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Identification and Characterization of a Novel Vitamin B_{12} (Cobalamin) Biosynthetic Enzyme (CobZ) from *Rhodobacter capsulatus*, Containing Flavin, Heme, and Fe-S Cofactors*

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One of the most intriguing steps during cobalamin (vitamin B_{12}) biosynthesis is the ring contraction process that leads to the extrusion of one of the integral macrocyclic carbon atoms from the tetrapyrrole-derived framework. The aerobic cobalamin pathway requires the action of a monooxygenase called CobG (precorrin-3B synthase), which generates a hydroxylactone intermediate that is subsequently ring-contracted by CobJ. However, in the photosynthetic bacterium *Rhodobacter capsulatus*, which harbors an aerobic-like pathway, there is no *cobG* in the main cobalamin biosynthetic operon although it does contain an additional uncharacterized gene called *orf*663. To demonstrate the involvement of Orf663 in cobalamin synthesis, the first dedicated 10 genes of the B_{12} pathway (including *orf*663), encoding enzymes for the transformation of uroporphyrinogen III into hydrogenobyrinic acid (HBA), were sequentially cloned into a plasmid to generate an artificial operon, which, when transformed into *Escherichia coli*, endowed the host with the ability to make HBA. Deletion of *orf*663 from this operon prevented HBA synthesis, demonstrating that it was essential for corrin construction. HBA synthesis was restored to this recombinant strain either by returning *orf*663 or by substituting it with *cobG*. Recombinant overproduction of Orf663, now renamed CobZ, allowed the characterization of a novel cofactor-rich protein, housing two Fe-S centers, a flavin, and a heme group, which like B_{12} itself is a modified tetrapyrrole. A mechanism for Orf663 (CobZ) in cobalamin biosynthesis is proposed.

Vitamin B_{12} (cobalamin) is a modified tetrapyrrole and belongs to the same class of compounds as heme, chlorophyll, siroheme, and coenzyme F_{430} (1). Modified tetrapyrroles are synthesized via a branched biosynthetic pathway, with 5-aminolevulinic acid the first common intermediate and uroporphyrinogen III the first branchpoint step in the pathway (1) (Fig. 1). The structural complexity of vitamin B_{12} means that its biosynthesis remains one of most enigmatic and exigent metabolic pathways in nature, requiring around 30 enzymes for the complete *de novo* construction of the coenzyme form (2). For cobalamin biosynthesis two distinct yet similar routes exist, known as the oxygen-dependent (aerobic) and oxygen-independent (anaerobic) pathways (3). These pathways diverge at precorrin-2 and merge again at adenosylcobyric acid. The major differences between these pathways include the timing of cobalt insertion, the requirement for molecular oxygen, and the nature of the extruded carbon fragment, which is lost during the ring contraction process (4). These biochemical differences are also reflected at the genetic level, where the presence of certain genes offers a diagnosis of the type of pathway in operation. Thus pathways that operate the aerobic route to B_{12} always contain the genes *cobN*, *-S*, and *-T*, which encode the subunits of a cobaltochelatase that can insert cobalt into a ring-contracted tetrapyrrole-derived macrocycle (5), and *cobG*, which encodes a monooxygenase required for synthesizing the hydroxylated γ-lactone derivative of precorrin-3 (6).

CobG[1] (precorrin-3B synthase) was identified initially from sequencing of the cobalamin biosynthetic operons in *Pseudomonas denitrificans* (7). It encodes a protein with a molecular mass of 46 kDa and displays some sequence similarity to sulfite reductase, including a motif for a Fe-S center (7). The role of CobG in cobalamin synthesis was demonstrated when it was shown that it converted precorrin-3A into a compound with an extra 16 mass units (precorrin-3B), corresponding to the inclusion of an oxygen atom (8). The structure of precorrin-3B was determined from multiple ^{13}C labeling studies coupled with infrared spectroscopy, revealing that the product of the reaction catalyzed by CobG contained a γ-lactone attached to ring A and a hydroxy group at C-20 (6, 8). Further oxygen labeling experiments proved conclusively that the latter was derived from molecular oxygen and that CobG was therefore a monooxygenase (6). Despite its discovery more than a decade ago, comparatively little is known about the mechanism of CobG.

However, in *Rhodobacter capsulatus* and a number of α-proteobacteria, genome sequencing projects have revealed the

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[1] The abbreviations used are: CobG, precorrin-3B synthase; CobZ, gene product of *orf*663 and an enzyme isofunctional protein to CobG, but containing flavin and heme group as well as Fe-S centers; HBA, hydrogenobyrinic acid.

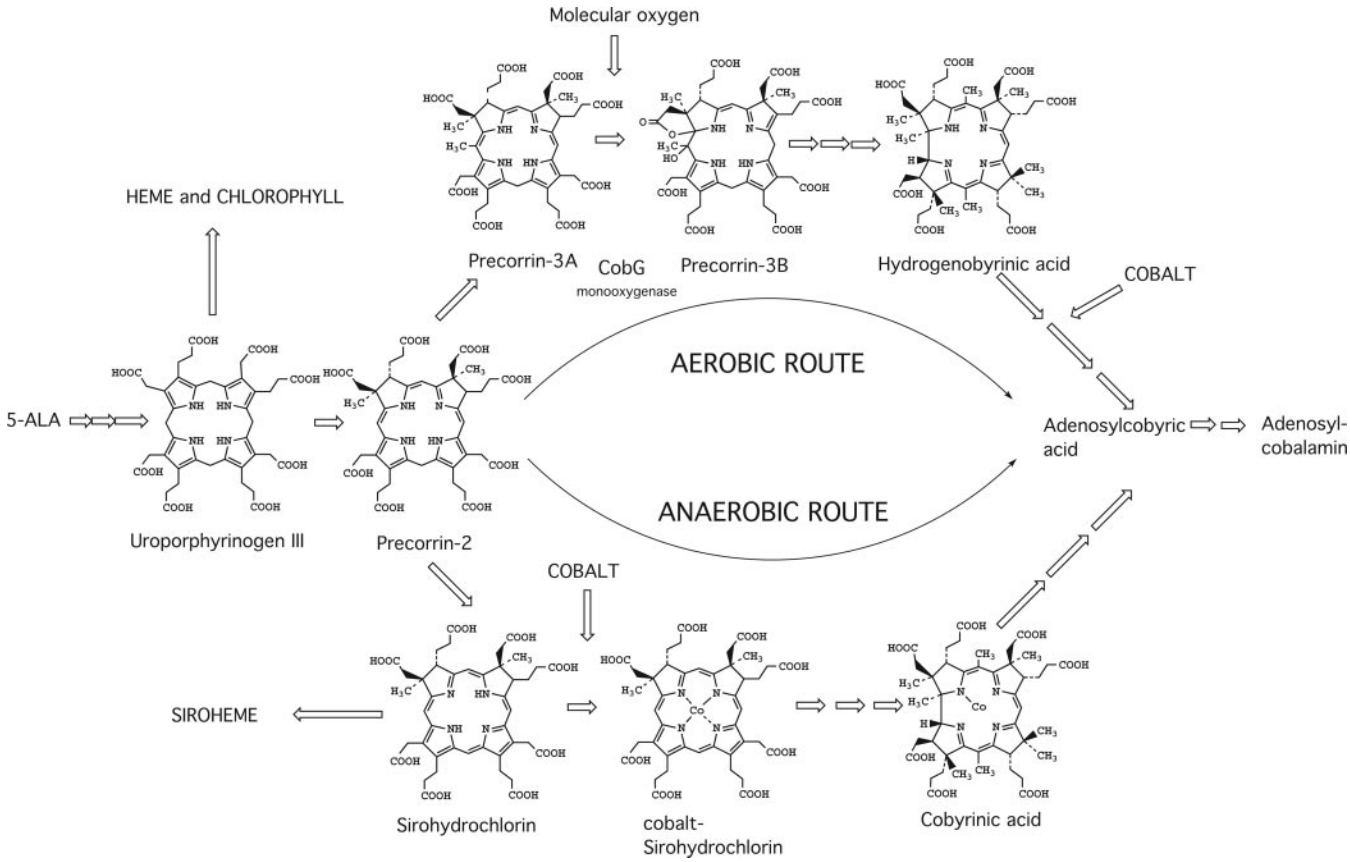

FIG. 1. **Tetrapyrrole biosynthesis outline, highlighting the aerobic and anaerobic routes for the construction of adenosylcobalamin.** The aerobic route proceeds via cobalt-free intermediates and requires molecular oxygen, whereas cobalt is inserted at a much earlier stage during the anaerobic pathway.

presence of cobalamin pathways that appear aerobic in character, but are missing an orthologue of *cobG*. In this project we sought to try and overproduce the enzymes found in the main *R. capsulatus* cobalamin operon (9) in *Escherichia coli* to genetically engineer a strain with a capacity to synthesize hydrogenobyrinic acid (HBA) *in vivo* and thereby identify the protein required for initiating the ring contraction process.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents

Most chemicals were purchased from Sigma. Other materials were provided by the following suppliers: restriction enzymes and modification enzymes were from Promega, Chilworth, Southampton, United Kingdom; pKK223.3, chelating-Sepharose fast flow resin and gel filtration columns were from Amersham Biosciences, Little Chalfont, Bucks, UK; pET14b was from Novagen, Madison, WI; tryptone and yeast extract were from Oxoid, Basingstoke, UK; sodium dithionite was from Roche, Poole, UK; and primers were from Invitrogen, Paisley, UK.

Engineering E. coli for the Production of HBA

For the production of HBA, *E. coli* strain BL21(DE3) was transformed with the two compatible plasmids pKK223–3:R*ccob*HIKLEMJAF*orf*663 and pBAD33:Pd*ecob*AI. Plasmid pKK223–3:R*ccob*HIKLEMJAF*orf*663 was constructed by inserting the appropriate amplified *R. capsulatus* genes into pKK223-3 (ColE1 ori, *lac*PO, Amp^R) using the "Link and Lock" method, which allows the consecutive cloning of genes with the reuse of the same restriction enzyme sites (see Fig. 2). The first gene to be cloned is amplified with primers containing an EcoRI at the 5′ and SpeI and BamHI sites at the 3′ end. The remaining genes are amplified with primers containing an XbaI at the 5′ end and SpeI and BamHI sites on the 3′ end. After cloning the first gene into the plasmid via the EcoRI and BamHI sites, the remaining genes, cut with XbaI and BamHI restriction enzymes, were cloned consecutively into the plasmid after it had been restricted with SpeI and BamHI. The SpeI- and XbaI-restricted fragments form compatible cohesive ends, which after ligation do not reform a restriction site. Thus by fusing the SpeI and XbaI sites, these sites can be reused in subsequent cloning steps (so long as the sites do not occur naturally within the gene).

Plasmid pBAD33:*cob*AI was constructed by cutting a DNA fragment bearing the two genes from pUC18:*cob*AI (10) with KpnI and PstI and ligating it into pBAD33 (11) (p15A ori, pBAD, Chl^R). An *orf*663 minus strain was constructed in a similar manner except that the large plasmid did not contain *orf*663. The *orf*663 minus strain was also transformed with a third compatible plasmid, pZS*24 (pSC101 ori, Plac/ara1, Kan^R) (12), bearing either *orf*663 or the *P. denitrificans cobG* gene.

Cell Growth and Isolation of HBA

E. coli strains were grown with aeration at 37 °C in LB medium containing 0.2% glucose plus the appropriate antibiotics (50 mg/liter ampicillin, 30 mg/liter chloramphenicol, 50 mg/liter kanamycin) to an A_{600} = 1 and the cells collected by centrifugation. The combined cell pellets from 4 liters of Luria-Bertani (LB) broth were resuspended in 1 liter of minimal medium consisting of M9 salts containing the same antibiotics plus the following additives: 0.2% glycerol, 0.2% L-(+)-arabinose, 0.01% yeast extract, 2.0 mM $MgCl_2$, 0.1 mM $CaCl_2$, 20 mg of 5-aminolevulinic acid, and 0.2 mM isopropyl β-D-thiogalactopyranoside. The cells in minimal medium were incubated with aeration at 37 °C for 24–48 h. The products found in both the culture medium and in the heated (80 °C for 15 min) cell lysate were isolated by binding them to DEAE-Sephadex. HBA was esterified and purified by TLC as previously described (13). Alternatively, HBA was purified in its free acid form and quantified by high performance liquid chromatography on a BDS Hypersil C-18 column (4.6 × 250 mm; Thermo Electron Corporation) run on a Agilent 1100 series LC/MSD Trap equipped with a diode array detector and eluted at a flow rate of 1 ml min^{-1} with a gradient of acetonitrile in 1 M ammonium acetate.

Protein Purification

E. coli cells transformed with the plasmid expressing the *R. capsulatus* Orf663 were grown overnight on LB medium, and were harvested and concentrated by centrifugation for 15 min at 5000 × *g* in a refrigerated Sorvall RC-5 centrifuge. The pellet was washed with 10 volumes of a 100 mM Tris-HCl buffer, pH 7.5, 2 M NaCl and then concentrated by

a second centrifugation run at 5000 × *g* for 15 min in a 100 mM Tris-HCl, pH 7.5, buffer, supplemented with a mixture of protease inhibitors (Sigma).

The cell pellet was broken by four passages through a French press operating at 12,000 psi, under an argon atmosphere, to obtain a homogenous cell lysate. The unbroken material was removed by centrifugation for 10 min at 5,000 × *g*. The pellet was kept. The supernatant was subjected to a further centrifugation at 88,000 × *g* for 1 h in a Sorvall Combi-plus ultracentrifuge. The supernatant was yellow in color and contained the soluble flavin-binding fragment of the Orf663. The flavin-binding fragment of the protein was further purified and concentrated by affinity chromatography on a His-bind column.

The pellet, dark-red in color, contained the full-length form of Orf663. This was salt-washed in 100 mM Tris-HCl, pH 7.5, 2 M NaCl buffer, and then centrifuged at 88,000 × *g* for 1 h. The supernatant was discarded and the pellet was resuspended and homogenized in a 100 mM Hepes-NaOH buffer, pH 7.5. The membrane fraction was then solubilized by the mild detergent *n*-dodecyl β-D-maltoside for 30 min with continuous gentle stirring. The final detergent concentration was 1% (w/v) and the protein concentration was adjusted to 4 mg/ml after measuring the absorption at 280 nm.

The material that had not been solubilized by the detergent treatment was removed by centrifugation for 1 h at 88,000 × *g*. The full-length form of Orf663 was then purified from the cleared supernatant by affinity chromatography on a His-bind column. The protein was eluted in a buffer containing 200 mM imidazole, 0.003% *n*-dodecyl β-D-maltoside. The protein was concentrated and the excess imidazole removed by dialysis. The samples for spectroscopic analysis were prepared after concentration.

Spectroscopic Measurements

Electron Paramagnetic Resonance—The continuous wave EPR spectra were recorded on a Bruker Alexis E580 spectrometer operating at X-band (9.7 MHz, 300 millitesla) equipped with an Oxford Helium flow cryostat. The precise experimental conditions are given in the figure legends. The temperature is controlled by an Oxford ITC unit. The samples for EPR measurements were either frozen in liquid nitrogen immediately after the preparation (oxidized), or incubated for 30 min in the presence of the reductant sodium dithionite (11.5 mM) under anaerobic conditions, and stored in liquid nitrogen.

UV-Visible Spectroscopy—The optical spectra were either recorded on a Hitachi (Model U-3310) spectrophotometer equipped with an attachment for the measurement of highly scattering solutions. The samples were kept under a gentle argon stream to maintain the anaerobic conditions.

Redox Potentiometry

Redox titrations were performed in a Belle Technology glove box under a nitrogen atmosphere, essentially as described previously (14). All solutions were degassed under vacuum with argon. Oxygen levels were maintained at less than 2 ppm. The protein solution (~5 μM) in 8 ml of 100 mM potassium phosphate, pH 7.0, containing 10% (v/v) glycerol, was titrated electrochemically according to the method of Dutton (15) using sodium dithionite as reductant and potassium ferricyanide as oxidant. Absorption *versus* potential data at appropriate wavelengths (reflecting maximal changes in spectral properties between oxidized and reduced enzyme) were fitted to the Nernst equation to derive the apparent midpoint reduction potential (*E*) for the 1 electron reduction of the bound heme iron.

RESULTS

After growing *R. capsulatus* under both chemoheterotrophic (aerobic) and phototrophic (anaerobic) conditions we observed that cobalamin accumulated to similar levels within the cells under both sets of conditions (11 pmol per A_{600} unit), indicating that this organism can make cobalamin even in the presence of low concentrations of molecular oxygen. This result coupled to the observation that *R. capsulatus* does not contain an orthologue of *cobG* (9), utilized by other bacteria operating an aerobic cobalamin biosynthetic pathway (16), suggests that it may employ an alternative system to help promote ring contraction. Significantly, within the main cobalamin operon there is a candidate gene (*orf*663) that may encode such a surrogate.

To determine whether Orf663 is involved in corrin synthesis, 10 *R. capsulatus* genes thought to be required for HBA synthesis from uroporphyrinogen III were introduced into pKK233.3 using a newly developed multicloning procedure that we have termed Link and Lock (Fig. 2) because genes can be linked together and locked in position with the use of a minimal number of restriction enzymes. The enzymes required to convert 5-aminolevulinic acid to uroporphyrinogen III are provided by the host, and the large plasmid was transformed into an *E. coli* strain harboring another plasmid bearing the *cobA* and *cobI* genes necessary to convert uroporphyrinogen III to precorrin-3A to provide sufficient amounts of this intermediate and to facilitate high flux through the pathway (10). The resulting strain was found to produce comparatively large quantities of HBA, the first intermediate on the pathway to cobalamin that is stable and can be easily isolated (17). HBA was isolated both as a free acid and in its esterified form and its structure confirmed on the basis of its UV-visible, mass, and NMR spectra (Fig. 3).

The branch of enteric taxa to which *E. coli* belongs is thought to have lost the ability to make cobalamin *de novo* some time ago, although some members of this group such as *Salmonella* sp. would appear to have reacquired the ability through horizontal transfer of the anaerobic *cob* operon (18). Indeed, it has also been shown that *E. coli* can make B_{12} *de novo* after transformation of the bacterium with a plasmid containing this *cob* operon (19). However, endowing *E. coli* with the capability to synthesize HBA is much more of a challenge, because HBA is a compound that *E. coli* has never made before. Moreover, HBA is made under aerobic conditions unlike the anaerobic conditions required for the genetically engineered *E. coli* to make cobalamin (19).

Having an *E. coli* strain with the ability to make HBA also allowed us to address the question of whether the *R. capsulatus* Orf663 was required for HBA synthesis. Deleting *orf*663 resulted in a strain that no longer accumulated HBA. Significantly, HBA synthesis could be restored either by reintroduction of *orf*663 or by addition of the *P. denitrificans cobG* gene. Thus, *cobG* and *orf*663 encode enzymes that are isofunctional. On the basis that the *R. capuslatus* Orf663 is a cobalamin biosynthetic enzyme and that it is quite distinct from the *P. denitrificans* CobG (see below), we propose that Orf663 should be called CobZ.

The protein encoded by *cobZ* (*orf*663) is therefore able to catalyze a reaction similar to that enhanced by CobG, *i.e.* it has the ability to generate a hydroxylactone derivative of precorrin-3A (Fig. 1) (6). Thus CobG and CobZ are alternative precorrin-3B synthases. However, CobZ (Orf663) must mediate this reaction in a manner very different from that employed by CobG, because the two proteins display no primary sequence similarity. Moreover, CobZ (866 amino acids) is considerably larger than CobG (459 amino acids).

A detailed bioinformatics analysis reveals that CobZ is composed of two distinct moieties, an N-terminal flavin-binding region that displays substantial similarity to succinate dehydrogenase/fumarate reductase and a C-terminal region that is an integral membrane protein with similarity to proteins of unknown function (originally called CitB but now called TcuB-see below (20)). At the junction between these two domains is a cysteine-rich region that has the same consensus motif for a 2[4Fe-4S] cluster as found in a number of complex redox proteins, such as heterodisulfide reductase.

In some organisms, CobZ displays similarity to two separate proteins that are found as part of an operon recently identified as being involved in the metabolism of tricarballylate (20), a compound that is catabolized by some mircroorganisms as a source of carbon and energy (21). The tricarballylate utilization operon (*tcu*) contains three genes, *tcuA*, *-B*, and *-C*, where *tcuA*

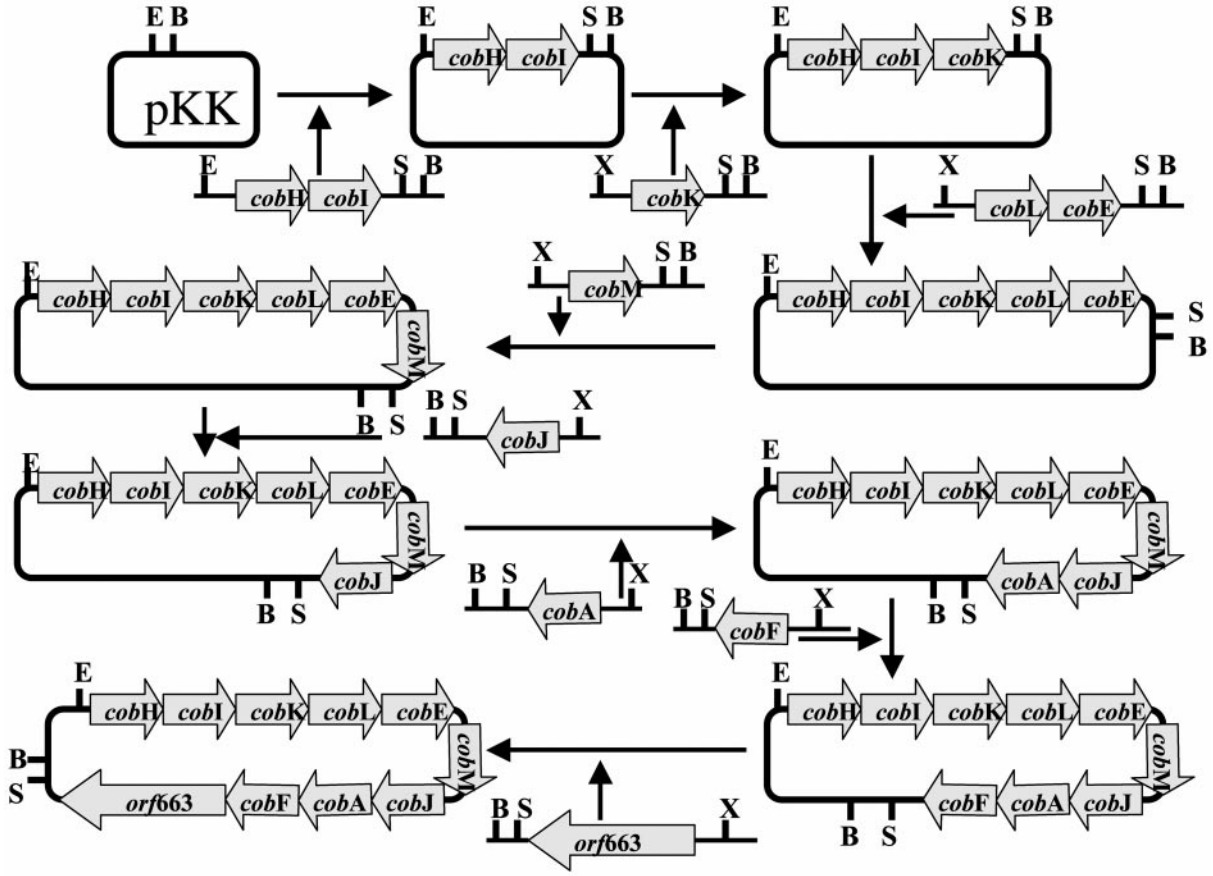

FIG. 2. **Plasmid construction using Link and Lock.** The first gene was cloned into pKK322 using EcoRI (*E*) and BamHI (*B*) sites. Subsequent genes were amplified using primers containing an XbaI (*X*) site at the 5′ end and SpeI (*S*) and BamHI sites at the 3′ end. The compatibility of SpeI and XbaI sites, which when ligated together do not reform a restriction site, means that these sites can be reused in successive rounds of cloning.

and *tcuB* align with the N and C termini of CobZ (20). Thus, TcuA is predicted to contain a flavin, whereas TcuB is predicted to be an integral membrane protein with several Fe-S centers. A detailed molecular genetics study of this operon provides convincing evidence that TcuA and -B are involved in the metabolism of tricarballylate, possibly via its oxidation to *cis*-aconitate (20). In this respect, TcuA and TcuB have been suggested to function in the dehydrogenation of tricarballylate.

To progress from an *in silico* predictive model of CobZ to experimental *in vitro* reality, *cobZ* was cloned to allow overproduction of CobZ as a recombinant protein in *E. coli* with an N-terminal His tag. Cells overproducing CobZ appeared to accumulate two proteins, one of the expected mass of 90 kDa and another of 50 kDa. Subsequent analysis revealed that the 50-kDa protein was a proteolytic fragment resulting from the cleavage of the 90-kDa protein product. This proteolysis is consistent with CobZ consisting of two distinct moieties, where the junction between them represents a region that is susceptible to cleavage.

The N-terminal proteolytic fragment was purified and shown to house a flavin molecule (Fig. 4*A*). Although the yellow coloration and UV-visible spectrum of this region of CobZ are typical of a flavin cofactor, it is not feasible to identify the particular type of flavin bound by this information alone. However, FMN has a 10-fold higher fluorescence at 600 nm than FAD thus allowing the flavin nucleotide to be differentiated by fluorescence spectroscopy. Moreover, FAD can be broken down into its FMN and AMP moieties, which therefore results in a 10-fold increase in the fluorescence (22). A purified sample of the CobZ N-terminal peptide was treated with trifluoroacetic acid and, once the precipitated protein had been removed by centrifugation, half was neutralized and its fluorescence spectrum measured at 600 nm. The other half of the sample was left to hydrolyze any FAD into its FMN and AMP components over a 4-h period, neutralized, and its fluorescence also measured. A comparison of the spectral readings revealed that the fluorescence had increased 10-fold. This result demonstrates that the flavin component of CobZ is FAD and also indicates that it is unlikely to be covalently bound to the protein.

The comparatively large quantities of the 50-kDa fragment obtained during the purification procedure allowed an initial investigation into the role played by the flavin cofactor. The purified 50-kDa protein was concentrated to ~2 mg/ml in buffer containing 200 mM imidazole and was titrated at 4 °C against an increasing concentration of sodium dithionite under anaerobic conditions. The titration against dithionite revealed that the flavin was reduced directly to the 2 electron reduced hydroquinone form, which can be deduced from the bleaching of the flavin chromophore with no evidence of any semiquinone formation during the reduction (Fig. 4*B*). A neutral or blue semiquinone would have been observed by the appearance of a band at 580–620 nm, whereas the anionic red semiquinone would have been identified by strong absorption features in the region around 380–400 nm. Moreover, the flavin can be returned to its fully oxidized state by addition of oxygen (data not shown) without any noticeable accumulation of semiquinone signal. This result is consistent with the flavin being involved in a 2-electron reduction process rather than two single electron steps, although we cannot rule out the possibility that semiquinone form(s) are transiently populated at this stage.

The truncated flavin module of the protein was also incubated with NADH, to see if this could act as a reductant. Many flavoproteins are reduced by NADH or NADPH as part of their

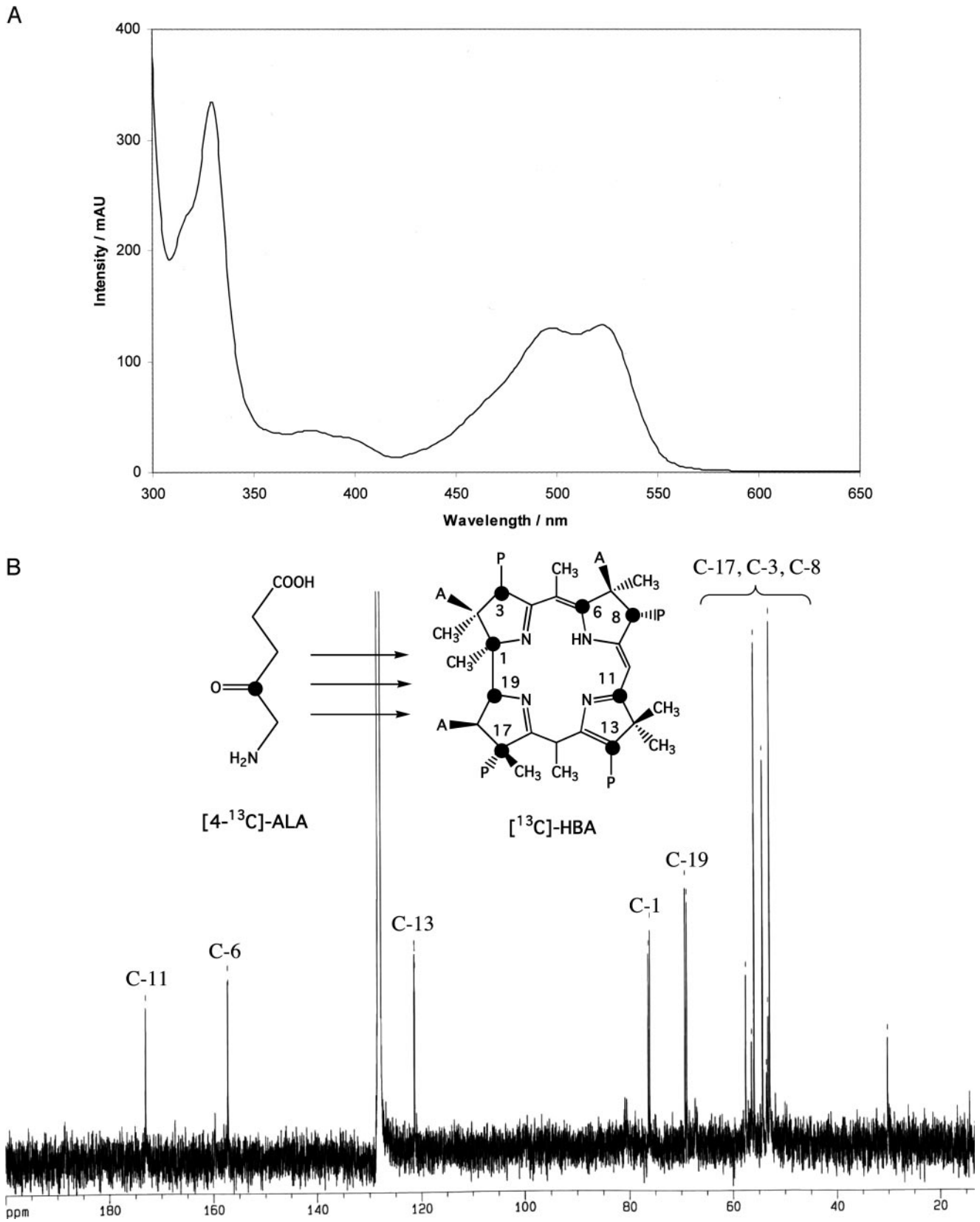

FIG. 3. **Characterization of HBA obtained from recombinant *E. coli*.** *A*, the UV-visible spectrum of isolated HBA. Mass spectral analysis confirmed the expected for HBA of 880.4 Da. *B*, NMR of HBA after synthesis from [4-^{13}C]5-aminolevulinic acid. The HBA was isolated as its heptamethylester.

catalytic cycle (*e.g.* cytochrome P450 reductase (23)). However, incubation of the N-terminal truncated version of CobZ with NADH did not lead to any noticeable reduction of the flavin as monitored by UV-visible spectroscopy. The N-terminal region of CobZ would therefore not appear to interact directly with NADH (or else be oxidized much faster than it is reduced by NADH), so it probably operates another method for the reduction of the flavin.

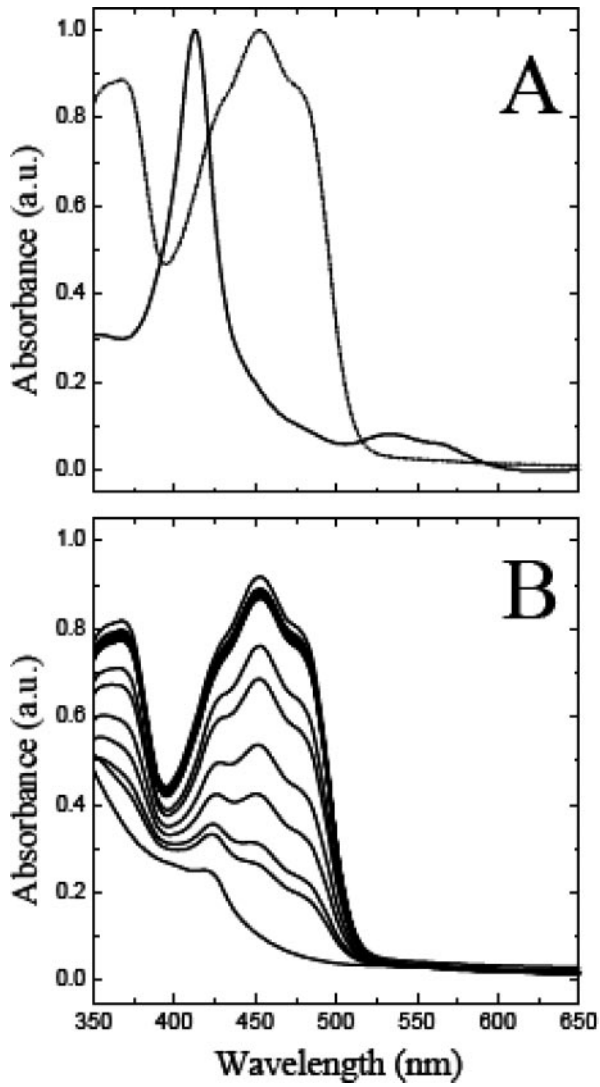

FIG. 4. **Characterization of CobZ.** *A*, UV-visible spectra of the N-terminal region of the protein, in comparison to the holoprotein. The spectrum of the N-terminal region of the protein has characteristics of a flavin (apparent absorption maxima at ~375 and 455 nm, with a pronounced shoulder on the longer wavelength band), whereas the holoprotein spectrum is dominated by the Soret band of a heme group with an absorption maximum at ~414 nm. *B*, equilibrium reductive titration of the flavin domain of CobZ (~80 μM) with dithionite. The UV-visible spectral changes during the reduction of the CobZ flavin with dithionite are shown at different points in the titration. The bleaching of the chromophore is observed with no obvious development of long wavelength absorption characteristics of neutral blue semiquinone, or of shorter wavelength features expected for the anionic semiquinone form.

The insolubility of CobZ is likely because of the fact that the C-terminal region is an integral membrane protein, as hydropathy plots predict that CobZ has at least six hydrophobic regions, all located within the C-terminal region. This would also be consistent with observations that the N terminus is cleaved, yielding a soluble 50-kDa fragment, whereas the rest (C-terminal) remains in the membrane. To test the hypothesis that CobZ may be a membrane protein, an extraction with detergent was undertaken. The extraction of CobZ was achieved with *n*-dodecyl β-D-maltoside and the protein was passed through a Ni^{2+}-affinity column to improve purity, because the intact protein was overproduced as an N-terminal His-tagged fusion. About 1–2 mg of purified CobZ could be obtained per liter of bacterial culture, which had been induced for a short 3-h period, to minimize degradation.

The purified CobZ was analyzed by UV-visible spectroscopy (Fig. 4*A*). The color of the protein appeared more orange/red than the bright yellow of the purified 50-kDa N-terminal region. The difference in color was presumed to be because of the Fe-S clusters. However, the UV-visible spectrum of CobZ revealed a very strong absorbance around 414 nm, consistent with the presence of an oxidized heme group. Reduction of the protein with dithionite resulted in a long wavelength shift of the Soret band from 414 to 425 nm, together with a considerable increase in the intensity of the α band absorption at 560 nm (Fig. 5). The EPR spectrum of the oxidized form of CobZ also shows the presence of a cytochrome in the low spin state (data not shown). The measured g values of $g_z = 2.94$, $g_y = 2.25$, and $g_x = 1.50$ are consistent with a low spin heme and suggest a *bis*-His coordination (24). However, we cannot rule out the possibility that the low spin state may be an artifact of the purification as the protein was purified in buffer containing imidazole. Nonetheless, extensive dialysis and gel filtration of the protein did not significantly alter the spectral characteristics of the heme, suggesting that the imidazole does not remain associated as a ligand. Significantly, the data demonstrate beyond all doubt that CobZ contains a heme group and that CobZ is therefore a novel flavin/heme-containing protein. Moreover, the heme group must be contained within the C terminus of the protein. The lack of any C*XX*CH motifs within this region, together with the absorption characteristic, suggests that it is not a *c*-type cytochrome and is more likely to harbor a *b*-type heme.

To examine further the properties of the redox centers in CobZ, an anaerobic potentiometric titration was performed, as we have done previously (*e.g.* Refs. 14 and 23). CobZ was degassed and transferred from its storage buffer into standard redox titration buffer (0.1 M potassium phosphate, pH 7.0, containing 10% (v/v) glycerol) by gel filtration within an anaerobic glove box. A spectral scan of the oxidized enzyme showed features typical of a bound heme cofactor, with a Soret band at 414 nm. Smaller absorption features in the region between 450 and 500 nm suggested some contributions from flavin and/or Fe-S centers (see below). It is possibly the case that some flavin is lost from the enzyme on purification and storage in the presence of mild detergent, and with buffer transfer for the redox titration, and that Fe-S cluster(s) are damaged by detergent treatment (see below). The potentiometric titration progressed smoothly with a clear shift in the heme Soret absorption peak to longer wavelength (from 414 to 425 nm) as the iron is reduced. Similarly, there is development of absorption of the α-band of the heme at 560 nm in the ferrous state (Fig. 5). An isosbestic point for the Fe^{3+}/Fe^{2+} Soret transition is at 419 nm. Data at the reduced Soret maximum (425 nm) were fitted to the Nernst function to produce a midpoint reduction potential of $E = -123 \pm 3$ mV for the Fe^{3+}/Fe^{2+} transition. Data fitting at longer wavelength is more complex because of likely large overlapping contributions from the different bound cofactors. However, it is notable that similar analysis of the data at 475 nm (where significant contributions from flavin and Fe-S centers are expected) suggests a more negative potential (about −145 mV). This estimate is undoubtedly a composite value from the contributions of the heme and the other cofactors (some of which may not be bound stoichiometrically). However, it does indicate that one or both of the other cofactors have a rather more negative reduction potential than the heme iron. Work is ongoing to resolve the potentials of the other centers in CobZ.

The EPR spectrum of the reduced form of CobZ produced evidence for the presence of two [4Fe-4S] centers. The complex EPR spectrum of the dithionite-reduced CobZ is presented in

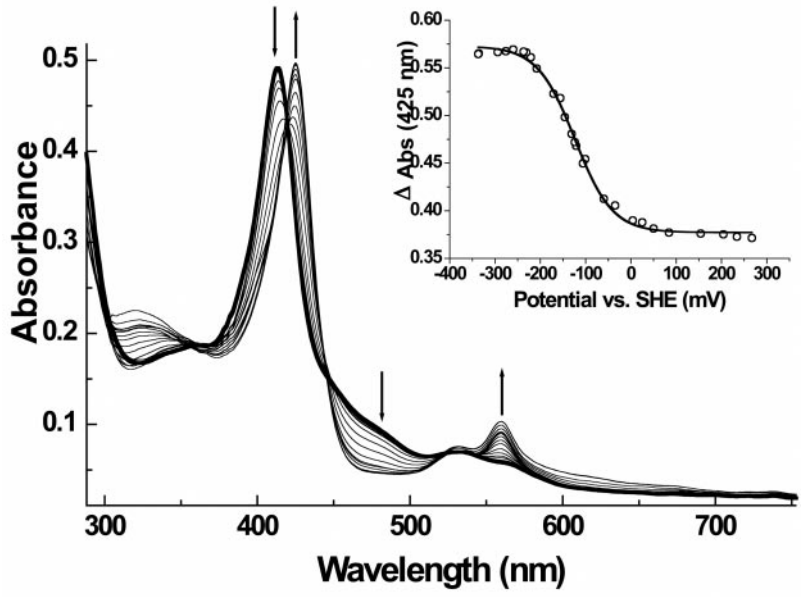

FIG. 5. **Potentiometric titration of the CobZ protein (~5 μM).** The spectrum is dominated by the contribution from the heme chromophore. In the oxidized form (*thick*, *solid line*) the Soret maximum is at 414 nm, shifting to ~425 nm in the ferrous form. Selected intermediate spectra in the redox titration are shown. An isosbestic point for the heme iron Fe^{3+}/Fe^{2+} transition is located close to 419 nm, and a strong feature in the Q-band region develops at 560 nm in the Fe^{2+} form. *Arrows* indicate direction of absorption change at various wavelengths during the reductive phase of the titration. The *inset* shows a fit of absorption *versus* potential data at 425 nm (at the Fe^{2+} Soret peak) to the Nernst function, producing a midpoint reduction potential of -123 ± 3 mV for the Fe^{3+}/Fe^{2+} transition of the heme iron.

Fig. 6, as detected at 15 K. The features at g_z and g_x shows a clear band splitting with peaks at $g_{z1} = 2.12$, $g_{z2} = 2.08$, and $g_{x1} = 1.93$, $g_{x2} = 1.91$ and $g = 1.89$. The EPR signals are completely suppressed at 70 K, an indication of fast magnetic relaxation, typical of metal centers, and the measured value of g are in the range reported for [4Fe-4S] clusters. Given the marked temperature dependence, the observed EPR spectrum can be safely assigned to a [4Fe-4S] cluster (25, 26). The fact that the transitions assignable to the g_z and g_x are each split into two closely lying peaks indicates the presence of two, interacting, Fe-S clusters (27). A similar behavior has been previously observed for proteins binding more than one [4Fe-4S] cluster, such as some ferredoxins, and the iron-sulfur center F_A and F_B bound to the PsaC subunit of Photosystem I (28, 29).

The presence of the split [4Fe-4S] EPR spectrum was detectable in all the reduced preparations of the purified CobZ. On the other hand in some, but not all of them, a rather large EPR signal could be detected in the oxidized form of the protein (data not shown). The main positive feature is located at $g = 2.023$, and two shoulders can be detected at $g = 1.99$ and $g = 1.97$. Again, this signal became undetectable at temperatures higher then 35 K, an indication of fast magnetic relaxation, typical of metal centers. The spectroscopic characteristics of this signal are consistent with that of a [3Fe-4S] center (26, 30). Because this signal was not detected in all the independent purifications of CobZ, it is attributed to damage of the naturally occurring iron-sulfur in the protein, induced by the detergent extraction.

DISCUSSION

In this paper we have demonstrated through a synthetic molecular genetics approach that CobZ is involved in the biosynthesis of vitamin B_{12} and that it is isofunctional with CobG. Moreover, CobZ has been shown to contain a flavin in the form of a non-covalently bound FAD, two Fe-S centers, and a *b*-type heme. So, how does this cofactor-rich CobZ mediate the synthesis of precorrin-3B, bearing in mind that the reaction has to be similar to that catalyzed by the monooxygenase CobG (6)? Within CobZ we have a duality of choices for oxygen-binding functional groups; both heme and flavin can act as monooxygenase hydroxylating agents. The UV-visible and EPR spectra of the heme group of the intact CobZ show that the heme is in a low spin state, although this result has to be interpreted with the caveat that the protein was purified in the presence of imidazole. However, it is unlikely that imidazole remains bound to CobZ following dialysis/gel filtration. To bind oxygen, the heme group would need to be in a high spin state (31). Thus, by a process of elimination, the flavin must be the oxygen activation site. Because the flavin group of CobZ may more likely undergo a 2-electron oxidation-reduction rather than two single electron steps (on basis of apparent failure to stabilize a semiquinone at equilibrium), a mechanism of precorrin-3A hydroxylation as outlined in Fig. 7 can be predicted. In this scenario, reduced flavin reacts with oxygen to form a 4a-hydroperoxide intermediate, which then reacts with precorrin-3A resulting in hydroxylation at C-20. In this model, the Fe-S and heme group would act to reduce the oxidized flavin back to a state where it can again react with oxygen, possibly by drawing on reducing equivalents from the quinol pool in the membrane. This process is likely to involve electron transfer via the membrane-bound heme group. The presence of such a dedicated electron transport chain to the flavin ensures that the flavin is reduced rapidly after removal of the 4a-hydroxy group. This high electron flux through the system ensures maximum reactivity with the available oxygen. Work is now in progress to provide experimental evidence for this proposed mechanism.

The role of CobZ in cobalamin synthesis is quite distinct from the role suggested for its *Salmonella enterica* homologues TcuA and -B, which have been proposed to be involved in the oxidation of tricarballylate to *cis*-aconitate, although it is interesting to note that the authors also invoke a membrane-linked electron transport system (20). In their case, they have hypothesized that the reduced flavin resulting from the oxidation of tricarballylate passes its reducing equivalents to TcuB via the Fe-S centers and then onto the cellular electron transport chain (20). In this respect, TcuA and TcuB are undertaking a reaction that is significantly different to the monooxygenase function that has been described for CobZ in this paper. However, it is known that such diverse reactions can occur in protein families, especially where cofactor chemistry is being modulated (32).

Finally, the presence of heme within a cobalamin biosynthetic enzyme extends a web of intrigue concerning tetrapyrrole inter-relationships within *R. capsulatus*. It is known that cobalamin is required for bacteriochlorophyll synthesis (33). As *R. capsulatus* contains a *metH*, cobalamin is required for methionine and, thus, *S*-adenosylmethionine synthesis (34), which in turn, is needed for heme synthesis (35). Now, it is apparent that heme is required for cobalamin biosynthesis.

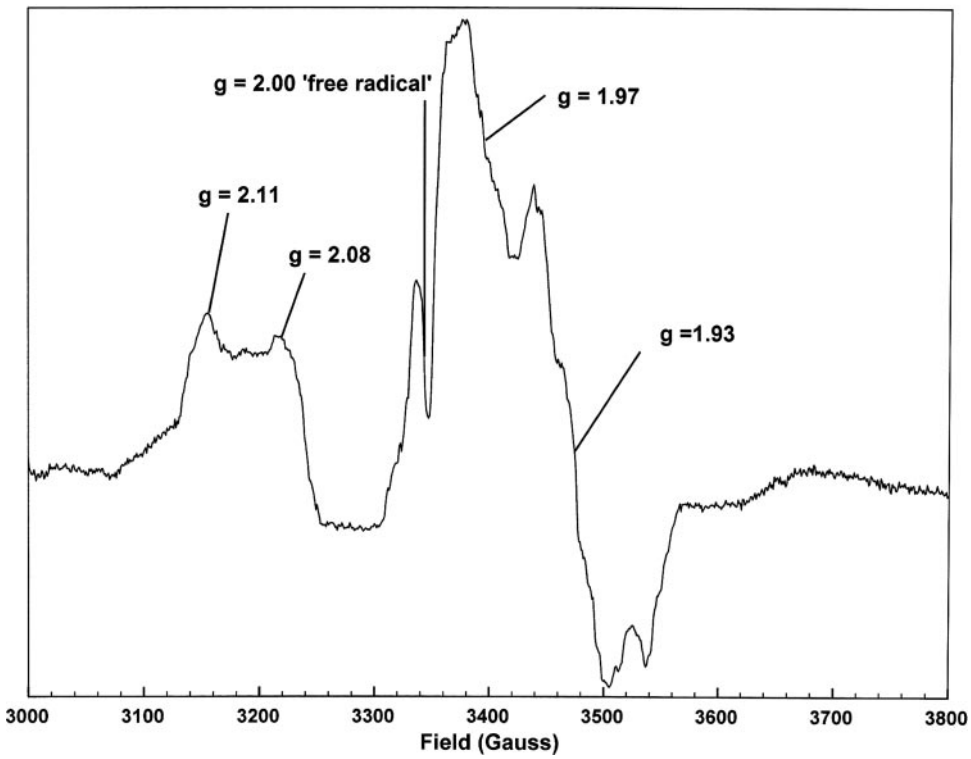

FIG. 6. **EPR spectrum of the reduced 4Fe-4S clusters, recorded at X-band and 15 K employing a microwave power of 2.5 milliwatts, modulation frequency of 100 kHz, and a modulation amplitude of 6 G.** The free radical signal indicated at $g = 2.00$ accounts for less than 1% of the total number of unpaired electrons.

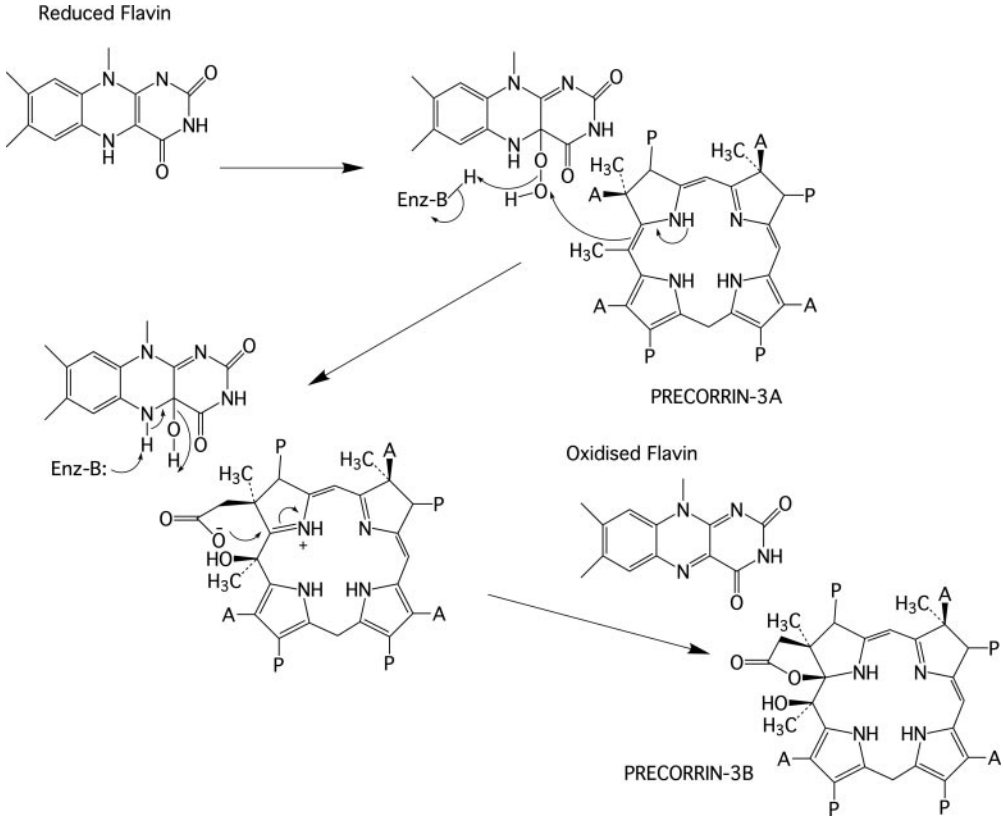

FIG. 7. **Proposed function of CobZ in the ring contraction process.** The reduced flavin reacts with molecular oxygen to generate a peroxide intermediate, which then combines with the precorrin substrate to spawn the precorrin-hydroxy lactone product and oxidized flavin. The oxidized flavin is subsequently reduced, probably via electrons channeled via the heme group and Fe-S centers.

Presumably, there is tight co-ordination of modified tetrapyrrole biosynthesis within this organism, but this unique interdependence may represent an early signaling mechanism that is still apparent in higher plants where intermediates in chlorophyll synthesis are involved in plastid to nucleus communication (36).

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