt insertion (*5, 29*). The *D. vulgaris* CbiK^P and CbiK^C cobaltochelatases were also found to have specific activites with iron lower than that measured with cobalt. Nevertheless, the two *D. vulgaris* chelatases were able to complement the *E. coli cysG* mutant strain showing that *in vivo*, they can both insert iron into sirohydrochlorin, thereby rescuing the cell sirohydrochlorin ferrochelatase deficient phenotype.

The presence of two putative genes encoding cobaltochelatases is also found in other genomes, including *D. desulfuricans* G20 (gene locus Dde2181 and Dde3103), *Clostridium acetobutylicum* (gene locus CAC1373 and CAC0583), *Methanospirillum hungatei* (gene locus Mhum0266 and Mhun0668), *Methanobrevibacter smithii* (gene locus Msm1280 and Msn1281) and in the Methanogenic archaeon RC-I (gene locus RCIX2006 and RCIX98). The fact that in *D. vulgaris* one of cobaltochelatases is periplasmic and the other is cytoplasmic suggests that the two chelatases may contribute to different processes.

In *P. gingivalis*, an outer membrane hemin-binding protein, termed IhtB, is proposed to be involved in iron transport and has ~38% sequence identity with the CbiK from *S. enterica (34)*. The *ihtB* gene is located in a gene cluster *ihtABCDE,* where the other genes are predicted to encode iron transporters of the ABC type (*34*). The protein was shown to be an outer membrane hemin-binding protein and it was suggested that the protein may be involved in the removal of iron from heme prior to uptake by *P. gingivalis* (*34*). The *P. gingivalis* protein was also shown to act as a functional cobaltochelatase in both *in vivo* and *in vitro* studies, although the organism does not appear to have a complete cobalamin biosynthetic pathway (*28*). Moreover, in *Dichelobacter nodosus cbiK* was shown to be under the control of the ferric uptake regulator (Fur) as was the gene for an orthologue of the periplasmic iron binding protein YfeA, itself a component of an ABC transporter system that is involved in iron uptake (*35*). It is interesting to note that the DVU0650 gene is also located in a putative operon, predicted by the Softberry FGENESB program (*36*), which contains genes for iron transport proteins (Figure 3). Cumulatively, this information provides a case for *D. vulgaris* CbiK^P being part of an iron or heme acquisition system. The presence of the protein in the periplasm means that it cannot participate in cobalamin biosynthesis as this process takes place in the cytosol. Thus, *D. vulgaris* has two distinct *cbiK* genes, which encode proteins that can both act as cobaltochelatases. The CbiK^C is likely to be the enzyme associated with cobalamin biosynthesis as this protein is produced in the cytoplasm without any transit peptide. CbiK^P is moved to the periplasm and is likely to be associated with the iron transporter system. The actual role of CbiK^P in iron transport will require further investigation.

Table 2: Genes Present in the Genome of *D. vulgaris* Hildenborough that Encode Putative Enzymes that Are Required for the Anaerobic Production of Vitamin B_{12}

gene locus number	gene name	enzyme
DVU1693	*gltX-1*	gltX-1 glutamyl-tRNA synthetase
DVU1461	*hemA*	glutamyl-tRNA reductase
DVU3168	*hemL*	glutamate-1-semialdehyde-2,1-aminomutase
DVU0856	*hemB*	delta-aminolevulinic acid dehydratase
DVU1890	*hemC*	porphobilinogen deaminase
DVU0734	*cysG-1*	uroporphyrinogen III synthase/methyltransferase
DVU1463	*cysG-1*	siroheme synthase, N-terminal domain protein
DVU0650	*cbiK*	cobalt chelatase
DVU1365	*cbiK*	cobalt chelatase
DVU0646	*cbiL/cobI*	precorrin-2 methyltransferase CobI
DVU2750	*cbiD*	cobalamin biosynthesis protein CbiD
DVU3169	*cbiG*	cobalamin biosynthesis protein CbiG
DVU3170	*cbiH/cobJ*	precorrin-3b c17-methyltransferase CobJ
DVU2748	*cbiF/cobM*	precorrin-4 C11-methyltransferase CobM
DVU2749	*cbiET/cobL*	precorrin-6y methylase cobL
DVU3087	*cbiC/cobH*	precorrin-8X methylmutase CobH
DVU3086	*cbiA/cobB-2*	cobyrinic acid *a*,*c*-diamide synthase CobB-2
DVU0405	*cbiA/cobB-1*	cobyrinic acid a,c-diamide synthase CobB-1
DVU1403	*cobA/cobO*	cob(I)alamin adenosyltransferase CobO
DVU0816	*cbiP/cobQ*	cobyric acid synthase CobQ
DVU2237	*cbiB/codCD*	cobalamin biosynthesis protein CbiB
DVU1007	*cobU/cobP*	cobinamide kinase/cobinamide phosphate guanylyltransferase CobU
DVU0914	*cobS/cobY*	cobalamin 5′-phosphate synthase/cobalamin synthase CobS
DVU3279	*cobT*	nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase CobT

Tetrapyrrole biosynthesis and modification in sulfate reducing bacteria are still poorly understood at the biochemical and molecular level. The production of guanylcobamide and hypoxanthylcobamide by *D. vulgaris* was reported a few years ago, and the addition of 5,6-dimethylbenzimidazole to *D. vulgaris* culture led to the production of cyanocobalamin (vitamin B_{12}) (*26*). Furthermore, analysis of the *D.*

vulgaris Hildenborough genome shows the presence of all the genes required for the anaerobic production of vitamin B_{12} (Table 2), with the exception of precorrin-6x reductase (*cbiJ*/*cobK*), which within the anaerobic metal-reducing δ-proteobacteria is only found in the genome of *Desulfuromonas* sp (*37*). Although the requirement of vitamin B_{12} and/or related corrinoids in *Desulfovibrio* for growth is still unclear, the ability of *D. vulgaris* to produce vitamin B_{12} was demonstrated in this work.

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