Site directed mutagenesis of L-pyrrolysine in dimethylamine methyltransferase

A Senior Honors Thesis

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by

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Abstract

Methanosarcina barkeri is an archaeon and is able to use methylamines as growth substrates for methanogenesis. In *M. barkeri*, the three methylamine methyltransferases that are involved in initiating methanogenesis from the methylamines have an in-frame amber codon (UAG) that does not terminate translation, but in fact encodes for the 22nd amino acid L-pyrrolysine. This thesis attempts to examine the rationale for the presence of pyrrolysine in one of these three methylamine methyltransferases.

A system that allowed the recombinant expression of the dimethylamine methyltransferase gene, *mtbB*, in the native organism *Methanosarcina acetivorans*, was developed (9). Site directed mutagenesis of *mtbB* was performed wherein UAG, which encodes for pyrrolysine, was substituted by GCA, which encodes for alanine (Jodie Lee). This allowed for functional studies of MtbB vs. the mutant MtbB, dubbed MtbB(O356A). An activity assay, which measured the ability of MtbB and MtbB(O356A) to methylate hydroxocobalamin, showed that MtbB(O356A) had lost its functionality. This indicated that pyrrolysine is an essential residue in MtbB and that it may play a catalytic role. However, preliminary data from a native gel electrophoresis experiment showed that the ability of MtbB(O356A) to bind its cognate corrinoid cofactor protein, MtbC, was greatly reduced; whilst the wild-type MtbB was able to form a complex with MtbC. This indicated that pyrrolysine is required for proper folding of MtbB and/or it is required for binding MtbC.

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1. Introduction

1.1 Methanogens

Methanogens belong to the domain Archaea and help breakdown bacterial fermentation products during environmental recycling of organic nutrients. They do so by the process of methanogenesis, by which methanogens produce about 1 billion tons of methane per year. As a green house gas, this methane has an impact on our climate. Methanogens are also found in variety of environments, such as sanitary landfills, fresh and saline sediments, and animal gastro-intestinal tracts. Hence, methanogens play an important role in our ecosystem.

1.2 *Methanosarcina barkeri* MS and L-pyrrolysine

M. barkeri is one such methanogen. But unlike many other methanogens, which thrive with canonical twenty amino acids, *M. barkeri* employs a rare amino acid in its enzymes that are involved in methanogenesis from methylamines. This amino acid is pyrrolysine.

When the genetic code was solved, it was determined that the sequence of nucleotides in the mRNA molecule is read sequentially in groups of three nucleotides (a codon). Subsequently it was shown that the 64 possible nucleotide combinations either encode one of the known 20 common amino acids or terminate translation. It was also realized that the genetic code is universal. At this time, it was believed that the ochre, amber and the opal codons universally lead to translation termination. However, this perception has changed. It is now known that some organisms, under certain circumstances, can in fact code for amino acids using the stop codons. Amino acid selenocysteine, encoded by the opal codon UGA, was the first such example.

Interestingly, it was discovered that the genes encoding methylamine methyltransferases in *M. barkeri* employ an in-frame amber codon (UAG) as a sense codon as well. Subsequent evidence for a dedicated pyrrolysyl-t-RNA synthetase (PylS) and tRNA^{Pyl}, and the unique electron density of the amber-codon encoded amino acid led to and proved the existence of the 22nd amino acid, L-pyrrolysine (1; 2; 3; 4). Figure 1 shows the structure of L-pyrrolysine. It is a modified lysine with an amide linkage to (4R,5R)-4-methyl-pyrroline-5-carboxylate.

1.3 Pyrrolysine and methanogenesis in *M.barkeri*

Pyrrolysine is found in the three methylamine methyltransferases in *M. barkeri*. Monomethylamine methyltransferase (MtmB), dimethylamine methyltransferase (MtbB) and trimethylamine methyltransferase (Mttb) each has a single residue of pyrrolysine incorporated into their sequence. As shown in Figure 2, MtmB, MtbB, and MttB are specific for their substrates monomethylamine (MMA), dimethylamine (DMA) and trimethylamine (TMA) respectively. Once substrate is bound, the methylated MtmB, MtbB or MttB methylate their cognate corrinoid proteins MtmC, MtbC or MttC respectively. Methylated MtmC, MtbC and MttC serve as substrates for MtbA, a methylcobamide:coenzyme M methyltransferase. MtbA demethylates the corrinoid proteins using zinc to form a nucleophilic coenzyme M thiolate, which, when reduced by methyl-CoM reductase, forms methane (5; 6; 7).

1.4 Proposed role of pyrrolysine in methanogenesis

A crystal structure of MtmB solved to 1.7 Å suggests that pyrrolysine may play a catalytic role. The structure depicts pyrrolysine at the bottom of a negatively charged cleft. Additionally, pyrrolysine reacts with nucleophilic compounds such as hydroxylamine (17).

Based on these observations Hao *et al* proposed a possible mechanism for the role of pyrrolysine in methylation of MtmC (2). The mechanism is described in Figure 3.

1.5 Project aim and hypothesis

In order to test the role of pyrrolysine in catalysis, the activity of wild-type MtbB was compared to that of a site-directed mutant MtbB, dubbed MtbB(O356A); wherein pyrrolysine encoded by codon 356 (UAG) was substituted with alanine (encoded by codon GCA). If pyrrolysine was in fact necessary in catalysis then MtbB(O356A), which lacked pyrrolysine, would show no activity and hence methanogenesis will not occur.
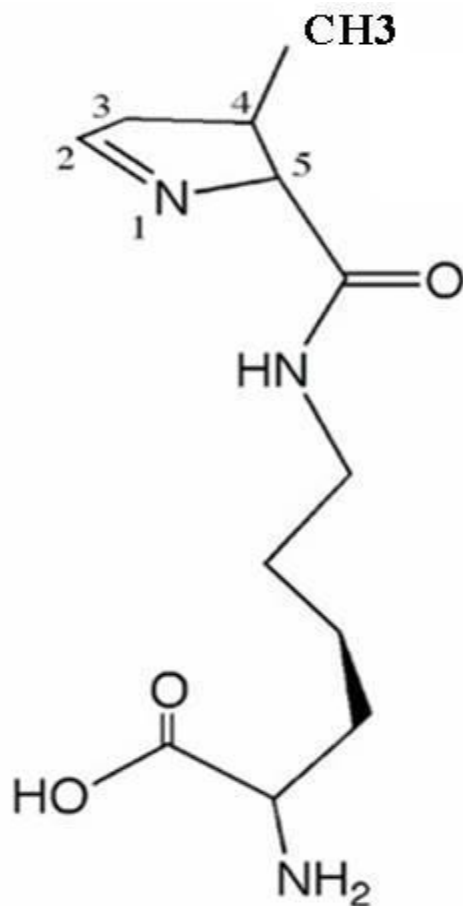
Figure 1. The structure of L-pyrrolysine as determined by X-ray crystallography and mass spectrometry. Pyrrolysine is a modified lysine with an amide linkage to (4R,5R)-4-methyl-pyrroline-5-carboxylate (2;17;18). It is found in the three methylamine methyltransferases-MtmB, MtbB and MttB, each involved in initiating methanogenesis from methylamines.
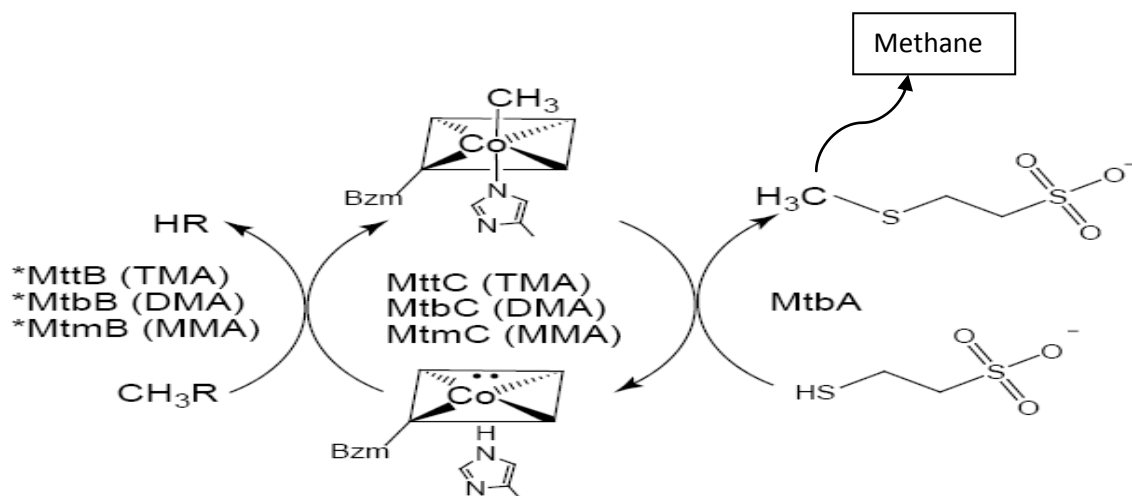
Figure 2. Schematic showing the enzymes involved in methanogenesis using substrates TMA, DMA and MMA. Methanogenesis involves demethylation of substrates (TMA, DMA or MMA) with the help of their respective methyltransferase (MttB, MtbB or MtmB).The initial reaction involves each methyltransferase (MttB, MtbB or MtmB) demethylating their respective methylamine and methylating their respective corrinoid protein (MttC, MtbC, or MtmC). The corrinoid protein must be in the active Co(I) state. Following methylation of the corrinoid cofactor, transfer of a methyl group from MttC, MtbC or MtmC to coenzyme M is catalysed by MtbA, to CoM; leading to eventual release of methane. Mttb, MtbB, and MtmB each have an amber codon encoded pyrrolysine residue. Figure is taken from Krzycki 2004 (16).
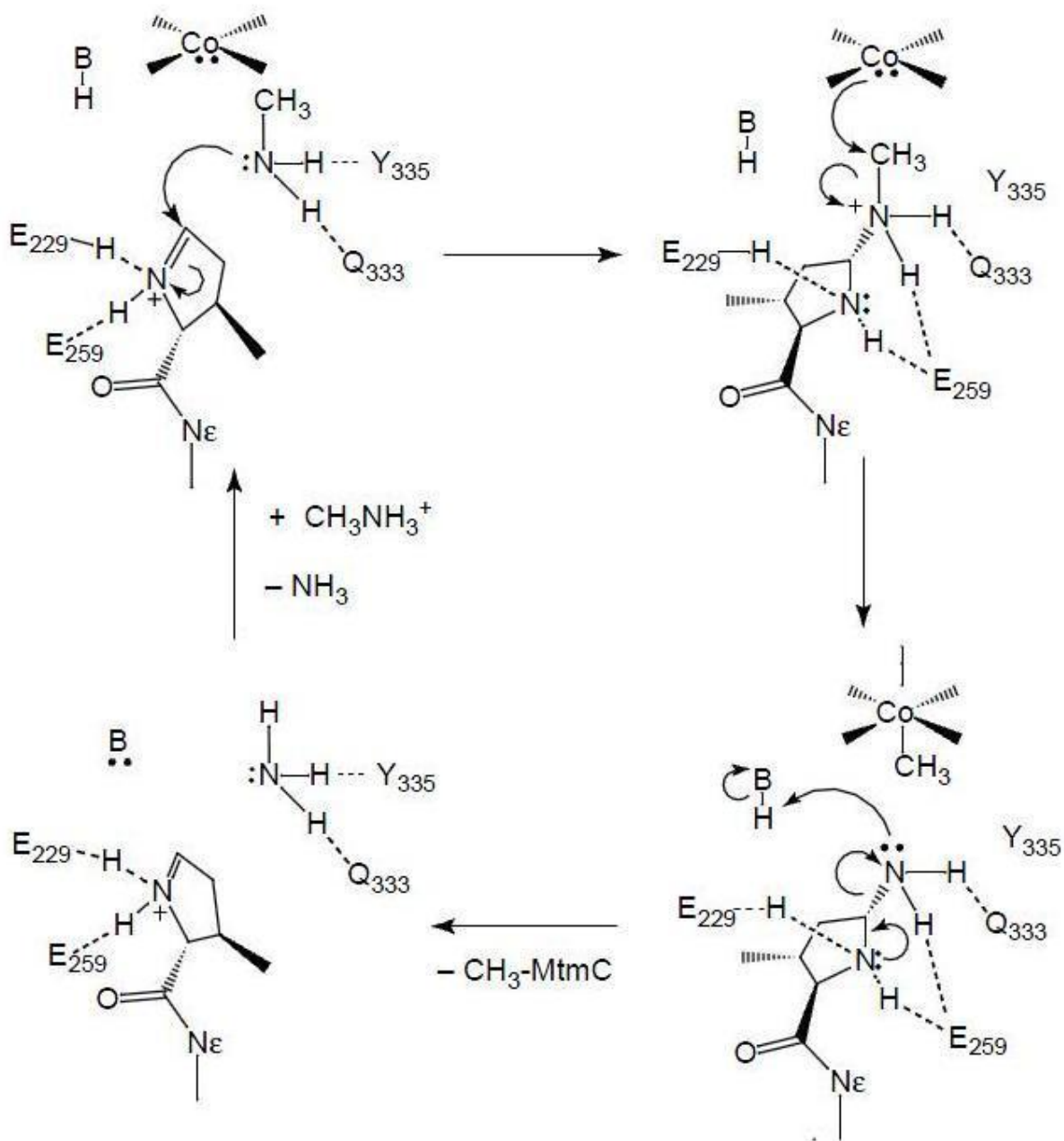
Figure 3. Proposed mechanism concerning the role of pyrrolysine in methylating MtmC. Monomethylamine ion (MMA ion) is attracted to the negatively charged cleft of MtmB and then deprotonated by an unknown base. In its un-ionized form MMA binding to MtmB is facilitated by Y_{335} and Q_{333} residues. The C-2 atom in the pyrroline ring attacks the lone electron pair of un-ionized MMA. This in turn re-orients the pyrroline ring and is subsequently followed by a nucleophilic attack by MtbC-Co(I), resulting in formation of methylated MtbC-Co(III). This figure is taken from Krzycki 2004 (16).

2. Materials and Methods

2.1 General Analytical Methods

2.1.1 Denaturing polyacrylamide gel electrophoresis

All SDS-polyacrylamide gels contained 12.5% polyacrylamide but were otherwise ran as described by Laemmli (8).

2.1.2 Protein assay

All protein estimations were done using bicinchoninic acid as per manufacturer's recommendations (Pierce, Rockford, IL). Bovine serum albumin (1 mg/ml) was used in serial dilutions to produce the standard curves.

2.2 Isolation of MtbB

The *M. barkeri mtbB* gene was cloned into a methanogen expression vector in order to produce a C-terminally hexahistidine-tagged MtbB protein (9). Site directed mutagenesis of the UAG codon for pyrrolysine in this gene to a GCA codon encoding alanine was also performed by Jodie Lee to generate a plasmid producing MtbB-O356A. Both plasmids were transformed into *Methanosarcina acetivorans*.

M. acetivorans cultures harboring the recombinant *mtbB* gene were grown on high salt media (HS) supplemented with 100 mM methanol and 2 µg/ml puromycin, in 15 L anaerobic carboys, at 37 °C until early stationary phase. Cells were pelleted aerobically and resuspended in an equal volume of 50 mM MOPS, pH 7.0. The cells were lysed at 11,000 psi using a French pressure cell. The cell lysate was ultracentrifuged at 40,000 rpm for 1 hour and 10 minutes, at 5 °C. The supernatant was passed through a 1 ml Ni-activated Hi-trap chelating column (GE

Healthcare Bio-Sciences Corp., Piscataway, NJ) in 50 mM sodium phosphate, pH 7.4, 500 mM

NaCl and 10 mM imidazole. This bound the hexahistidine-tagged MtbB to the column. The

column was eluted at rate of 1 ml/min using a linear gradient from 10-500 mM imidazole (both

in 50 mM sodium phosphate buffer, pH 7.4 and 500 mM NaCl), over a total gradient volume of

40 ml. The elution was monitored by absorbance at 280 nm (A_{280}).

2.3 Isolation of MtbB(O356A)

MtbB(O356A) was isolated in a manner similar to the unmutated MtbB. Elution from a 5

ml Ni-activated Hi-trap chelating column (GE Healthcare Bio-Sciences Corp, Piscataway, NJ)

was monitored by A_{280}. The column was run at a flowrate of 5.0 ml/min over a 200 ml 10-500

mM imidazole gradient in the buffer described above. Fractions eluting between 50-100 ml of

the gradient were pooled. Using an Amicon Ultra filter (Millipore, Billerica, MA) with a 10,000

Da cutoff, the pooled fractions were then concentrated 10-fold by centrifuging at 3000 rpm.

2.4 Isolation of MtbC

In order to isolate MtbC, the recombinant MtbB strain was grown in HS medium as

above, but supplemented with 100 mM trimethylamine hydrochloride, rather than methanol.

Cells were pelleted aerobically then resuspended in an equal volume of 50 mM MOPS, pH 7.0.

The cells were lysed at 11,000 psi using a French pressure cell. The cell lysate was

ultracentrifuged at 40,000 rpm for 1 hour and 10 minutes, at 5 °C. The supernatant was passed

through a 5 ml Ni-activated Hi-trap chelating column (GE Healthcare Bio-Sciences Corp.,

Piscataway, NJ) in 50 mM sodium phosphate, pH 7.4, 500 mM NaCl and 10 mM imidazole. A

pool of the pink colored, enriched *M. acetivorans* MtbC eluted after about 15 ml of the buffer

had passed through the matrix. These pink fractions were pooled and diluted 10-fold with 50 mM MOPS pH 7.0. The diluted sample was loaded on to a Mono-Q column (GE Healthcare Bio-Sciences Corp, Piscataway, NJ). The column was then run at a rate of 1 ml/min, for a total volume of 84 ml, with a linear gradient from 0-500 mM NaCl in 50 mM MOPS, pH 7.0. Elution of the MtbC was monitored by A_{280}. MtbC was concentrated 2-fold as described for MtbB(O356A). In order to exchange the high salt buffer in which MtbC eluted, the concentrated MtbC was diluted 4 fold with 50 mM MOPS, pH 7.0, and then concentrated 4 fold as described before. This process was repeated four times, effectively exchanging the salt buffer to 50 mM MOPS, pH 7.0.

3. Assays

3.1 DMA-dependent cobalamin methylation by MtbB or MtbB(O356A)

The reactions were performed in a N_2-flushed anaerobic cuvette with a 2-mm path length. The reactions contained 84 µg of MtbB, 15 mM Ti(III) citrate (prepared as described earlier), and 2.5 mM hydroxocobalamin in 50 mM MOPS, pH 7.0, in a total volume of 200 µl (7). The spectrophotometer was blanked against the same cuvette containing only Ti(III)citrate and buffer. The reactions were initiated by the addition of DMA and were performed at 37 °C under dim red light for duration of 70 minutes. The conversion of cob(I)alamin to methylcobalamin was quantified at 540 nm using an extinction coefficient of 4.4 mM^{-1} cm^{-1} (10). Reagent mix with all reagents except DMA was used as a negative control.

To determine the activity of MtbB mutant, 84 µg of MtbB(O356A) was used in lieu of MtbB. All other assay conditions were kept the same as in the previous method.

3.2 Methylation of MtbC by MtbB or MtbB(O356A)

Attempts to measure MtbB dependent methylation of MtbC spectrally were made by following the formation of methyl-Co(III)-MtbC at 532 nm, and the corresponding loss of Co(I)-MtbC at 386 nm. Assays for reduction of the corrinoid cofactor of MtbC from the Co(II)-form to the Co(I)-form were conducted in 1.6 mM mM Ti(III)citrate, 6 µg of RamA, 3.3 mM ATP and 6 mM $MgCl_2$. The 1.6 mM Ti(III)citrate was obtained from a 98 mM stock prepared in an anaerobic bag by sequentially mixing 100 µl of a commercial preparation of Ti(III) in 10% HCl with 445 µl of 500 mM sodium citrate, then 445 µl of a saturated Tris-base solution (10).

3.3 Visualization of MtbBC complex formation by native gel electrophoresis

To determine whether the hexahistidine-tagged MtbB or MtbB(O356A) can form a complex with MtbC, either 6.8 µg of MtbB or 8.4 µg of MtbB(O356A) were incubated at 25 °C, for 30 minutes, with 2.8 µg of MtbC. These fractions were electrophoresed in a native acrylamide gel at 30 mA until the bromophenol dye ran to the end of the gel. The separating layer of the gel consisted of 7.5% bis-acrylamide (37.5:1 acrylamide:bisacrylamide) buffered with 0.38 M Tris-HCl, pH 8.8; the stacking layer comprised of 3.75% bis-acrylamide (37.5:1 acrylamide:bisacrylamide) buffered with 0.13 M Tris-HCl, pH 6.8. The electrophoresis buffer was composed of 25 mM Tris and 200 mM glycine with a final pH of 8.3. Proteins were identified using a standard coomassie stain (0.1% (w/v) Coomassie Brilliant Blue R250, 40% methanol, 10% acetic acid), and destained in a 40% methanol and 10% acetic acid solution (9).

4. Results

4.1 Protein purification

Three proteins were purified for this work, the wild type DMA methyltransferase (MtbB), the mutated form of MtbB in which the pyrrolysine was replaced with alanine MtbB(O356A), and the DMA corrinoid protein, MtbC.

MtbB was isolated from a strain of recombinant *M. acetivorans* bearing a derivative of the pDL05c plasmid (9) in which the mtmB1 gene was replaced with the mtbB1 gene from M. barkeri modified to encode a C-terminal hexahistidine tag. The His-tagged MtbB protein could be isolated from *M. acetivorans* extracts by nickel affinity column as shown in Figure 4A. The MtbB eluted at about 180 mM imidazole. In the figure, the second peak corresponds to MtmB. The first peak appears to be a mixture of many different proteins and/or complexes. The results were confirmed using a standard SDS-PAGE (Figure 4B and 4C). Fraction collected from the first peak in the elution profile is shown in Figure 4B. In Figure 4C, lanes marked 13, 14, 15, 16, 17, 18, 19 and 20 correspond to their elution volumes (ml). Fractions 15-20 were pooled. The purification of MtbB(O356A) was carried out in a similar fashion, with the protein eluting between 125 and 250 mM imidazole (Fig. 5A). Figure 5B shows the SDS-PAGE analysis of the pooled fraction of the eluting peak when loaded with 8 µg of the concentrated MtbB(O356A). In all a total of 0.7 mg of MtbB was isolated. This indicates a final yield of 0.1 mg/ml of MtbB from 8 ml of *M. acetivorans* extracts.

The *M. acetivorans* MtbC protein was isolated from recombinant *M. acetivorans* in which *M. barkeri* mtbB1 was expressed during growth with trimethylamine. The *M. acetivorans*

MtbC protein formed a complex with the nickel-bound MtbB, and eluted later than the majority

of proteins from the extract. This pink fraction was pooled and applied to a Mono-Q anion

exchange column (Fig. 6A). MtbC was concentrated 2X as described for MtbB(O356A) and

buffer exchanged to 50 mM MOPS pH 7.0. Upon subsequent analysis through SDS-PAGE

(Figure 6B) it was determined that the first peak in the elution profile corresponded to MtbC

purified to near-homogeneity. The final protein concentration of MtbC isolated was 0.83 mg/ml.

The UV-visible spectra of MtbC thus isolated (Figure 7A) can be compared to the one published

by Ferguson et al (Figure 7B) (7). MtbC was under aerobic conditions and in 50 mM MOPS pH

7.0 buffer for both spectra. While the spectra are highly similar, the lower ratio for the 350 nm

peak to the 280 nm peak for the current preparation suggests some loss of the corrinoid cofactor
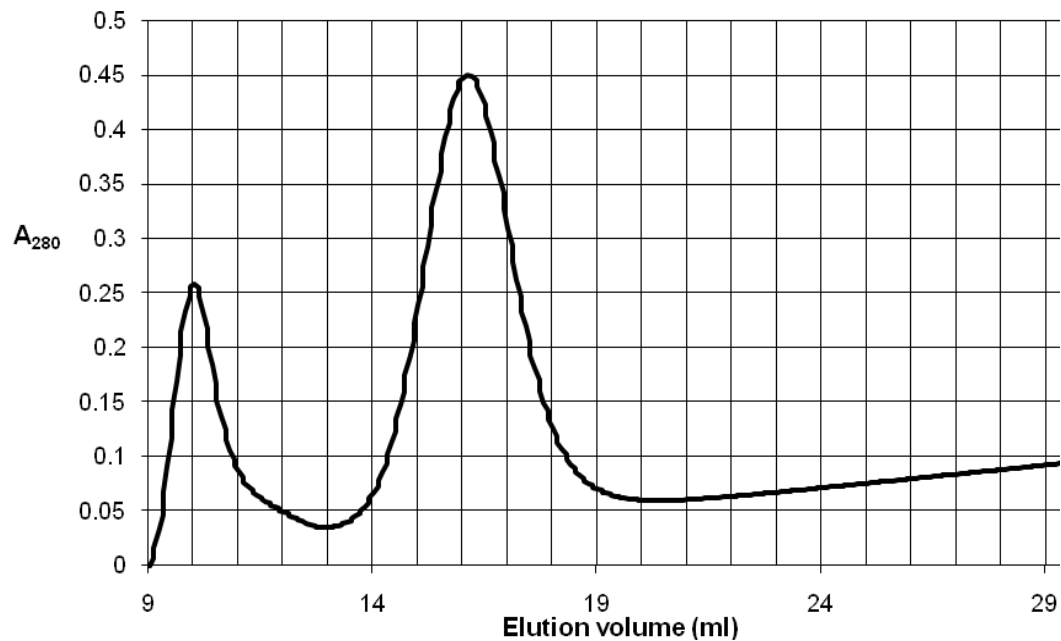
from this preparation.

Figure 4A. Typical elution profile when protein is monitored by absorbance at 280 nm during the isolation of recombinant MtbB from *M. acetivorans* extracts using a Ni-activated chelating column. The column was eluted with a linear gradient from 10 to 500 mM imidazole over a total gradient volume of 40 ml. The first peak appears to be a mixture of proteins and/or complexes. The second peak corresponds to MtbB (54 kDa), as verified by SDS-PAGE (see next figure).
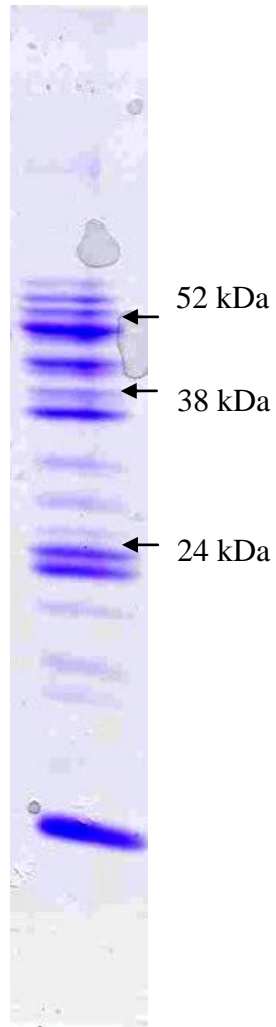
Figure 4B. Representative SDS-PAGE gel of the first peak shown in Figure 4A. Arrows point to their respective molecular weight in kDa.
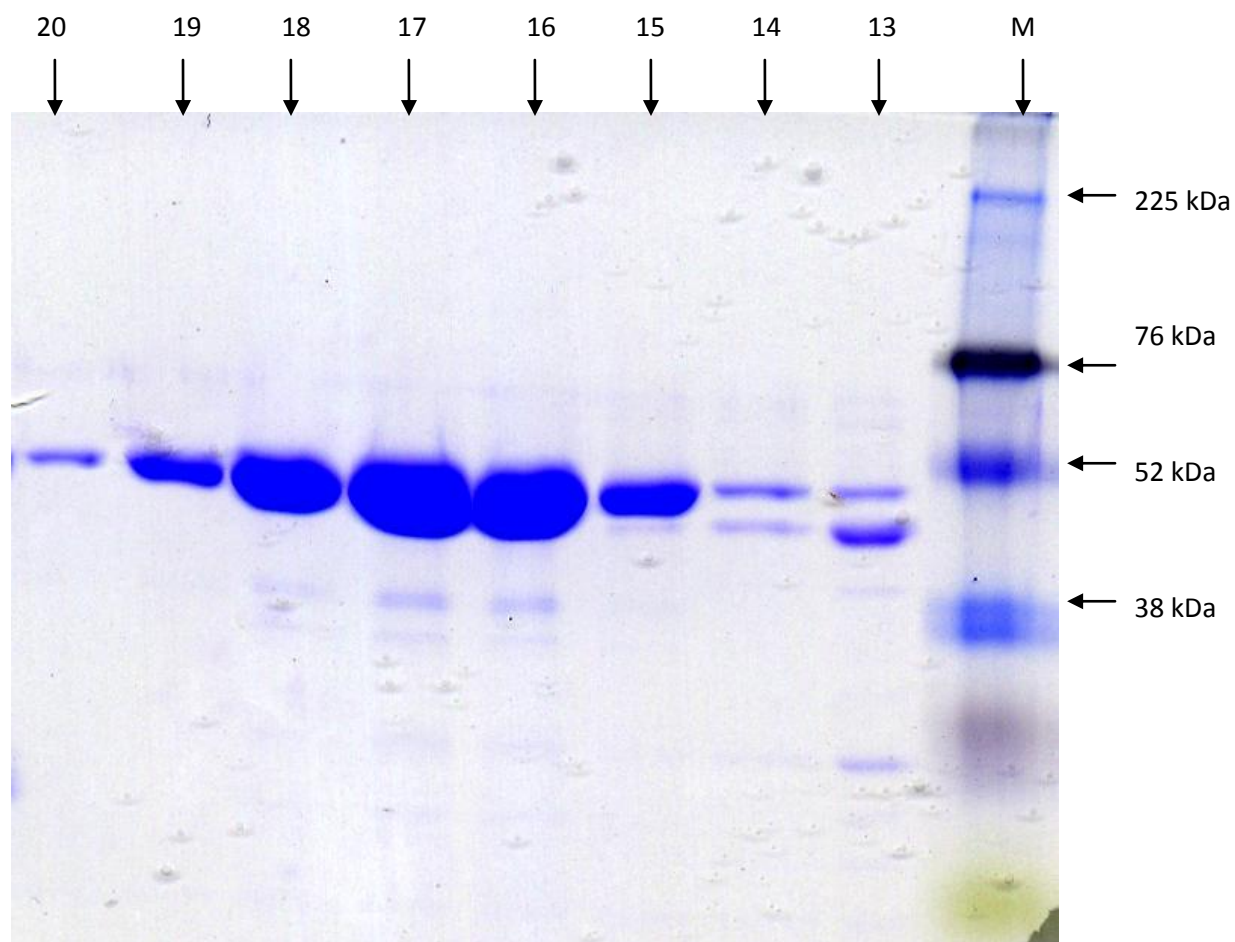
Figure 4C. SDS-PAGE gel of fractions comprising peak 2 of Figure 4A. M corresponds to molecular weight marker, and the numbers 13-20 correspond to their elution volume in ml. 8 μg of sample was loaded into each lane. Fractions 15-20 were pooled and determined to have a final concentration of 1.11 mg/ml. The major band comigrated with authentic MtbB in other gels (not shown).
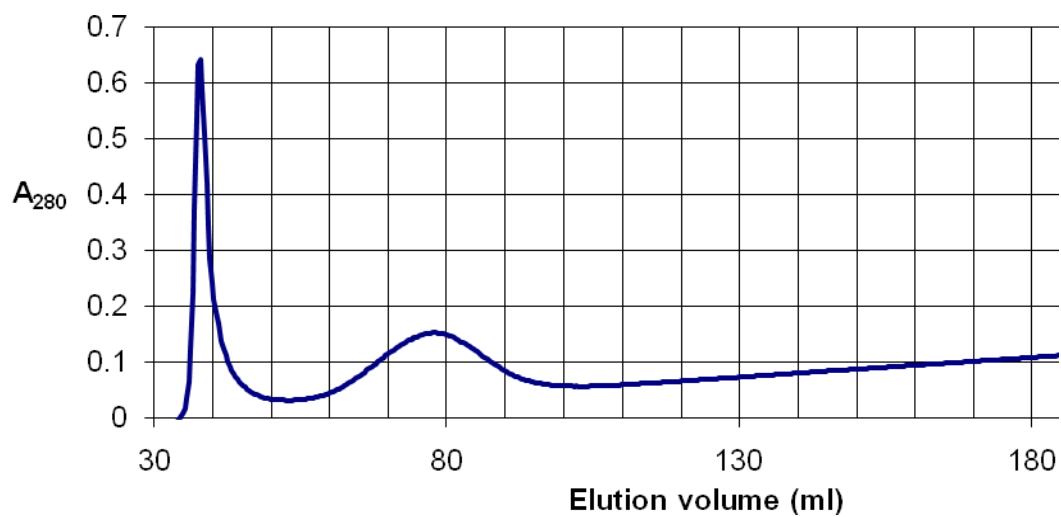
Figure 5A. Elution profile of protein (monitored by absorbance at 280 nm) during the isolation of MtbB(O356A) using a 5 ml Ni-activated chelating column. The column was eluted with a linear gradient ranging from 10 mM imidazole to 500 mM imidazole, for a total volume of 200 ml. The second peak corresponds to MtbB(O356A) as verified by SDS-PAGE.
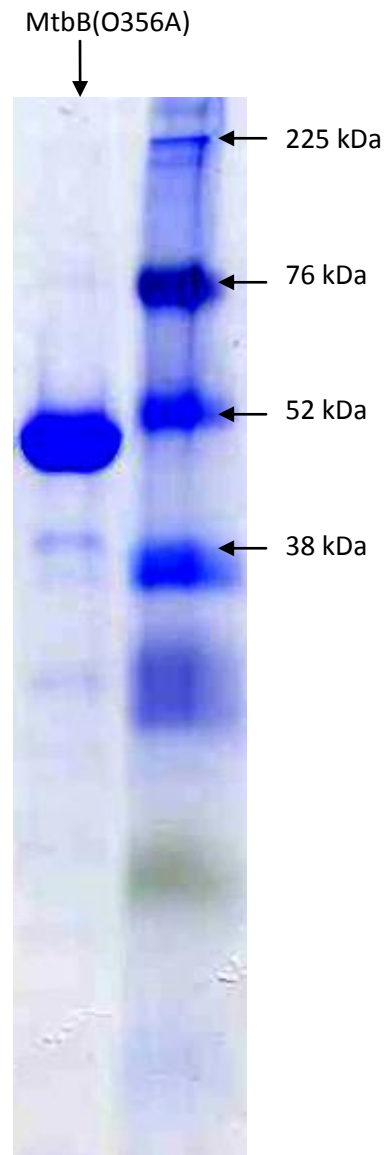
Figure 5B. SDS-PAGE gel of purified MtbB(O356A). M corresponds to standard molecular weight marker and MtbB(O356A) corresponds to 10-fold concentrated fractions from the second peak in Figure 5A. 8 µg of MtbB(O356A) was loaded onto the gel.

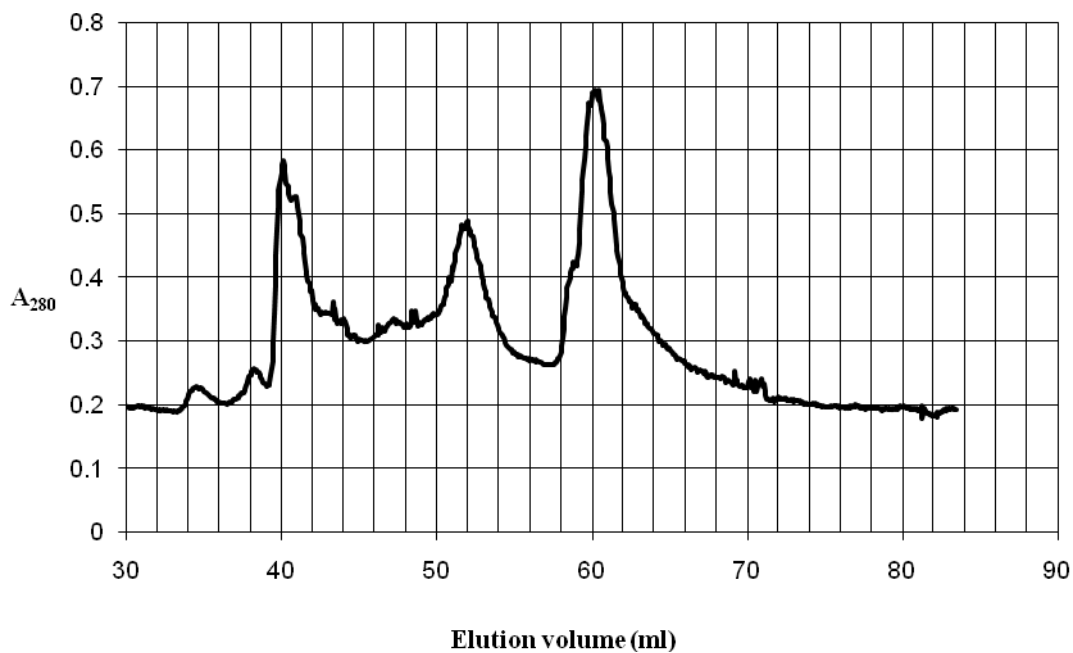**Figure 6A**. Elution profile (protein followed by absorbance at 280 nm) obtained from the isolation of MtbC by a MonoQ column following the initial nickel affinity column. The first major peak (0.6 AU) was shown to be MtbC by SDS-PAGE.

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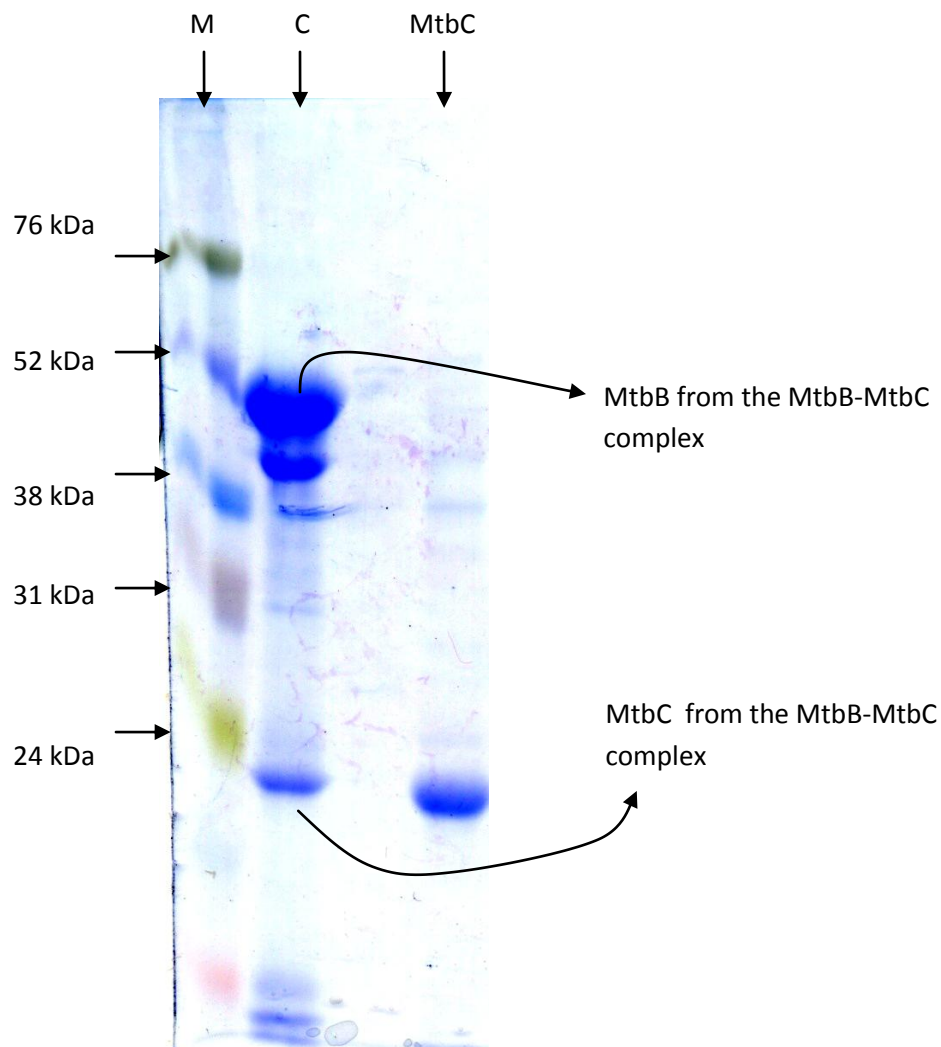
Figure 6B. SDS-PAGE gel of purified MtbC from the preceding Mono-Q column. M corresponds to a molecular weight marker assortment, C corresponds to a complex of MtbB and MtbC used as a control and MtbC is a fraction from the first peak in Figure 6A. 8 μg of the sample was loaded into the gel. The fraction was later concentrated 2X and buffer exchanged to MOPS pH 7.0. (Note: The band at approximately 38 kDa under lane C is likely methylcobamide:coenzyme M methyltransferase (MtbA) which is known to elute with the MtbB-C complex)

A
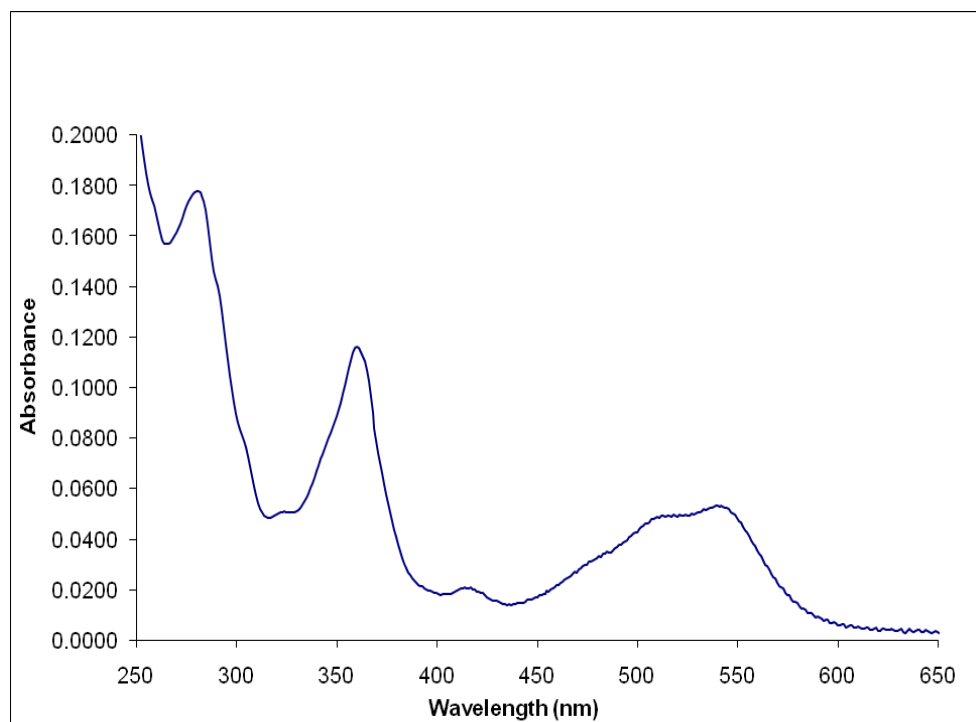
B

Figure 7. Comparision of UV-visible spectra of the purified MtbC vs. a previously published spectra. A, spectrum of purified MtbC (0.57 mg/ml) isolated through MonoQ column. B, spectrum of MtbC (0.4 mg/ml) as published by Ferguson *et al*, 2000. MtbC was in aerobic 50 mM MOPS, pH 7.0 for both spectra.
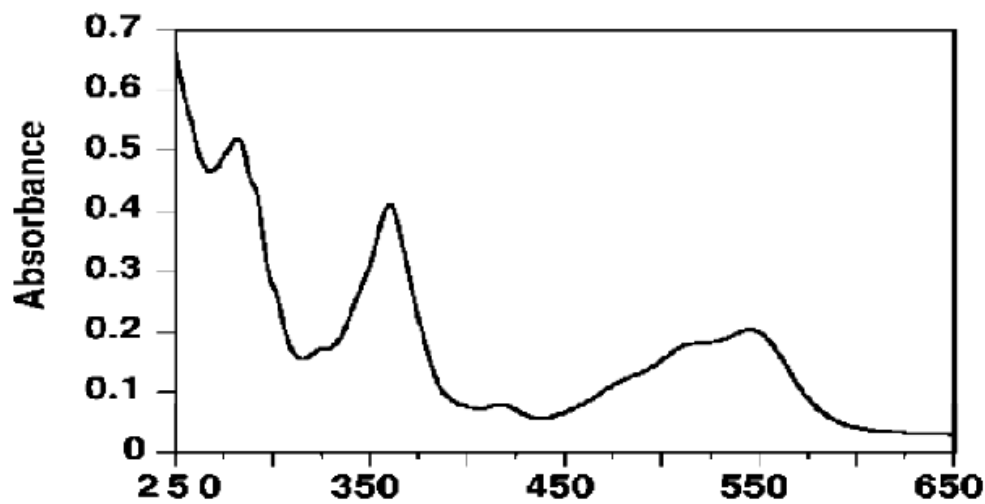
4.2 MtbB (O356A) and wild type MtbB differ markedly in methylating cob(I)alamin

As shown in Fig. 2, the dimethylamine methyltransferase, MtbB, demethylates DMA and methylates the corrinoid prosthetic group of MtbC. The corrinoid prosthetic group must be in its reduced Co(I) state to accept the incoming methyl group. In addition to methylating its cognate corrinoid protein MtbC, MtbB can also methylate free cob(I)alamin (7). This cofactor is only slightly different than the MtbC cofactor. This activity was first used to compare DMA-dependent co(I)balamin methylation rates between MtbB and MtbB lacking pyrrolysine.

Hydroxocobalamin was reduced to the Co(I) state by Ti(III)citrate in an anaerobic cuvette. Following addition of MtbB, the reaction was initiated by addition of DMA. Formation of methyl-cob(III)alamin was monitored spectrophotometrically. As the reaction progressed a steady shift from the cob(I)alamin peak at 553 nm to methyl-cob(III)alamin peak at 532 nm was observed. An isosbestic point at 578 nm was also observed, and indicated the formation of methyl-cob(III)alamin from cob(I)alamin without a detectable intermediate (Figure 8A). These results closely match to the ones published by Ferguson *et al* for this assay (7). The rate of conversion of cob(I)alamin to methyl-cob(III)alamin was measured at 540 nm, the isosbestic point of Co(I)/Co(II)-balamin (11) and was calculated over a period of 12 minutes, starting at the 8 minute mark and ending at the 20 minute mark. MtbB methylated 2.5 mM hydroxocobalamin at a rate of 48 nmol/min/mg of MtbB (Figure 8C).

In contrast, no apparent methylation of co(I)balamin was observed when MtbB(O356A) was used. Over the 70 minute duration of the experiment, absorbance changes were minimal (Figure 8B). The cob(I)alamin peak at 553 nm increased by 0.0056 AU and the methylcob(III)alamin peak at 532 nm increased by 0.007 AU. This generalized increase suggests

that no real shift to 532 nm occurred. The isosbestic point at 578 nm was not observed either. Based on these results no significant methylation of cob(I)alamin can be detected for the O356A mutant under conditions of the assay.

On the surface, these results are consistent with a necessary role for pyrrolysine in the dimethylamine methyltransferase (MtbB). It is either involved in catalysis and hence directly mediates the transfer of the methyl group from its substrate DMA to its corrinoid cofactor protein MtbC; it is important in maintaing the structure of MtbB; and/or it stablizes the interaction of MtbB with MtbC.
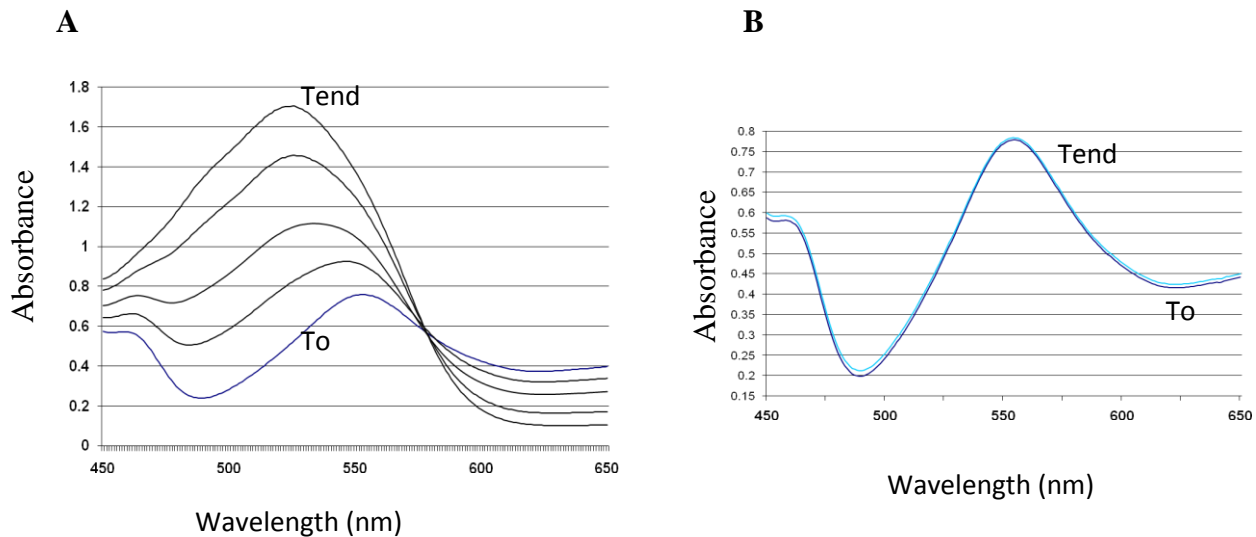
A

B

Figure 8A and 8B. UV-Vis spectra of DMA dependent methylation of free cob(I)alamin by MtbB (A) and MtbB(O356A) (B). **A-** Absorbance spectra of reduced cobalamin, over a 70 minute incubation, at 37 °C, in a reagent mix of 84 μg MtbB, 15.6 mM Ti(III)citrate, 0.5 M DMA, 2.5 mM hydroxocobalamin and 50 mM MOPS, pH 7.0. Spectra marked To and Tend represent start and end points of the assay. The remaining spectra were taken at 17 minute intervals. **B-** Absorbance spectra of reduced cobalamin in the same reagent mix as described for A, exception being that 84 μg of MtbB(O356A) was used in lieu of MtbB. Spectra marked To and Tend mark the start (0 minutes) and end (70 minutes) points respectively.
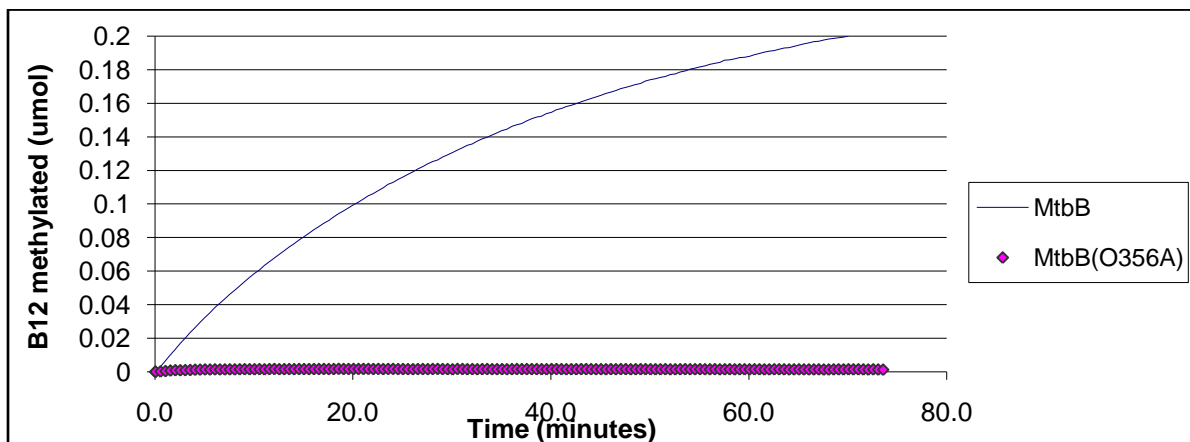

Figure 8C- Comparision of rate of methylation of reduced cobalamin by MtbB or MtbB(O356A). Rates were calculated using an extinction coefficient of 4.4 $mM^{-1} cm^{-1}$ and at absorbance 540 nm. 2.5 mM hydroxocobalamin was methylated at a rate of 48 nmol/min/mg of MtbB. The rate of cob(I) alamin methylation with DMA by MtbB(O356A) was essentially undetectable.

4.3 Assay of direct methylation of MtbC is incomplete because of inability to maintain the corrinoid cofactor of MtbC in the Co(I) state

As described above, MtbC is the natural substrate of MtbB for methylation with DMA. The rate of this methylation is at least 100-fold higher than the rate observed for methylation of cob(I)alamin. MtbC methylation would therefore provide a more sensitive assay for DMA dependent methyltransferase activity, and allow a more accurate comparison of wild type and mutant MttB activities. In order to use this assay, MtbC must first be reduced to the Co(I) state. RamA (isolated by Tsuneo Ferguson), is an ATP-dependent redox active protein required for the reduction of the corrinoid prosthetic group, and was used for this purpose (12). In two attempts, evidence of reduction of Co(II) MtbC to Co(I) MtbC could be obtained, as the major peak of Co(I) MtbC could be observed upon addition of RamA, ATP, and Ti(III)citrate. Unfortunately in these assays, the peak at 386 nm would subsequently decrease, suggesting that completely anaerobic conditions were not maintained in these experiments. As complete reduction of the corrinoid prosthetic group was not accomplished, the assay was not pursued further due to time limitations.

4.4 MtbB, but not MtbB(O356A) is able to form a putative complex with conjugate corrinoid protein MtbC

MtbB is thought to form a complex with its cognate corrinoid protein MtbC, but this has not been observed directly. In order to detect a complex, and provide evidence as to whether the O356A mutation affects complex formation, possible complex formation was tested by a native gel assay used previously to examine MtmB binding to MtmC (9). Approximately 8 µg of MtbB(O356A) and 2.6 µg of MtbC were incubated at room temperature for 30 minutes and the

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sample electrophoresed through a native gel. A slower migrating protein band formed when wild

type MtbB was co-electrophoresed following incubation with MtbC, but not when MtbB or

MtbC were incubated alone. This is preliminary evidence that a stable complex between MtbB

and MtbC is detectable. In contrast, MtbB(O356A)'s ability to interact with MtbC was

apparently impaired relative to the wild type protein (Figure 9). Minimal formation of the

putative complex indicates that MtbB(O356A) had lost most if not all of its ability to bind MtbC.

This is consistent with either gross mis-folding of MtbB(O356A) or that pyrrolysine is necessary

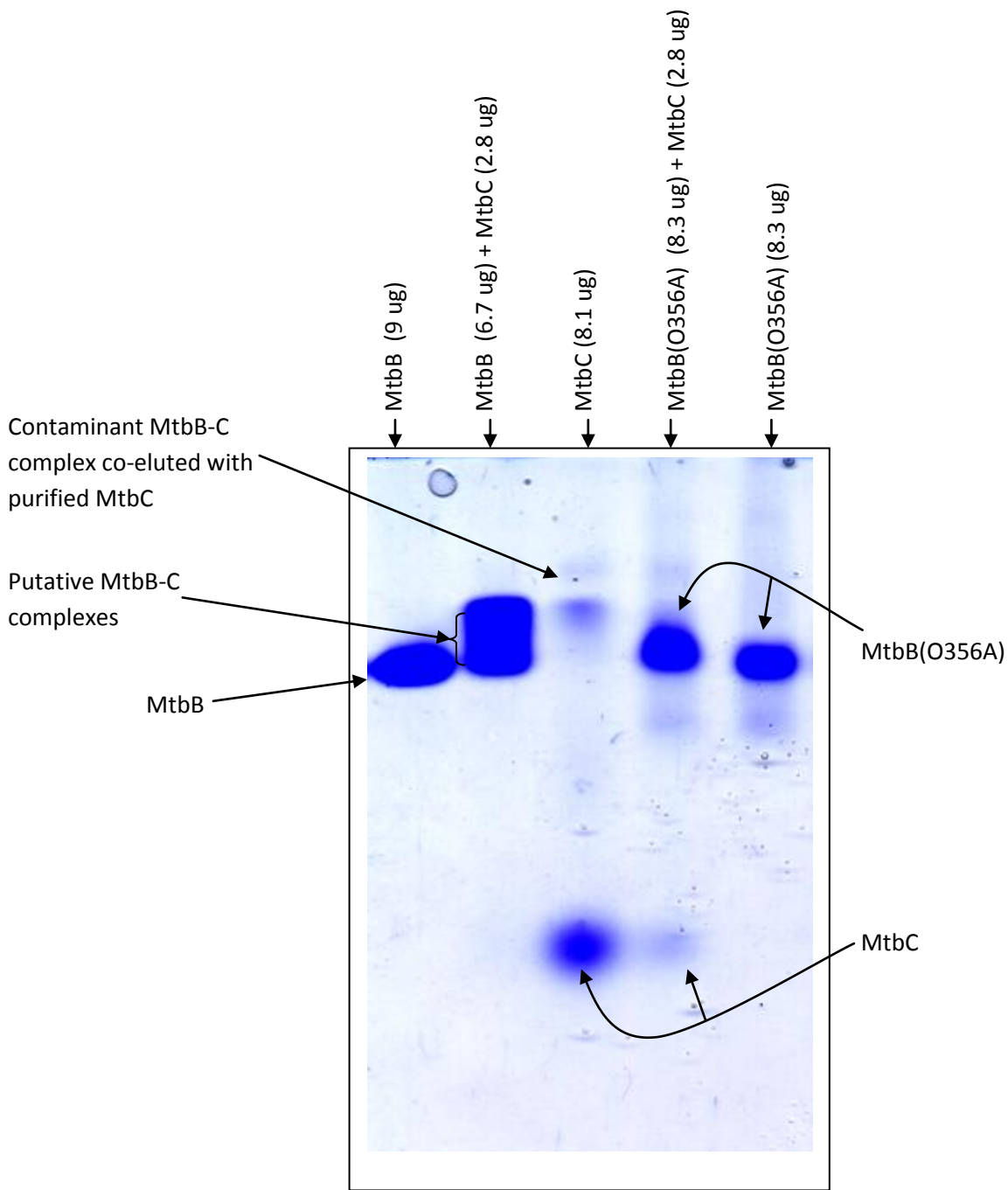for interaction of MtbB and its conjugate corrinoid MtbC.

Figure 9. A native polyacrylamide gel to determine the ability of the mutant MtbB(O356A) to form a slower migrating band in the presence of MtbC, indicating a possible complex with MtbC. Lane names are assigned to correlate with their contents and numbers in brackets correspond to the protein quantity in μg. The proteins were incubated at room temperature for 30 minutes and electrophoresed at 30 mA until the loading dye ran to the end of the gel. The separating layer of the gel consisted of 7.5% bis-acrylamide (37.5:1 acrylamide:bisacrylamide) buffered with 0.38 M Tris-HCl, pH 8.8; the stacking layer comprised of 3.75% bis-acrylamide (37.5:1 acrylamide:bisacrylamide) buffered with 0.13 M Tris-HCl, pH 6.8. The wide band under the MtbB+MtbC lane likely indicates the formation of higher complexes at various ratios of MtbB to MtbC. MtbB(O356A) formed much less of a putative complex with MtbC.

5. Discussion

The in-frame amber codons in the *Methanosarcina* spp. are unique in the sense that they are almost exclusively found in the family of genes encoding methylamine methyltransferases, exception being a putative family of transposases (13; 14). This leads us to believe that the amber encoded pyrrolysine likely plays an exclusive role in methylamine methyltransferases. In fact *in vivo* study with *pylT* (encodes for tRNA^{Pyl}) deletion in *M. acetivorans* resulted in cells unable to metabolize methylamines as substrates, while growth on methanol was not affected (15). Hence, pyrrolysine's exclusiveness in the methylamine methyltransferases and the apparent cessation of enzymatic activity upon its deletion compels us to pursue in finding its role and hence understanding the evolution of the genetic code in *Methanosarcina* spp to use methylamines.

Based on the current experiments pyrrolysine is an essential residue in the dimethylamine methyltransferase MtbB. When the pyrrolysine residue in MtbB was substituted with alanine, the activity of the MtbB(O356A) was negligible relative to that of MtbB. This is consistent with a catalytic role for the amino acid. However, the native gel migration patterns suggest that the mutant MtbB(O356A) has lost most if not all of its ability to interact with MtbC in this preliminary assay for complex formation. Since this result is quite different than that of wild type MtbB, it indicates either of the two possibilities: a) pyrrolysine is essential for proper folding of MtbB or b) pyrrolysine is directly involved in binding MtbC. A circular dichroism spectrum comparing the profiles of the hexahistidine-tagged MtbB and MtbB(O356A) might be helpful in determining if the O356A mutant has any gross folding problems. There also exists a formal possibility that the slower migration patterns are not representative of a MtbBC complex, but are composed of only one or the other protein. A gel filtration chromatography column,

followed by SDS-PAGE analysis could be employed to determine if the ability of MtbB(O356A) to form a higher order homomultimer or heteromeric complex with MtbC has been compromised and whether the putative complexes indeed constitute MtbB(O356A) and MtbC. A western blot of the native gel with anti-MtbB and anti-MtbC antibodies is recommended for future experiments as well.

It should also be noted that only one prep of MtbB(O356A) was used to arrive at the present conclusion that pyrrolysine to alanine substitution in MtbB results in its inability to bind MtbC. An additional native gel experiment with a new prep of MtbB(O356A) is desirable. I am especially concerned with the current result because past experiments have shown that the monomethylamine methyltransferase MtmB with the same site directed mutation (pyrrolysine to alanine) can still bind its conjugate corrinoid protein MtmC (9). It is true that there is no sequence similarity between either of the methyltransferases, but both contain pyrrolysine residue. Moreover, the pyrrolysine residue in MtmB is indeed found in the negatively charged catalytic cleft, and the proposed mechanism of catalysis through the imine bond in pyrrolysine is substantiated by the fact that the when the imine bond in the pyrroline ring is reduced by sodium borohydride MtmB loses its catalytic activity. Similar experiments with MtbB have also revealed that reduction of the imine bond correlates with loss of MtbB methylation of cob(I)alamin (10).

In light of these data, it is disconcerting to arrive at the conclusion that the loss of activity of MtbB(O356A) mutant occurred because of gross mis-folding of the protein. If indeed it is found that pyrrolysine to alanine mutation is unstable, then it becomes necessary to consider alternate mutations wherein pyrrolysine is substituted with leucine, isoleucine or lysine. After confirming that the suggested mutations cause no gross mis-folding of MtbB, the assays should be repeated.

Assay of methylation of the corrinoid cofactor protein MtbC should take precedence over the methylation of cob(I)alamin. As mentioned earlier MtbC is the natural substrate for MtbB and hence gives a 100 fold higher rate of methylation when compared to cob(I)alamin methylation. However, maintaining the corrinoid cofactor of MtbC in the Co(I) state is crucial for the assay. During my attempts of the assay, MtbC-Co(II) reduction by RamA to MtbC-Co(I) was indeed observed but the Co(I) state could not be held stable in the anaerobic cuvette. This implies oxidation of the reagent mix and hence more vigorous anaerobic techniques are recommended. The amount of Ti(III)citrate used may also be increased to maintain the reduced environment in the cuvette.

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