Bacillus megaterium Has Both a Functional BluB Protein Required for DMB Synthesis and a Related Flavoprotein That Forms a Stable Radical Species

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Abstract

Despite the extensive study of the biosynthesis of the complex molecule B12 (cobalamin), the mechanism by which the lower ligand 5,6-dimethylbenzimidazole (DMB) is formed has remained something of a mystery. However, recent work has identified and characterized a DMB-synthase (BluB) responsible for the oxygen-dependent, single enzyme conversion of FMN to DMB. In this work, we have identified BluB homologs from the aerobic purple, nonsulfur, photosynthetic bacterium Rhodobacter capsulatus and the aerobic soil bacterium Bacillus megaterium and have demonstrated DMB synthesis by the use of a novel complementation assay in which a B12 deficient strain, substituted with the precursor cobinamide is recovered either by the addition of DMB or by the recombinant expression of a bluB gene. The DMB-synthetic activity of the purified recombinant BluB enzymes was further confirmed in vitro by providing the enzyme with FMNH2 and oxygen and observing the formation of DMB by HPLC. The formation of a 4a-peroxyflavin intermediate, the first step in the oxygen dependent mechanism of DMB biosynthesis, is reported here and is the first intermediate in the enzyme catalysed reaction to be demonstrated experimentally to date. The identification and characterization of an FMN-binding protein found on the cobi operon of B. megaterium, CbiY, is also detailed, revealing an FMN-containing enzyme which is able to stabilize a blue flavin semiquinone upon reduction with a 1-electron donor.

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Introduction

5,6-Dimethylbenzimidazole (DMB), a derivative of benzimidazole, forms the alpha-axial ligand of cobalamin and is the critical distinguishing factor between cobalamin and pseudocobalamin (in which an N7-linked adenine forms the lower ligand) [1,2]. There has historically been an interest in differentiating between the biosynthesis of cobalamin and pseudocobalamin since, as the name suggests, the latter cannot be used by humans, whilst the former is an important dietary cofactor [3].

Earlier research by Renz [4] had shown that at least two pathways for DMB synthesis exist. The first of these is an aerobic pathway that sees the transformation of FMN into the base. Here it has long been known that flavin mononucleotide (FMN) is a substrate in the de novo biosynthesis of DMB in B12-producing micro-organisms [5] and that the C2 carbon of DMB is derived from the C1' carbon of the flavin [6]. It has only relatively recently been shown that a single protein, BluB, is responsible for the complete conversion of reduced FMN into DMB [1,2]. An alternative anaerobic route also exists where several building blocks including erythrose, formate, glutamine, glycine, and methionine are required for the synthesis of the base [4].

In the aerobic biosynthesis the formation of DMB from flavin is a complex process involving the contraction of the isoalloxazine ring system and cleavage of the ribityl tail [6,7]. This transformation, for which no enzymological precedent exists, sees the destruction of the flavin and production of DMB along with the predicted by-products urea and erythrose-4-phosphate [6,8] and gives rise to the suggestion that BluB could be categorized as a ‘flavin destructase’.

Intriguingly, the enzyme displays a quite unusual requirement for reduced FMN as a substrate but also requires oxygen for the formation of DMB to proceed [1]. The BluB from Sinorhizobium meliloti was the first to be characterized and the crystal structure solved [1]. The crystal structure revealed that the active site of BluB is relatively small and sandwiched between the two units of a dimer. This diminutive active site is thought to contribute to the selectivity of the enzyme for molecular oxygen [1,2]. Despite the requirement for a reduced flavin substrate, the enzyme is not itself a flavin reductase, but rather it seems likely that the flavin is reduced independently elsewhere, before delivery to BluB [1].
Cobalamin biosynthesis is mediated by one of two pathways, either by a late cobalt insertion (aerobic) pathway or by an early cobalt insertion (anaerobic) pathway. The differences between the pathways are in the timing of the insertion of the cobalt and mechanism of ring contraction [9]. The BluB proteins studied to date have been derived from organisms utilising the late cobalt insertion pathway [1,2,10]. In this study we have chosen to isolate DMB-synthases from bacterial species utilising each of these pathways. Thus, the organisms we have selected, *B. megaterium* and *R. capulatus*, represent the early and late cobalt insertion pathways respectively [11,12]. By identifying the BluB orthologs from these established examples of the two pathways of B12 biosynthesis we have been able to demonstrate that BluB is not specific to the late cobalt insertion pathway. Rather, we have been able to isolate BluB from each pathway and shown that both are single-enzyme DMB-synthases, and that both require oxygen to catalyze the conversion of FMN into DMB.

### Results

**Identification of a BluB Homologue in *Bacillus Megaterium***

The amino acid sequences of three BluB proteins from organisms harbouring the oxygen-dependent pathway towards cobalamin, *Rhodobacter capulatus* [15], *Rhodospirillum rubrum* [2] and *Sinorhizobium meliloti* [1] were used as search templates to mine the genome of *B. megaterium* (strain DSM319). Two potential gene candidates, **BMD_2595** and **BMD_0969** were identified. The first, **BMD_2595**, also known as *cbiY*, is located at the end of the *cobI* operon [11] and displays a sequence identity of between 18 to 20% to the other BluB enzymes. **BMD_0969**, which shall be referred to as BluB(Bm) hereafter, is not located in proximity to any genes known to be involved in cobalamin biosynthesis and displays a higher sequence identity of between 29 to 40% to the other bluB templates (Table S1). In *R. capulatus* the bluB gene is located in a large operon that codes for the majority of genes responsible for the biosynthesis of cobalamin [14]. To investigate the activity of these two proteins, the *bluB* and *cbiY* genes together with the *bluB*, gene of *R. capsulatus* were individually amplified by PCR and cloned into pET14b for recombinant overproduction of the encoded products as N-terminal His-tag fusions in *E. coli*.

**Complementation of BluB Activity in *Salmonella Enterica***

*S. enterica* is able to facilitate the coenzyme B12-dependent utilization of ethanolamine in the presence of vitamin B12 under aerobic conditions, thus enabling its growth on ethanolamine enriched media if vitamin B12 is available. However it can make vitamin B12 from exogenous cobanamide only if DMB is also provided [15,16].

Cobanamide is a coenzyme B12 precursor that lacks the lower nicotine loop (Nomenclature, I.-I. C. o. B., 1974). *S. enterica* strain JE9385 carrying the empty cloning vector pBAD24 is unable to grow on ethanolamine in the presence of vitamin B12 under aerobic conditions, thus enabling its growth on ethanolamine both when uninhibited and when induced by the addition of arabinose (Figure 1A & B, closed squares). Transformation of JE9385 with the *B. megaterium* *bluB* gene construct permitted growth (Figure 1B, closed triangles), although in the absence of arabinose this growth was slightly delayed (Figure 1A, closed triangles). The *B. megaterium* *cbiY* gene did not allow growth of JE9385 on ethanolamine at any tested arabinose concentration (Figure 1A & B, black diamonds).

Further confirmation that the *B. megaterium* BluB encodes a DMB synthase was achieved in a second bioassay. Here a *S. enterica* metE cobC double mutant was employed as a reporter for cobalamin as under highly aerobic conditions, this mutant strain cannot grow in glucose minimal medium with 15 nM cobinamide (Figure 1C & D, open circles and open triangles). In contrast replacement of cobinamide with vitamin B12 resulted in rapid growth (Figure 1C, black squares), while the *B. megaterium* BluB gene was unable to rescue any growth (Figure 1C, black triangles). Addition of 1 mM arabinose allowed growth of the strain containing the *B. megaterium* *bluB* (Figure 1D, black triangles), but had no significant effect on the growth of the strain containing *bluB* from *R. rubrum* (Figure 1D, black squares). The *B. megaterium* *cbiY* gene did not allow growth of a *metE cobC* mutant at any tested arabinose concentration (Figure 1C & D, black diamonds).

**Purification of Recombinant BluB and CbiY Enzymes***

The *B. megaterium* BluB and CbiY proteins and the *R. capsulatus* BluB were overproduced in *E. coli* with N-terminal His-tags and purified by metal affinity chromatography under aerobic conditions. SDS-PAGE analysis showed all three fusion proteins migrated as single bands with molecular masses between 25 to 27.3 kDa for BluB(Rc), 27.3 kDa for BluB(Bm), and 25.7 kDa for CbiY(Bm) (Figure 2). While the purified fractions of both BluB proteins (at concentrations of up to 904 μM) were colourless, fractions containing CbiY(Bm) (100 μM) were found to be bright yellow.

**In vitro Dimethylbenzimidazole (DMB) Synthase Activity***

DMB synthase activity was also investigated using an in vitro HPLC based assay. The purified proteins were buffer exchanged (PD-10 column) and incubated with 100 μM FMN and 8 mM NADH. Protein concentrations ranged from 1 to 500 μM. NADH is required to reduce FMN to FMNH2 which is the substrate for the BluB reaction. The reaction was incubated in the presence of oxygen [2,19] for 18 hours. Following this, the reaction mixture containing CbiY(Bm) remained bright yellow and was comparable to the control that contained no enzyme. However, incubations containing BluB(Bm) and BluB(Rc) were pale yellow in colour. The products of the reaction were analyzed by HPLC by comparison to authentic standards of DMB and FMN. DMB formation was observed in reaction mixtures containing either BluB(Rc) or BluB(Bm), whereas no DMB could be detected in incubations containing CbiY(Bm) (Figure 3). Quantification of the amount of DMB formed during the reaction revealed that the *R. capsulatus* enzyme was slightly more active than the *B. megaterium* BluB. No DMB could be detected if the reaction mix was incubated in the absence of oxygen. These findings indicate that the BluB enzyme from *B. megaterium*, which is known to use the early cobalt insertion (anaerobic) pathway for adenosylcobalamin synthesis, has a requirement for oxygen to complete the biosynthesis of cobalamin.
BluB Forms a Peroxyflavin Intermediate

BluB catalyzes the O2-dependent conversion of FMNH2 to DMB. The first step in the proposed mechanism (Figure 4) is the activation of molecular oxygen forming the peroxyflavin intermediate. To confirm the presence of such an intermediate in the BluB catalyzed reaction, FMNH2 (formed from the chemical reduction of FMN with sodium dithionite), which is the substrate for BluB [1,2], was incubated with different ratios of purified BluB from R. capsulatus or B. megaterium in an anaerobic chamber. Peroxyflavin formation was initiated by rapidly mixing the protein solution containing FMNH2 with buffer containing oxygen in a stopped-flow apparatus and the reaction was monitored using a multi-wavelength diode array detector. For this purpose, oxygenated buffer (equilibrated in atmospheric air) was sealed in a syringe and taken into the anaerobic chamber. In the case of the BluB(Rc) enzyme, rapid formation of a peroxyflavin intermediate, with an absorption maximum at 380 nm, was observed (Figure 4). The absorbance peak at 320 nm can be attributed to sodium dithionite (used to reduce the flavin prior to beginning the stopped flow experiment). This peak is also present in the control spectra (Figure S1), and as such cannot be attributed to the formation of a stable intermediate. It is possible to calculate the theoretical spectrum of the peroxyflavin by subtracting the control spectrum from the spectrum observed experimentally (Figure S2). The initial peroxyflavin formation step was complete within 20 ms of mixing and was followed by a much slower decay of the optical signal (in the order of seconds) which seems to occur in a biphasic manner, this is possibly due to there being both productive and non-productive decay of the peroxyflavin (Figure 4). The apparent rate of peroxyflavin formation and decay was unchanged over a range of enzyme concentrations (Figure S3). Diminishing oxygen concentration, accomplished by re-equilibration of the oxygen containing buffer in the anaerobic chamber, had little effect on the rate of formation. However, under these conditions no peroxyflavin could be detected with B. megatarium BluB.

Characterisation of CbiY(Bm)

Following the results of the in vitro and microbiological DMB synthase assays, the function of CbiY remained to be determined. In order to investigate the role it may play within the biosynthesis of cobalamin it was decided to characterize the protein further. Unlike the BluB enzymes from R. capsulatus and B. megaterium, CbiY is not required for DMB synthesis.
fractions containing purified CbiY were bright yellow in colour, indicating that the protein binds a flavin cofactor. The UV-visible spectrum of the protein confirmed the presence of a flavin with absorbance maxima at 362 and 450 nm and shoulders at 420 and 472 nm. To distinguish between FMN and FAD, the cofactor was removed from the protein by heat denaturation and subjected to analysis by HPLC. The retention time of the extracted flavin was consistent with the bound cofactor being FMN (data not shown).

Flavoproteins are typically involved in electron transfer processes, and often interact with NAD(P)(H) coenzymes. On addition of either NADH or NADPH to CbiY under anaerobic conditions, the yellow colour of the CbiY FMN cofactor was bleached, consistent with the 2e⁻ reduction of the flavin to its hydroquinone state. When the protein was titrated with a one electron reducing agent (sodium dithionite) the reduction of the flavin proceeded through a semiquinone intermediate, as observed by the development of a broad absorption band centred at ~600 nm which is characteristic of a neutral (blue) semiquinone. The midpoint reduction potentials for the FMN cofactor housed within CbiY were determined by subjecting the protein to a spectroelectrochemical potentiometric titration using sodium dithionite as the reductant and potassium ferricyanide as the oxidant. The protein remained stable for the duration of the titration and no hysteresis was observed between titrations in oxidative and reductive directions.

Absorption versus potential data were fitted at different wavelengths in order to obtain robust estimates for the midpoint potentials of the CbiY FMN ox/sq (E₁) and sq/hq (E₂) transitions, as well as for the overall 2-electron reduction of the FMN (ox/hq, E₁₂). The CbiY FMN was essentially completely converted from fully oxidized to completely hydroquinone in the range from 0 to −200 mV versus the normal hydrogen electrode (NHE). Figure 5A shows spectra from the redox titration, demonstrating the progressive decrease in absorption at the major flavin band during reduction of the flavin, and on the increase and subsequent decrease in the absorption feature centred at ~600 nm reporting on the formation and decay of a neutral (blue) semiquinone. Data
Characterisation of BluB

A

![Graph showing absorbance vs wavelength and absorbance at 380 nm vs time.]

B

**FMN**

[Chemical structure of FMN]

**Reduction**

[Chemical structure showing reduction process]

**O₂**

[Chemical structure showing reaction with oxygen]

**C4a-peroxylavin**

[Chemical structure of C4a-peroxylavin]

**DMB**

[Chemical structure of DMB]
fitting at 610 nm (near the peak of the blue semiquinone) to a 2-electron Nernst function provided data consistent with those from analysis at the isosbestic points, with $E_1 = -95 \pm 4$ mV and $E_2 = -153 \pm 8$ mV (Figure 5B inset). Data fitting using the same function at a wavelength near the oxidized flavin maximum (443 nm) also provided midpoint potential values for the $E_1 (-91 \pm 4$ mV) and $E_2 (-142 \pm 10$ mV) couples that were consistent with the other data sets (Figure 5B).

A much smaller peak at $\sim 385$ nm shows a similar pattern of formation and decay, and possibly reflects a small proportion of anionic (red) semiquinone. Clear isosbestic points are observed at 487 nm and 430 nm for the ox/sq and sq/hq couples, respec-
tively. Data fitting at these wavelengths provides values for $E_2$ (sq/ hq) = $-149.5 \pm 2$ mV, and for $E_1$ (ox/sq) = $-92.5 \pm 5$ mV, respectively (Figures 5C and 5D).

The formation of a neutral semiquinone radical was further confirmed by EPR and ENDOR studies of the one electron reduced protein at X- and W-band. For EPR samples, ChbY (between 1 and 10 mM in 100 mM phosphate buffer pH 7.0 containing 10% glycerol) was anaerobically reduced with sodium dithionite until the proportion of semiquinone reached its maximum. The protein was then transferred into quartz EPR tubes and frozen in liquid nitrogen. The X-band cw-EPR spectrum was recorded at 100 K and exhibited a line-width of 19 G and a $g_{an}$ of 2.0036 which is consistent with the formation of a neutral flavin semiquinone radical [20]. The same sample at W-band gave rise to the axial EPR spectrum of Figure 6A. The apparent $g$ values marked on the figure would give a gap of 2.0036, however, it is known from studies at yet higher fields that flavin semiquinones display rhombic $g$ anisotropies which can only be determined by spectrum simulation [21]. As neither EPR spectrum of the protein bound flavin radicals displays resolved hyperfine structure, due to the anisotropies and multiplicities of the hyperfine interactions [20,21], the Davies pulsed ENDOR spectrum [22] of the radical was acquired at W-band. The spectrum, Figure 6B, shows several prominent features which arise through the coupling of protons covalently attached to the 7,8-dimethyl isalloxazine ring and are consistent with previous studies of neutral flavosemiquinone radicals [23]. The most intense feature is the matrix-ENDOR signal in the middle of the spectrum which extends from around 140.4 to 144.4 MHz and is centred on the free proton Larmor frequency. This signal arises from an overlap of multiple resonances with only small hyperfine couplings that originate from protons surrounding the flavin in the binding pocket, either protein protons or water protons along with some weakly coupled protons from the isalloxazine ring [23]. The remaining features can be assigned as indicated in Figure 6B, based on comparison with other known neutral flavin semiquinone radicals [23]. The H6$\beta$ protons constitute the methyl group attached at C(8) and these give rise to an axial hyperfine coupling with $A_{6} = 8.3$ MHz and $A_{11} = 9.7$ MHz. The H6 hyperfine coupling, normally assigned as being indicative of the isotropic hyperfine coupling [23], is 5.7 MHz.

Discussion

BluB proteins from *Rhodobacter capsulatus* and *Bacillus megaterium* have been identified.

BluB proteins from both *R. capsulatus* and *B. megaterium* were identified by comparison to the *S. meliloti* BluB. Surprisingly the results of this exercise highlighted two possible candidates for a BluB orthologue in *B. megaterium*. Both genes were expressed in trans in a strain of *S. enterica* that required cobinamide and DMB for growth on ethanolamine. When provided with cobinamide, expression of BluB$_{Bm}$ was shown to be sufficient to rescue the growth of the strain. ChbY$_{Bm}$, however, proved unable to rescue DMB-dependent growth. These results show that, BluB$_{Bm}$ is able to mediate the synthesis of DMB. No activity was found associated with ChbY$_{Bm}$.

Using an in vitro HPLC assay it was also shown that isolated recombinant BluB$_{Bm}$ and BluB$_{Re}$, both synthesise DMB from a reduced flavin precursor in the presence of oxygen, further demonstrating that, like the previously identified BluB proteins from *R. rubrum* [2] and *S. meliloti* [1,10], these proteins are single-enzyme DMB synthases. These findings support the concept that a single gene product is responsible for catalysing the complex biosynthesis of DMB from FMNH$_2$.

The isolation of functional BluB proteins from *R. capsulatus* and *B. megaterium* demonstrate that this enzyme is not specific to the late cobalt insertion pathway and that the single-enzyme conversion of a FMN to DMB is apparently common to organisms of both B$_{12}$ synthetic pathways. As the processes require molecular O$_{2}$, the anaerobic pathways employing BluB must operate in organisms that are at least microaerophilic.

The Biosynthesis of DMB Proceeds via a 4a-peroxyflavin Intermediate

The formation of DMB is a chemically complex process involving the breakage of three bonds as well as the formation of a fourth, all of which are catalysed by a single ‘flavin destructase’ enzyme. The complexity of the process has led to speculation as to the possible mechanism of the BluB catalysed DMB biosynthesis [1,24,25]. All the mechanisms proposed so far share a common first stage intermediate, a peroxyflavin, thought to be formed by the interaction of molecular oxygen with the reduced flavin. The mechanism of 4a-peroxyflavin formation is strongly supported by crystallographic data demonstrating the presence of an apparent ‘peroxyanion hole’ formed by the O2– hydroxyl group of the ribityl tail of the flavin and the backbone amide of an active site residue in BluB from *S. meliloti*. These H-bonds could serve to not only position the molecular oxygen for attack at the C4a position of the flavin but also to stabilize the peroxyflavin intermediate [1]. Using stopped-flow spectroscopy we have, for the first time, demonstrated experimentally that BluB$_{Re}$ catalysis proceeds via a 4a-peroxyflavin intermediate. When reduced flavin and BluB$_{Re}$ are combined with molecular oxygen a characteristic, relatively short-lived peak, is observed at 300 nm.

The 4a-peroxyflavin is generated rapidly and is followed by a much slower decay phase (in the order of seconds). Peroxyflavin was not observed in reactions undertaken in the absence of oxygen, demonstrating that the formation of peroxyflavin requires molecular oxygen. The lifetime of the 4a-peroxyflavin is relatively brief in comparison to the overall rate of DMB formation, suggesting that the formation of a peroxyflavin intermediate is not the rate limiting step in the biosynthesis of DMB. A control experiment indicated that there is no evidence for the formation of a peroxyflavin in the absence of the enzyme (Figure S1).

The formation of a peroxyflavin intermediate was not observed under similar conditions in stopped flow experiments with BluB from *B. megaterium*. It is possible that either the formation of the 4a-peroxyflavin intermediate in the case of *B. megaterium* DMB synthesis occurs over a much longer time scale than is observed with the *R. capsulatus* homolog (and thus may not accumulate if the rate of reoxidation of the flavin is relatively fast) or conversely that the formation and degradation of the 4a-peroxyflavin occurs much more rapidly in *B. megaterium*, thereby prohibiting the detection of the UV-visible signal as it occurs within the dead time of the stopped-flow instrument. Considering that the overall rate of formation of DMB for BluB$_{Bm}$ was found to be slower than that observed for BluB$_{Re}$, the former explanation is the more likely.

CbiY from *Bacillus Megaterium* is not a BluB Protein

Interestingly, when searching for the BluB-encoding gene in the genome of *B. megaterium*, in addition to BluB$_{Bm}$, another gene with high sequence similarity to known BluB proteins was also identified (Table S1). This gene *cbiY* is located on the *cobI* operon and was therefore implicated in cobalamin biosynthesis in *B. megaterium* [11].
The experimental results reported herein have demonstrated that CbiY is not a DMB synthase. It is unable to rescue the DMB-dependent growth of a transformed *S. enterica* strain, and it lacks the ability to utilise FMN to form DMB *in vitro*. In addition, unlike any BluB proteins characterised to date, it was found to bind a flavin cofactor. The flavin cofactor could be removed from the holoenzyme by denaturing the protein by heat treatment, indicating that it is non-covalently bound, and was identified as FMN (Figure S4). CbiY was shown to stabilize a neutral, blue semiquinone species when reduced anaerobically with sodium dithionite. The H8α hyperfine coupling determined from the W-band ENDOR experiment is large, $A_{\text{iso}} = 8.9$ MHz, compared to typical H8α hyperfine couplings in protein-bound neutral flavin semiquinones of 7–7.4 MHz. A recent study [26] has attributed this phenomenon to solvent exposure of the dimethylbenzene portion of the flavin. Such a flavin environment is typical of flavodoxins that act as ‘promiscuous’ single electron transfer proteins, stabilising the flavin semiquinone [27].

CbiY is first the flavoprotein to be identified in the *cobI* operon of *B. megaterium*. Other examples of flavoproteins involved in the biosynthesis of B12 have been previously reported in bacterial species utilising the late cobalt insertion pathway of B12 synthesis, for example CobZ and CobR. CobZ from *R. capsulatus* is an FAD-binding mono-oxygenase involved in the ring contraction step of

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**Figure 6. ENDOR spectrum of the CbiY bound flavin radical.** (A) W-band (94 GHz) cw-EPR spectrum of the CbiY flavin radical. Experimental parameters: microwave power 65 nW, modulation frequency 100 kHz, modulation amplitude 1 G, temperature 120 K. Inset is the spectrum of the CbiY flavin radical recorded at X-band EPR. (B) W-band Davies pulsed ENDOR spectrum of the CbiY bound flavin semiquinone radical recorded at $T = 120$ K. The pulse sequence $\pi-T-\pi/2-\tau$-acq was employed with $\tau = 400$ ns, $T = 20$ $\mu$s and $\tau = 1.2$ $\mu$s. A 18 $\mu$s radiofrequency pulsed was applied during $T$, 1 $\mu$s after the initial $\pi$ pulse. The field was set to $g = 2.0039$ (33498 G). doi:10.1371/journal.pone.0055708.g006
cobalamin biosynthesis. CobZ, however, unlike CbiY, shows no stabilization of a flavin semiquinone [28]. Although CobR, a flavoprotein from Brucella melitensis, exhibits coHyrin reductase activity by a mechanism that proceeds via a single electron transfer, no stable semiquinone is observed with this enzyme [29]. The stabilization of a neutral radical species, although well documented in several flavoproteins [30,31,32], would be none the less novel for an enzyme implicated in B₁₂ synthesis. Furthermore it is unusual to observe such substantial formation of a semiquinone species, particularly considering that the redox potentials of the ox/qₐ and sq/qₜ transitions are only ~60 mV apart, indicating that the reduction of the flavin cofactor is possibly kinetically (rather than thermodynamically) controlled. This unusual stabilization of a radical species suggests that CbiY may be involved in a single-electron transfer pathway. However, no experimental evidence for a reductase role for CbiY has yet been obtained; it is not considered to be a flavin reductase because it does not reduce flavin in the presence of NADH (data not shown).

Thus it is unclear, as yet, what role CbiY may perform in vivo. The gene is located in a cobalamin biosynthetic operon, however, no direct requirement for CbiY in the biosynthesis of cobalamin has ever been demonstrated. On the basis of this evidence it is plausible that CbiY is not involved in the biosynthesis of cobalamin but instead could play a role in another downstream process. This arrangement of a single electron transfer, no stable semiquinone is observed with this enzyme [29].

Materials and Methods

Chemicals and Reagents

Chemicals were purchased from Sigma-Aldrich if not mentioned otherwise. Other materials and reagents were provided by Promega UK (restriction and modification enzymes, the vector pGEM®-T easy), New England Biolabs UK (restriction and modification enzymes), Novagen (the vector pET14b), Amersham Biosciences UK (chelating Sepharose fast flow resin), Oxoid UK (tryptone, yeast extract and agar-agar), Difco (nutrient broth), Fisher/Invitrogen UK (oligonucleotides).

Molecular Biology Techniques

Strains and plasmids used in this work are given in Tables 1 and 2. Escherichia coli strain JM109 (Promega) was utilized for all DNA manipulations according to established laboratory methods [34]. The QIAprep® Miniprep kit (Qiagen) and the Wizard Plus SV Plasmid Miniprep kit (Promega) were used to isolate plasmid DNA. DNA fragments were extracted from 0.7–1% (w/v) agarose gels using a QIAquick gel extraction kit (Qiagen). PCR products were purified using a QIAquick PCR purification kit (Qiagen) or the Wizard SV Gel and PCR Clean-Up System kit (Promega). Cloned DNA fragments were sequenced by GATC Biotech or at the Biotechnology Center of the University of Wisconsin (Madison, USA).

Cloning of bluB from Rhodobacter capsulatus, of bluB and cbiY from Bacillus megaterium and of bluB from Rhodospirillum rubrum.

The R. capsulatus bluB was PCR amplified from genomic DNA from R. capsulatus strain SB1003 with primers containing restriction sites for NdeI and BamHI (given in italics). The sequence of the forward primer was 5’- cacatagtaacgtaacagcagcag-3’ and that of the reverse primer was 5’- cctggactgcgttgcgcctccattg-3’. The amplified fragment was cloned initially into pGEM®-T easy (Promega). For pET14b-BluB(Rc), the gene was then subcloned from this new plasmid into pET14b (Novagen) cut with NdeI and BamHI. BluB from B. megaterium was amplified by PCR with genomic DNA from B. megaterium strain DSM319 as a template. The primers used contained the restriction sites for BglII and XhoI (given in italics) and the sequences were 5’- cacatagtaacgtaacagcagcag-3’ and 5’- catatgtcttttcatcttca-3’. The resulting PCR product was cloned into the BamHI/BglII digested pET14b (Novagen) to yield pET14b-BluB(Bm). For pBluB25-ShqB, the coding sequence of bluB from B. megaterium was PCR amplified from plasmid pET14b-BluB(Bm) as a template. The primers contained the restriction sites for EcoRI and HindIII (given in italics) and had the sequences 5’- aggtagctattcttgacagcttgagcag-3’ and 5’- agtagctttcatctttttcatcata-3’. The resulting product encoding BluB with a silent T6C mutation eliminating the natural EcoRI site in the gene, was cloned into the EcoRI and HindIII sites of plasmid pBAD24 [17] to yield plasmid pBluB25 ShqB. The cbiY gene from B. megaterium was amplified by PCR using genomic DNA from B. megaterium strain DSM319 as a template. The restriction sites for NdeI and BamHI were inserted into the primers (given in italics). The primer sequences were 5’- gccttgactgaacttcaccag-3’ and 5’- gccttgactgaacttcaccag-3’. The PCR fragment was finally cloned into the NdeI and BamHI sites of pET14b (Novagen). For pCbiY6 NdeI, the coding sequence of cbiY from B. megaterium was amplified by PCR using genomic DNA from B. megaterium strain DSM319 as a template. The primers contained the restriction sites for EcoRI and XhoI and had the sequences 5’- aggtagctattcttgacagcttgagcag-3’ and 5’- aggtagctattcttgacagcttgagcag-3’. The resulting PCR fragment was cloned into the EcoRI and XhoI sites of plasmid pBAD24 to yield plasmid pCbiY6 NdeI. The bluB gene from R. rubrum was amplified by PCR using genomic DNA from R. rubrum UR2 as a template. The primers contained the restriction sites for EcoRI and XhoI and had the sequences 5’- aggtagctattcttgacagcttgagcag-3’ and 5’- aggtagctattcttgacagcttgagcag-3’. The resulting PCR fragment was cloned into the EcoRI and XhoI sites of plasmid pBAD24 to yield plasmid pBluB25 ShqB.

Overproduction of Recombinant Proteins and Protein Purification Techniques

For the overproduction of recombinant proteins, E. coli strain BL21star(DE3)pLysS was transformed with the appropriate pET14b derivative. The recombinant strain was grown under aerobic conditions at 37℃ in Luria Bertani (LB) medium supplemented with 100 mg 1⁻¹ ampicillin and 34 mg 1⁻¹ chloramphenicol. When the cells reached an optical density (OD) at 578 nm of ~0.6, the recombinant protein overproduction was induced by the addition of isopropyl-1-thio-β-d-galactopyranosid (IPTG) to a final concentration of 400 μM and the cells were grown overnight at 19℃. They were harvested by centrifugation (3,500 x g for 15 min and 4℃, Beckmann Coulter, MLA-9,1000). The cell pellets were resuspended in binding buffer (20 mM Tris pH 8.0 containing 300 mM NaCl and 10 mM imidazole). Cells were lysed by sonication (Sonics VibraCell).
The gel was stained and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) at room temperature and then kept on ice. Fractions were collected at 100 mM NaCl. Proteins were purified in the presence of oxygen with coomassie brilliant blue [34]. Size standard was Dalton VII (Sigma-Aldrich) with marker proteins of 66, 45, 36, 29, 24, 20.1 and 14.2 kDa.

Fast Protein Liquid Chromatography (FPLC)

The desalted protein fractions were loaded onto a pre-packed high flow, high resolution Superdex 200 column (10/300 GL) equilibrated in Buffer A (20 mM Tris-HCl (pH 8.0) containing 100 mM NaCl). The proteins were eluted at 0.5 ml min\(^{-1}\) into 1 ml fractions with buffer A on an Akta\(^{\text{®}}\) FPLC chromatography system at 19°C. Fractions were analyzed by SDS-PAGE (Laemmli system at 19°C) and the purest fractions were pooled.

**Table 1.** Strains used in this study.

| Strain | Genotype | Reference or source |
|--------|-----------|---------------------|
| *Escherichia coli* | endA1, recA1, gyrA96, thi, hisD17 (h<sup>+</sup> m<sup>+</sup>), relA1, supE44, Δ(lac-proAB), ΔF<sub>r</sub> traD36, proAB, lacI<sub>q</sub>ZΔM15 | Promega |
| BL21 star(DE3)pLysS | F<sup>−</sup>, ompT, hsdS6r<sup>5</sup>, m<sup>+</sup>, gal, dcm, metE131, (DE3), pLysS (Cm<sup>+</sup>) | Invitrogen |
| OH5s/F<sup>−</sup> | F<sup>−</sup>/endA1 hisD17 (h<sup>+</sup> m<sup>+</sup>) glnIV44 thi-1 recA1 gyrA (Nal<sup>R</sup>) relA1 Δ(lacI234YA-argF)U169 deoR (q80IdlA)Δ(lacZΔM15) | [42] |
| *Salmonella enterica* | | |
| JE2119 | S. enterica metE205 ara-9 cobC1175::Tn10ΔΔI<sup>+</sup> | J. Escalante-Semerena lab collection |
| JE7087 | S. enterica metE205ΔΔI<sup>+</sup> ara-9 | J. Escalante-Semerena lab collection |
| JE9384 | S. enterica metE205ΔΔI<sup>+</sup> ara-9/pBluB9<sup>Rr</sup> | This work |
| JE9385 | S. enterica metE205ΔΔI<sup>+</sup> ara-9/pBAD24<sup>+</sup> (bla<sup>+</sup>) | This work |
| JE12053 | S. enterica metE205ΔΔI<sup>+</sup> ara-9/pBluB25<sup>Bm</sup> (8. megaterium BluB<sup>+</sup> bla<sup>+</sup>) | This work |
| JE12130 | S. enterica metE205ΔΔI<sup>+</sup> ara-9/pBluB1Y<sup>Bm</sup> (8. megaterium cbi<sup>+</sup> bla<sup>+</sup>) | This work |
| JE10515 | S. enterica metE205ΔΔI<sup>+</sup> ara-9 cobC1175::Tn10ΔΔI<sup>+</sup>/pBAD24<sup>+</sup> (bla<sup>+</sup>) | This work |
| JE10516 | S. enterica metE205ΔΔI<sup>+</sup> ara-9 cobC1175::Tn10ΔΔI<sup>+</sup>/pBluB25<sup>Rc</sup> (R. rubrum BluB<sup>+</sup> bla<sup>+</sup>) | This work |
| JE12054 | S. enterica metE205ΔΔI<sup>+</sup> ara-9 cobC1175::Tn10ΔΔI<sup>+</sup>/pBluB25<sup>Bm</sup> (8. megaterium BluB<sup>+</sup> bla<sup>+</sup>) | This work |
| JE12131 | S. enterica metE205ΔΔI<sup>+</sup> ara-9 cobC1175::Tn10ΔΔI<sup>+</sup>/pBluB1Y<sup>Bm</sup> (8. megaterium cbi<sup>+</sup> bla<sup>+</sup>) | This work |

**Table 2.** Plasmids used in this study.

| Plasmid | Description | Reference or source |
|---------|-------------|---------------------|
| pET14b | N-terminal his<sub>6</sub>-tag sequence followed by thrombin site, recombinant gene expression under control of T7 RNA dependant promoter, amp<sup>+</sup> | Novagen |
| pGEM<sup>®</sup>-T Easy | PCR product cloning vector | Promega |
| pBAD24 | recombinant gene expression under control of the P<sub>bad</sub>-promoter, amp<sup>+</sup> | [17] |
| pET14b-BluB<sup>Bm</sup> | BluB<sup>Bm</sup> BglII/Xhol cloned with pET14b BamHI/Xhol, amp<sup>+</sup> | This work |
| pGEM<sup>®</sup>-T Easy-BluB<sup>Bm</sup> | BluB<sup>Bm</sup> cloned with pGEM<sup>®</sup>-T Easy, amp<sup>+</sup> | This work |
| pET14b-BluB<sup>Rc</sup> | BluB<sup>Rc</sup> Ndel/BamHI cloned with pET14b Ndel/BamHI, amp<sup>+</sup> | This work |
| pET14b-cbiY<sup>Bm</sup> | cbiY<sup>Bm</sup> Ndel/BamHI cloned with pET14b Ndel/BamHI, amp<sup>+</sup> | This work |
| pBluB9<sup>gy</sup> | BluB<sup>gy</sup> EcoRI/Xhol cloned with pBAD24 EcoRI/Xhol, amp<sup>+</sup> | This work |
| pBluB25<sup>Bm</sup> | BluB<sup>Bm</sup> EcoRI/HindIII cloned with pBAD24 EcoRI/HindIII, amp<sup>+</sup> | This work |
| pCbiY6<sup>Bm</sup> | cbiY<sup>Bm</sup> EcoRI/Xhol cloned with pBAD24 EcoRI/Xhol, amp<sup>+</sup> | This work |

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BluB in vitro Activity Assay

The formation of dimethylbenzimidazole (DMB) was monitored in 1 ml reactions containing 100 μM flavin adenine dinucleotide (FMN) and 40 mM nicotinamide adenine dinucleotide (reduced form) (NADH) in 20 mM Tris-HCl (pH 8.0) containing 100 mM NaCl and 1 mM diithiothreitol (DTT) at room temperature in the presence of oxygen in the dark for 18 h. The amount of the purified enzymes in the reaction mixtures varied between 10 μg and 4.8 mg. Control reactions were performed in the absence of either FMN, NADH, or enzyme. Reactions were stopped by boiling the samples for 5 min followed by a centrifugation step at 14,000 rpm at 4 °C for 10 min. The products of the reaction were separated and identified by high performance liquid chromatography on an ACE 5 AQ column (4.6 × 250 mm; Advanced Chromatography Technologies) run on an Agilent 1100 series HPLC equipped with a diode array detector. Intermediates were eluted with a flow rate of 1 ml min⁻¹ and a gradient of methanol in 5 mM ammonium acetate pH 6.5 as described previously [2]. DMB and FMN were identified by comparison of their absorbance spectra and retention time compared with authentic DMB and FMN standards.

Formation of the BluB Peroxyflavin Intermediate

After aerobic overproduction and purification of the recombinant BluB enzymes, the elution fractions where transferred into an anaerobic glove box (Bell Technology), maintained at less than 2 ppm oxygen. The protein was desalted and liberated from oxygen by gel filtration using a Sephadex G25 PD10 column (GE Healthcare) equilibrated in oxygen free 20 mM Tris-HCl (pH 8.0) containing 100 mM NaCl. The protein (42–500 mg) was preincubated with 171 μM reduced FMN (produced by the reduction of FMN with sodium dithionite (DT)) and rapidly mixed with non-dissolved (air saturated) buffer in an Applied Photophysics SX20 stopped-flow spectrometer at 20 °C. The reaction was monitored by photodiode array detector. Formation of the peroxylavin derivative, with an absorption maximum at 380 nm, was complete within 20 ms of mixing.

Identification of Flavin Cofactor Bound to CbiY

The bound flavin cofactor was extracted from CbiY [Bm] by incubation of the protein at 100 °C for 10 minutes. The precipitate was removed by centrifugation leaving a yellow supernatant, 10 μl of which was injected on to an Ace 5 AQ column (4.6 × 210 mm, 5 μm, Advanced Chromatography Technologies) run on an Agilent 1100 series HPLC equipped with an online diode array and fluorescence detectors linked to a Bruker microTOF-Q II mass spectrometer at a flow rate of 0.2 ml min⁻¹. Flavin cofactors were separated isocratically using 25% v/v methanol in 20 mM ammonium bicarbonate (pH 7.5) as the eluent. The absorbance at 455, fluorescence emission at 525 nm (excitation wavelength 445 nm) and the mass spectrum was monitored. Identification of species was based on the comparison of the retention time and mass spectrum with that of commercial standards.

Determination of Flavin Redox Potentials

Potentiometric redox titrations were performed to determine the midpoint reduction potentials for the flavin cofactor housed within CbiY [Bm]. Spectroelectrochemical potentiometric titrations were performed in a anaerobic glove box (Belle Technology) under a nitrogen atmosphere as initially described by Dutton and as detailed in our previous studies of other flavoproteins [36,37,38]. Absorption versus potential data were fitted at various wavelengths to establish the reduction potentials for both the 2-electron reduction couple of the CbiY flavin (oxidized/hydroquinone, E1/2), and for the individual oxidized/semiquinone (ox/sq) (E2) and semiquinone/hydroquinone (sq/hq) (E2) couples. Data were fitted at both 443 nm (at the oxidized flavin maximum) and at 465 nm (at the major oxidized flavin shoulder), and at 487 nm (isosbestic point for the flavin ox/sq transition) and at 430 nm (isosbestic point for the flavin sq/hq transition). Data were fitted using 1- and 2-electron Nernst equations, as described previously [39].

EPR and ENDOR Spectroscopy

EPR and ENDOR spectra were obtained using either a Bruker ELEXYS E500/580 EPR spectrometer operating at X-band or a Bruker ELEXYS E600/680 EPR spectrometer operating at W-band. Temperature control was effected using Oxford Instruments ESR900, CF935 and CF1200 cryostats interfaced with an ITC503 temperature controller. Experimental conditions were as given in the figure caption.

Complementation Assays

S. enterica strains were grown aerobically overnight in nutrient broth supplemented with 50 μg ml⁻¹ ampicillin. For derivatives of S. enterica strain JE7087, overnight cultures were subcultured (1:50 vol:vol inoculum) into no-carbon E (NCE) medium [40] supplemented with 90 mM ethanolamine, 1 mM MgSO4 1x trace minerals [41] (53 μM nitrilotriacetic acid, 12.5 μM MnSO4 4H2O, 171 μM NaCl, 4 μM FeSO4 7H2O, 4 μM CoSO4 7 μM CaCl2 2H2O, 3.5 μM ZnSO4 0.4 μM CaSO4 5H2O, 2 μM H2BO3, 12 μM Na2MoO4 2H2O, 11 μM Na2SeO4 10H2O, 2 μM NiSO4 6H2O, 150 mM dicyanocobinamide, and 50 μg ml⁻¹ ampicillin. Aerobic growth of JE7087 derivatives took place in 200 μl volumes in 96-well microtiter dishes (Falcon). Optical density at 650 nm was measured for 36 h at 37 °C. Growth curves were obtained using an ELx808 Ultra Microplate reader (Bio-Tek Instruments). For derivatives of S. enterica strain JE2119, overnight cultures were rinsed twice with sterile saline and subcultured (1:50 vol:vol inoculum) into NCE medium containing 11 mM glucose, 1 mM MgSO4 15 mM dicyanocobinamide, 1x trace minerals, and 50 μg ml⁻¹ ampicillin. Aerobic growth of JE2119 derivatives took place in 5 ml volumes in 125 ml sidearm flasks which were agitated at 280 rpm at 37 °C. Growth was monitored with a Summerson colorimeter (Klett). Each growth curve was performed in triplicate.

Supporting Information

Figure S1 Control stopped flow experiment. FMNH2 (291 μM) combined in the stopped flow with oxygenated buffer A. Spectra were collected every 2.5 s. An increase in absorbance at 430 nm (at the oxidized flavin maximum) and a loss of absorbance (black) over time probably due to photobleaching of the flavin.

Figure S2 Subtraction spectrum. Dashed line shows the initial spectrum of BluB and FMNH2 combined with oxygenated buffer (291 μM FMNH2 was incubated with 684 μM (RC)BluB and prior to stopped flow mixing with oxygenated buffer A. Spectrum recorded 2.5 s after mixing), dotted line shows the initial spectrum of the control sample (291 μM FMNH2 combined in the stopped flow with oxygenated buffer A. Spectrum recorded 2.5 s after mixing) and the solid line shows the resulting spectrum if the control spectrum is subtracted from the sample spectrum.
Figure S3 Rates of peroxylaflavin formation and degragation; variable BluB concentration. The figure shows the apparent rate of the formation of c4a-peroxylaflavin in the presence of a variable cofactor concentration of [RC]BluB (black circles). The concentration of FMNH was 1.25 μM and the apparent rate was also shown here (white circles and black triangles). Temperature was maintained at 25°C throughout.

(DOC)

Figure S4 Analysis of the flavin cofactor bound to CbiY. HPLC chromatogram and ESI+ve mass spectrum of the flavin cofactor isolated from CbiY. Retention time and MS were consistent with the bound cofactor being FMN (m/z 457 [M+H]+).

(DOCX)

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Table S1 Analysis of sequence alignments. The table shows the consensus sequence given in [%] and the identity given in [%] of the BluB proteins from R. capsulatus, R. rubrum, S. meliloti, and B. megaterium (A) and consensus sequence given in [%] and identity given in [%] of CbiY from B. megaterium compared with BluB proteins of R. capsulatus, R. rubrum, S. meliloti, and B. megaterium (B).

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Author Contributions

Conceived and designed the experiments: SER ADL JCE-S MJW. Performed the experiments: HFC RB HKL MJG KJM SER ADL. Analyzed the data: RB JCE-S AWJ WM MJW ADL. Contributed reagents/materials/analysis tools: RBJCE-SER MJW. Wrote the paper: HFC MJG SER MJW ADL.
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