MiaB, a Bifunctional Radical-*S*-Adenosylmethionine Enzyme Involved in the Thiolation and Methylation of tRNA, Contains Two Essential [4Fe-4S] Clusters†

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ABSTRACT: The radical-*S*-adenosylmethionine (radical-AdoMet) enzyme MiaB catalyzes the posttranscriptional methylthiolation of *N*-6-isopentenyladenosine in tRNAs. Spectroscopic and analytical studies of the reconstituted wild-type and C150/154/157A triple variant forms of *Thermotoga maritima* MiaB have revealed the presence of two distinct $[4Fe\text{-}4S]^{2+,1+}$ clusters in the protein. One is coordinated by the three conserved cysteines in the radical-AdoMet motif (Cys150, Cys154, and Cys157) as previously reported, and the other, here observed for the first time, is proposed to be coordinated by the three N-terminal conserved cysteines (Cys10, Cys46, and Cys79). The two $[4Fe\text{-}4S]^{2+}$ clusters have similar UV−visible absorption, resonance Raman, and Mössbauer properties but differ in terms of redox properties and the EPR properties of the reduced $[4Fe\text{-}4S]^{1+}$ clusters. Reconstituted forms of MiaB containing two [4Fe-4S] clusters are more active than previously reported. Comparison of MiaB with other radical-AdoMet enzymes involved in thiolation reactions, such as biotin synthase and lipoate synthase, is discussed as well as a possible role of the second cluster as a sacrificial S-donor in the MiaB-catalyzed reaction.

Modified nucleosides are found in transfer RNAs (tRNAs)[1] of all three domains of life and are crucial to their biological activity (*1*). Complex maturation processes have been shown to be involved in the posttranscriptional modification of tRNA nucleosides at different positions, most of these being located either in the "wobble" position of the anticodon or in the position immediately adjacent to the 3′ end of the anticodon (*2*). Several of these modifications involve thiolation of tRNA nucleosides, and in *Escherichia coli* four different thiolated nucleosides have been characterized: 4-thiouridine (s^4U), 2-thiocytidine (s^2C), 5-methylaminomethyl-2-thiouridine (mnm^5s^2U), and 2-methylthio-*N*-6-isopentenyladenosine (ms^2i^6A). Sulfur incorporation in tRNA nucleosides is proposed to proceed via two distinct pathways (*3*, *4*). The first one is a non-redox substitution of an oxygen atom by sulfur, as in the case of the biosynthesis of s^4U. The reaction is dependent on IscS, a cysteine desulfurase enzyme, and thus begins with the liberation of a sulfur atom from cysteine by IscS followed by its subsequent transfer to its tRNA-modifying enzyme, ThiI, in the form of a cysteine persulfide (*5*−*7*). The same type of mechanism has been demonstrated for mnm^5s^2U biosynthesis. In this case, a chain of proteins (TusA−E), mediates sulfur transfer from IscS to MnmA, the tRNA-modifying enzyme (*8*). The second pathway is illustrated by the reaction leading to the synthesis of 2-methylthio-*N*-6-isopentenyladenosine (ms^2i^6A) which involves a highly challenging aromatic C−H to C−S bond conversion. The reaction involves the MiaB protein which utilizes an [Fe-S] center and *S*-adenosylmethionine (AdoMet) to achieve the thiolation of nucleosides (*9*, *10*).

Located at position 37 next to the anticodon on the 3′-position, ms^2i^6A-37 is found in almost all eukaryotic and bacterial tRNAs that read codons beginning with U except $tRNA^{I,V}Ser$ (*11*). The first step in ms^2i^6A-37 biosynthesis is catalyzed by MiaA and involves the addition of an isopentenyl group at the N-6 nitrogen of adenosine, converting adenosine-37 to i^6A-37; see Scheme 1 (*12*−*14*). The second step, catalyzed by MiaB, consists of both sulfur insertion and methylation at position 2 of i^6A-37, forming ms^2i^6A-37. Initially, iron, cysteine, and AdoMet were shown to be involved in the methylthiolation of i^6A (*15*−*17*), but it was not until the *miaB* gene was identified and the corresponding protein shown to contain the highly conserved CysXXX-CysXXCys motif that MiaB was speculated to be a radical-activating enzyme that binds iron (*18*). Subsequently, the MiaBs from *E. coli* and *Thermotoga maritima* were shown to be radical-AdoMet enzymes requiring AdoMet and a

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[1] Abbreviations: tRNA, transfer ribonucleic acid; i^6A-37, the modified nucleoside *N*-6-isopentenyladenosine at position 37; ms^2i^6A-37, the modified nucleoside 2-methylthio-*N*-6-isopentenyladenosine at position 37; AdoMet, *S*-adenosylmethionine; WT, wild type; LipA, lipoate synthase; BioB, biotin synthase; AdoH, 5′-deoxyadenosine.

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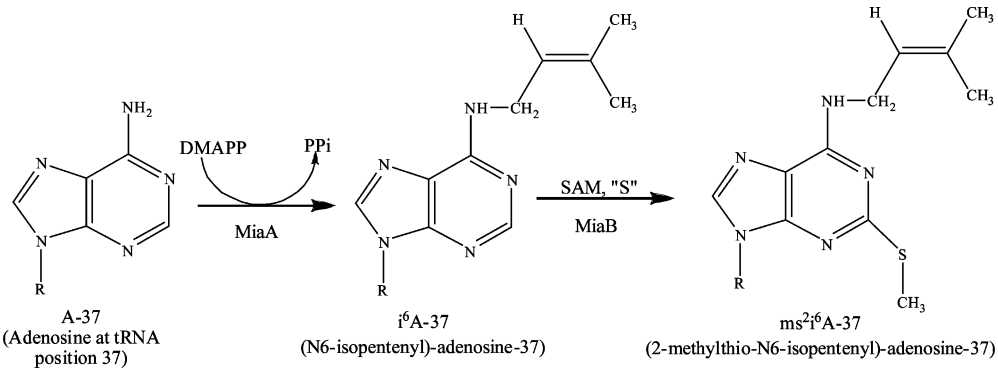
Scheme 1



[4Fe-4S] cluster chelated by the CysXXXCysXXCys (Cys150, Cys154, Cys157 in *T. maritima* MiaB) motif for catalytic activity (*10*, *19*, *20*).

Radical-AdoMet enzymes are the members of a superfamily of proteins consisting of over 600 putative enzymes (*21*). These enzymes share a common mechanism that utilizes a $[4Fe\text{-}4S]^{2+,1+}$ cluster to initiate a radical reaction by mediating reductive cleavage of AdoMet to yield a 5′-deoxyadenosyl radical. This radical then abstracts a hydrogen atom from an appropriately placed substrate, creating a substrate-based carbon radical (*22*−*24*). In the formation of ms^2i^6A-37, the H atom is supposed to be abstracted at position 2 of the base moiety, and then the activated carbon is thiolated and methylated. Recent studies revealed that MiaB is responsible for both the thiolation and methylation steps by using two molecules of AdoMet. The first AdoMet is used for radical generation and substrate carbon activation, while the second AdoMet is utilized in the more traditional role as the methyl donor (*10*).

Like MiaB, several other radical-AdoMet enzymes catalyze the thiolation of substrates, including biotin synthase (BioB) (*25*), lipoic acid synthase (LipA) (*26*), and possibly HydE or HydG (*27*). The source of the inserted sulfur has been the cause of much debate in the literature (*9*). Interestingly, BioB, LipA, and HydE have been shown to contain a second Fe-S cluster in addition to the radical-AdoMet [4Fe-4S] cluster: a [2Fe-2S] cluster in BioB (*28*, *29*) and an additional [4Fe-4S] cluster in LipA and HydE (*27*, *30*). These additional clusters have been proposed to be the physiological S-donor to the substrate in BioB and LipA. A recent 3.4 Å resolution crystal structure of BioB revealed a novel [2Fe-2S] cluster bound by three cysteine residues and one arginine residue and showed that a bridging S atom of the [2Fe-2S] is positioned close to the S-insertion site of the substrate, dethiobiotin (*31*). When Se was introduced into BioB and MiaB or ^{34}S into LipA, forming [2Fe-2Se/^{34}S] or [4Fe-4Se/^{34}S] clusters, Se/^{34}S was shown to be incorporated into the respective substrates (*10*, *32*, *33*). Thus far, only one other radical-AdoMet enzyme, MOCS1A/MoaA, is known to contain a second cluster (*34*, *35*). MOCS1A/MoaA is not involved in thiolation of substrate, and a recent crystal structure places its substrate, 5′-GTP, within binding distance of the non-radical-AdoMet cluster, implying that the cluster may act to position the substrate for H-abstraction by the 5′-deoxyadenosyl radical (*36*).

In this work, *T. maritima* MiaB is also shown to contain two Fe-S clusters, an N-terminal $[4Fe\text{-}4S]^{2+,1+}$ cluster in addition to the radical-AdoMet $[4Fe\text{-}4S]^{2+,1+}$ cluster, and activity measurements indicate that both clusters are required for optimal activity. The spectroscopic and redox properties of each [4Fe-4S] cluster have been investigated by comparing the UV−visible absorption, EPR, resonance Raman, and Mössbauer properties of wild-type MiaB, containing both clusters, to those of C150/154/157A MiaB, containing only the N-terminal [4Fe-4S] cluster. Possible roles for the latter are discussed.

MATERIALS AND METHODS

General Procedures. All DNA manipulations were as described previously. Enzymes, oligonucleotides, and culture media were purchased from Invitrogen, Cergy-Pontoise, France. The DNA purification kit, Flexiprep, was from Amersham-Pharmacia, Inc. DNA fragments were extracted from agarose gel and purified with the High Pure PCR product purification kit (Roche, Inc.). DNA sequencing was performed by Genome Express Co. (Grenoble, France).

Strains. E. coli DH5α was used for routine DNA manipulations. *E. coli* BL21CodonPlus(DE3)-RIL (Stratagene), which contains extra copies of genes encoding tRNA with codons rarely used in *E. coli* (argU, ileY, leuW tRNA genes), was used to produce the recombinant protein MiaB and triple mutant form.

Construction of the C10A Single Variant and the C150/154/157A Triple Variant of T. maritima MiaB. The cloning of the wild-type MiaB into expression plasmid pT_7-7 has already been described (*20*). Site-directed mutagenesis was performed on plasmid pT_7-MiaBTm with QuikChange site-directed mutagenesis kits from Stratagene according to the manufacturer's protocol. The oligonucleotides used as mutagenic primers are as follows: For the C10A single mutant, forward, 5′-cataaagaccttcggcgctcagatgaacgagaac-3′; reverse, 5′-gttctcgttcatctgagcgccgaaggtctttatg-3′. For the C150/154/157A triple variant, forward, 5′-gtcacgatcatccacggcgctgacaggttcgctacctatgccatcgttccctac-3′; reverse, 5′-gtagggaacgatggcataggtagcgaacctgtcagcgccgtggatgatcgtgac-3′. The identity of the cloned DNA sequence coding for the MiaBC10A and MiaBC150/154/157A mutated proteins was confirmed by sequencing of the entire genes.

Expression and Purification of WT and C150/154/157A T. maritima MiaB. All expressions were conducted in LB medium at 37 °C in the *E. coli* BL21CodonPlus(DE3)-RIL as previously described (*20*). All proteins were purified under aerobic conditions and contained only trace quantities of Fe-S clusters as judged by UV−visible absorption spectroscopy. Apoproteins were obtained by overnight exposure to EDTA

(10 mM) under reducing conditions (10 mM sodium dithionite). Following purification using a gel filtration column (G-25) equilibrated with 50 mM Tris-HCl buffer, pH 8, with 200 mM NaCl, the protein was washed and concentrated using a Centricon with a 30 kDa membrane.

Reconstitution of As-Prepared and Apo Forms of WT and C150/154/157A T. maritima MiaB. Fe-S cluster reconstitutions of WT and C150/154/157A *T. maritima* MiaB were carried out under strictly anaerobic conditions in a Vacuum Atmospheres glovebox containing less than 2 ppm O_2. Following incubation of the as purified or apoproteins with 10 mM DTT for 10 min, a 10-fold molar excess of Fe^{II}-$(NH_4)_2(SO_4)_2$ was added, followed by the addition of a 10-fold molar excess of Na_2S or a 20-fold excess of L-cysteine in the presence of catalytic amount of cysteine desulfurase (IscS). After 3 h, the reconstitution mixture was loaded onto 10 mL HiTrap Q columns previously equilibrated with buffer A (50 mM Tris-HCl, pH 8.0, plus 50 mM NaCl) and eluted with a 0−100% gradient of buffer B (100 mM Tris-HCl, pH 8.0, plus 1 M NaCl). The brown fractions were concentrated over a YM30 (Amicon) membrane and washed in buffer C (100 mM Tris-HCl, pH 8.0, plus 200 mM NaCl). Identical outcomes were achieved whether starting from apo- or as purified protein. The same procedure was used to prepare Mössbauer samples except that ^{57}Fe-enriched Fe^{II}-$(NH_4)_2(SO_4)_2$ was used. ^{57}Fe-enriched $Fe^{II}(NH_4)_2(SO_4)_2$ was prepared from ^{57}Fe metal (>95% isotopic enrichment) as previously described (*37*).

Analysis of tRNA Nucleoside Composition by HPLC. i^6A-37-tRNAs and the in vitro assay solutions were digested to nucleosides by the method of Gehrke et al. by using nuclease P1 and bacterial alkaline phosphatase (*38*). The digested tRNAs (50−100 μg) were loaded onto a Zorbax SB-C-18 column connected to a HP-1100 HPLC system. The short gradient profile developed by Gehrke and Kuo was used to separate the different nucleosides. The i^6A-37 and ms^2i^6A-37 were quantified as reported (*10*).

In Vitro Enzyme Assay. The assay mixture (50 μL) contained, in 100 mM Tris-HCl, pH 7.5, 1 mM SAM, 2 mM dithionite, 100−150 μg of bulk tRNAs, and 50 μM reconstituted MiaB protein. The reaction was carried out at 50 °C under anaerobic conditions, 50 μL of water was added to the assay mixture, and tRNAs were extracted by phenol treatment and then ethanol precipitated. tRNAs were digested and analyzed by HPLC for ms^2i^6A content as described above (*10*).

Protein, Fe, Labile Sulfide, and Activity Assays. Protein concentrations were determined by the DC protein assay (Bio-Rad), using BSA as a standard. All sample concentrations are based on protein determinations. Iron concentrations were determined colorimetrically using bathophenanthroline under reducing conditions, after digestion of the protein in 0.8% $KMnO_4$/0.2 M HCl (*39*). Labile sulfide was determined according to standard procedure (*40*). The bulk of tRNA containing i^6A-37-tRNA substrate was prepared, and the activity assays were conducted as previously described (*10*).

Spectroscopic Characterization of Fe-S Centers. UV−visible absorption spectra were recorded under anaerobic conditions in screw-top 1 mm cuvettes using a Shimadzu UV-3101PC spectrophotometer. Resonance Raman spectra were recorded using an Instruments SA U1000 spectrometer fitted with a cooled RCA 31034 photomultiplier tube with 90° scattering geometry. Spectra were recorded digitally using photon-counting electronics, and improvements in signal-to-noise ratio were achieved by signal-averaging multiple scans. Band positions were calibrated using the excitation frequency and are accurate to ± 1 cm^{-1}. Lines from a Coherent Sabre 10-W argon ion laser were used for excitation, and plasma lines were removed using a Pellin Broca prism premonochromator. For each sample, the laser power at the samples was ~200 mW, and slit widths were adjusted for each excitation wavelength to give 8.0 cm^{-1} spectral resolution. Scattering was collected from the surface of a frozen 17 μL droplet of sample using a custom-designed anaerobic sample cell attached to the cold finger of an Air Products Displex model CSA-202E closed cycle refrigerator. This enables samples to be cooled down to 18 K, which facilitates improved spectral resolution and prevents laser-induced sample degradation. X-band EPR spectra were recorded on a Bruker ESP-300E EPR spectrometer equipped with an ER-4116 dual-mode cavity and an Oxford Instruments ESR-9 flow cryostat. Resonances were quantified under nonsaturating conditions by double integration against a 1 mM CuEDTA standard. Mössbauer spectra were recorded at 4.2 K in a 50 mT magnetic field applied parallel to the γ-radiation using the spectrometer previously described (*41*) and analyzed with the program WMOSS (Web Research). The zero velocity of the spectra refers to the centroid of the room temperature spectrum of a metallic iron foil.

RESULTS

Evidence for a Second Cluster in WT MiaB. Samples of WT MiaB purified under aerobic conditions contain a substoichiometric amount of Fe and S atoms (*20*). However, a functional form of WT MiaB containing 4 Fe and 4 S ions can be prepared by standard anaerobic cluster reconstitution procedures. In previous reports reconstitution of clusters in the apo form using an excess of ferrous ion and Na_2S in the presence of DTT resulted in a protein containing an average of 4 Fe and 4 acid-labile S atoms per monomer, suggesting the presence of only one [4Fe-4S] in the MiaB enzyme (*10*, *20*). However, the procedure included a step in which the reconstituted enzyme was treated by EDTA in order to chelate the excess of iron and to remove it by a gel filtration step (G-25). We reasoned that this rather drastic treatment might have removed more than adventitiously bound iron. A new procedure using ferrous ion and sulfur in the form of Na_2S or provided by the cysteine/IscS system (as described in the Materials and Methods section) excluded the EDTA treatment. Purification was achieved either by a gel filtration column (G-25) or by anion-exchange chromatography (HiTrap Q) inside a glovebox at <2 ppm O_2. Under these conditions, the samples of WT MiaB protein contained up to 8 Fe per protein, suggesting the presence of two [4Fe-4S] clusters. As there are only three other cysteines that are conserved in all MiaB proteins (*20*), Cys10, Cys 46, and Cys79 in *T. maritima* MiaB, these residues are clearly the best candidates for the ligands to a second [4Fe-4S] cluster. To confirm the presence of such a second center and investigate its properties, all three of the cysteine ligands to the radical-AdoMet [4Fe-4S] cluster were replaced with alanine. The C150/154/157A triple variant of *T. maritima* MiaB was expressed and purified primarily as a protein containing traces of iron and acid-labile sulfide as described

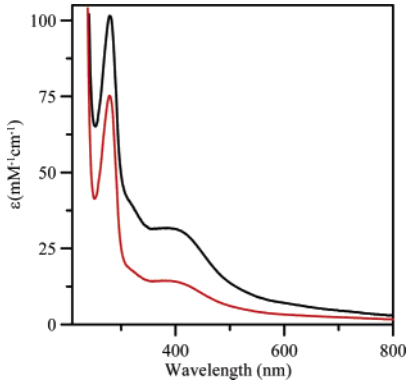


FIGURE 1: UV−visible absorption spectra of reconstituted wild-type (black) and C150/154/157A (red) *T. maritima* MiaB. The buffering medium was 100 mM Tris-HCl, pH 8.0, with 200 mM NaCl, and the sample concentrations were 108 and 194 μM for wild type and the C150/154/157A variant, respectively. The spectra were recorded in 1 mm cuvettes, and the molar extinction coefficients are based on protein concentrations.

in the Materials and Methods section. Anaerobic cluster reconstitution yielded a protein containing approximately 4 Fe atoms per MiaB monomer, in agreement with the presence of only one [4Fe-4S] cluster and with the three conserved cysteine residues, Cys10, Cys46, and Cys79, acting as cluster ligands.

Analytical and Spectroscopic Characterization of WT and C150/154/157A MiaB. Iron and protein analyses of the reconstituted proteins used for spectroscopic analyses indicated the presence of approximately 7.8 ± 0.6 Fe per MiaB for WT (average of three preparations) and 3.8 ± 0.5 Fe per C150/154/157A triple variant (average of three preparations). In both cases EPR studies showed the presence of varying amounts of reduced cluster (0−0.5 $S = {}^1/_2$ [4Fe-4S]$^{1+}$ per MiaB based on EPR quantitation; data not shown). The UV−visible spectra of both proteins in the fully oxidized state are displayed in Figure 1. The UV−visible spectrum of WT MiaB (Figure 1, black line) comprises a broad shoulder centered at 400 nm that is characteristic of [4Fe-4S]$^{2+}$ clusters and has an A_{400}/A_{280} ratio of 0.31. Moreover, the molar extinction coefficient at 400 nm ($\epsilon_{400} = 32$ mM^{-1} cm^{-1}) is characteristic of two [4Fe-4S]$^{2+}$ clusters per MiaB, as biological [4Fe-4S]$^{2+}$ centers typically have $\epsilon_{400} = 15-17$ mM^{-1} cm^{-1} on a per cluster basis. The UV−visible absorption spectrum of C150/154/157A MiaB (Figure 1, red line) is also characteristic of a [4Fe-4S]$^{2+}$ cluster (A_{400}/A_{280} ratio = 0.19), and the molar extinction coefficient, $\epsilon_{400} = 14$ mM^{-1}cm^{-1}, is indicative of ~0.9 [4Fe-4S]$^{2+}$ clusters per protein, in excellent agreement with the Fe determinations.

The resonance Raman spectra of reconstituted WT and C150/154/157A MiaB shown in Figure 2 confirmed the presence of [4Fe-4S]$^{2+}$ clusters in both samples and facilitated a more detailed comparison of the vibrational and structural properties of the N-terminal and radical-AdoMet [4Fe-4S]$^{2+}$ centers. The resonance Raman spectra of both samples in the Fe−S stretching region are uniquely characteristic of [4Fe-4S]$^{2+}$ clusters and are readily assigned on the basis of the detailed vibrational studies that are available for [4Fe-4S]$^{2+}$ centers in ferredoxins and model complexes (*42*, *43*). Moreover, the near-congruent resonance Raman spectra for both samples indicate structurally very similar [4Fe-4S]$^{2+}$ centers, each ligated by three cysteine ligands, in the N-terminal and radical-AdoMet binding sites.

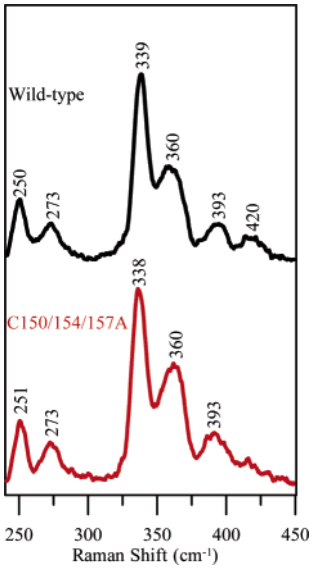


FIGURE 2: Resonance Raman spectra of reconstituted wild-type (black) and C150/154/157A (red) *T. maritima* MiaB. The resonance Raman spectra were recorded with 458 nm excitation using samples in the form of a frozen droplet at 17 K that were in 100 mM Tris-HCl buffer, pH 8.0, with 200 mM NaCl and ~3 mM in MiaB. Each scan involved photon counting for 1 s at 0.5 cm^{-1} increments with 8 cm^{-1} spectral resolution, and each spectrum is the sum of ~100 scans. Bands originating from lattice modes of ice and a linear ramp fluorescent background have been subtracted from each spectrum.

This is further supported by Mössbauer spectra of samples reconstituted using ^{57}Fe; see Figure 3. The spectra of both WT and C150/154/157A MiaB are dominated by quadrupole doublets (green lines) arising from the $S = 0$ [4Fe-4S]$^{2+}$ clusters that are well fit as a superposition of two equal intensity doublets representing the two valence-delocalized Fe^{2+}/Fe^{3+} pairs, one with $\delta = 0.46$ mm/s and $\Delta E_Q = 1.27$ mm/s and the other with $\delta = 0.44$ mm/s and $\Delta E_Q = 1.03$ mm/s. The Mössbauer spectra also indicated that 29% and 42% of the ^{57}Fe in the MiaB WT and C150/154/157A variant samples, respectively, are present as $S = {}^1/_2$ [4Fe-4S]$^{1+}$ clusters, the spectrum of which (red lines) is well simulated by using the parameters reported for reduced *Bacillus stearothermophilus* [4Fe-4S] ferredoxin (*44*). On the basis of Fe and protein determinations of the samples used for Mössbauer analysis (7.8 Fe/MiaB for WT and 4.3 Fe/MiaB for the C150/154/157A triple variant), the Mössbauer data indicate that 0.56 and 0.46 clusters/MiaB monomer in the WT and triple variant, respectively, are in the [4Fe-4S]$^{1+}$ state. The presence of [4Fe-4S]$^{1+}$ clusters in these samples is supported by parallel EPR studies of the same samples (Figure 4C), which revealed identical near-axial $S = {}^1/_2$ resonances with $g_{\|} = 2.06$ and $g_{\perp} = 1.94$. Spin quantification indicated 0.53 spin/MiaB for WT and 0.43 spin/MiaB for the C150/154/157A triple variant, in excellent agreement with Mössabuer results. In agreement with the fast relaxation properties that are characteristic of $S = {}^1/_2$ [4Fe-4S]$^{1+}$ clusters, these resonances are only observable at temperatures below 50 K. The complete absence of low-field resonances in the $g = 5$ region indicative of $S > {}^1/_2$ species, at low temperatures (4.2 K) and high microwave powers (50 mW), demonstrates that the [4Fe-4S]$^{1+}$ clusters in as-prepared reconstituted samples have exclusively $S = {}^1/_2$ ground states.

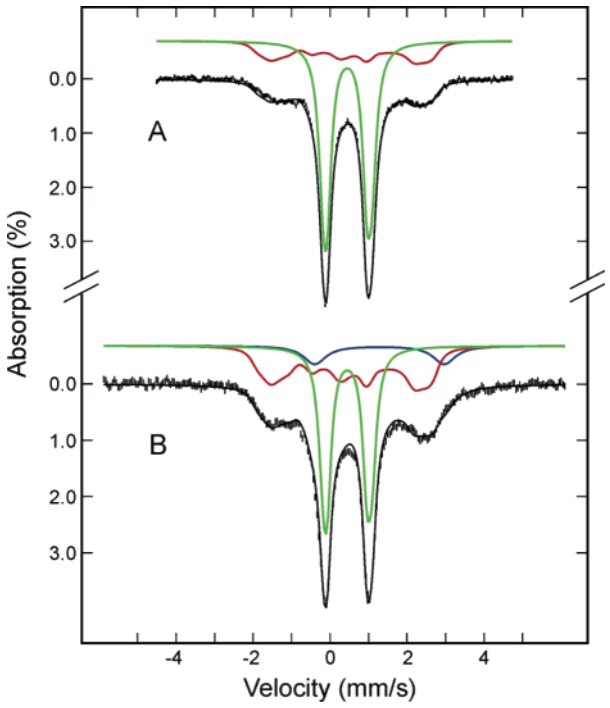


FIGURE 3: Mössbauer spectra (hatched marks) of reconstituted wild-type (A) and C150/154/157A *T. maritima* MiaB (B). The spectra were recorded at 4.2 K in a magnetic field of 50 mT oriented parallel to the γ-radiation. The black lines are composite simulations constructed from $S = 0$ $[4Fe\text{-}4S]^{2+}$ clusters comprising two equal intensity doublets with $\delta = 0.46$ mm/s and $\Delta E_Q = 1.27$ mm/s and $\delta = 0.44$ mm/s and $\Delta E_Q = 1.03$ mm/s (green) and $S = {}^1/_2$ $[4Fe\text{-}4S]^{1+}$ simulated with the parameters reported for reduced *B. stearothermophilus* ferredoxin (red), an extraneous ferrous species (blue). The spectrum of the reconstituted WT MiaB (125 μM) is simulated with 29% of the ^{57}Fe in $[4Fe\text{-}4S]^{1+}$ clusters and 71% in $[4Fe\text{-}4S]^{2+}$ clusters. The spectrum of the reconstituted C150/154/157A MiaB (293 μM) is simulated with 42% of the ^{57}Fe in $[4Fe\text{-}4S]^{1+}$ clusters, 48% in $[4Fe\text{-}4S]^{2+}$ clusters, and 10% in extraneous ferrous species.

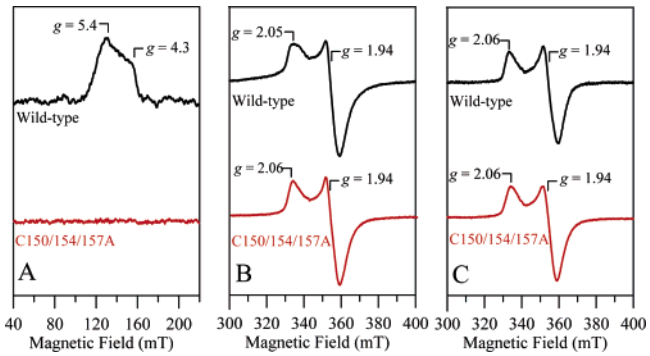


FIGURE 4: X-band EPR spectra of reconstituted wild-type (black) and C150/154/157A (red) *T. maritima* MiaB: (A) dithionite-reduced in the $S = {}^3/_2$ region; (B) dithionite-reduced in the $S = {}^1/_2$ region; (C) as-prepared Mössbauer samples in the $S = {}^1/_2$ region. The samples are described in Figures 1 and 3 and were reduced anaerobically with a 10-fold excess of sodium dithionite. Spectra were recorded at 20 K with a microwave power of 10 mW in the $S = {}^1/_2$ region and at 4.2 K with a microwave power of 50 mW in the $S = {}^3/_2$ region. The modulation amplitude was 0.64 mT, and the microwave frequency was 9.59 GHz for all spectra.

Differences in the properties of the reduced N-terminal and radical-AdoMet $[4Fe\text{-}4S]^{1+}$ clusters in MiaB are apparent in EPR studies of dithionite-reduced samples; see Figure 4A,B. The N-terminal $[4Fe\text{-}4S]^{1+}$ center in dithionite-reduced reconstituted C150/154/157A MiaB is exclusively $S = {}^1/_2$ and fully reducible by dithionite, as evidenced by spin quantifications and the absence of low-field resonances in the $g = 5$ region. The $S = {}^1/_2$ resonance ($g_{\|} = 2.06$ and $g_{\perp} = 1.94$) is identical to that observed in as-prepared reconstituted samples of both WT and C150/154/157A MiaB and corresponds to 0.9 spin/MiaB. In contrast, dithionite-reduced reconstituted MiaB WT shows a broad low-field resonance centered at $g = 5.4$ at low temperatures and high microwave powers that is indicative of a near-rhombic $S = {}^3/_2$ $[4Fe\text{-}4S]^{1+}$ center and a significantly broadened resonance in the $S = {}^1/_2$ region that accounts for ~1.1 spins/MiaB. The $S = {}^1/_2$ resonance has principal g-values similar to those of the N-terminal $S = {}^1/_2$ $[4Fe\text{-}4S]^{1+}$ center but is broader and has developed ill-defined "wings" to lower and higher field. Such EPR characteristics are indicative of weak intercluster magnetic interactions between paramagnetic clusters separated by 12−20 Å (*45*). Hence, in common with other radical-AdoMet $[4Fe\text{-}4S]^{1+}$ clusters (*46*, *47*), the $[4Fe\text{-}4S]^{2+}$ cluster in the radical-AdoMet binding site of MiaB is at least partially reducible by dithionite at pH 8 and most likely is present as a mixed-spin species with both $S = {}^1/_2$ and ${}^3/_2$ components. The picture that emerges from the EPR studies of dithionite-reduced samples is that reconstituted WT MiaB contains two $[4Fe\text{-}4S]^{1+}$ clusters separated by 12−20 Å. The N-terminal $[4Fe\text{-}4S]^{2+,1+}$ cluster has a significantly higher redox potential resulting in partial reduction in the absence of dithionite, and the reduced $[4Fe\text{-}4S]^{1+}$ cluster is exclusively $S = {}^1/_2$ with $g_{\|} = 2.06$ and $g_{\perp} = 1.94$. The radical-AdoMet $[4Fe\text{-}4S]^{2+,1+}$ cluster has a lower redox potential but is reduced at least partially by dithionite at pH 8, and the reduced $[4Fe\text{-}4S]^{1+}$ cluster exists as a mixture of forms with $S = {}^1/_2$ and ${}^3/_2$ ground states.

Activity of WT, C10A, and C150/154/157A MiaB. WT and C150/154/157A MiaB were assayed for their ability to catalyze the production of ms^{2}i^{6}A from i^{6}A substrate. For the in vivo assay, a *miaB*$^-$ *E. coli* strain was transformed with expression plasmids for WT and mutants of MiaB. After 16 h growth, cells were collected, and their tRNAs were purified, digested, and analyzed by HPLC for the presence of ms^{2}i^{6}A as previously described (*10*). In order to evaluate the functional importance of the second, N-terminal, cluster in this in vivo assay, we also prepared an expression plasmid for the C10A MiaB mutant in which the first conserved cysteine was changed to alanine. In the in vitro assay, pure proteins were assayed anaerobically in the presence of a slightly purified preparation of the tRNA substrate and AdoMet, using dithionite as a source of reducing equivalents (see Materials and Methods). It is important to note that no external sulfur source is added in the assay mixture and the enzyme is thus not expected to turn over under these conditions.

As expected, the C150/154/157A MiaB, which lacks the radical-AdoMet [4Fe-4S] cluster, was inactive in the in vivo assay as evidenced by the absence of a peak at 57 min in the chromatogram corresponding to ms^{2}i^{6}A (Figure 5A). Likewise, transformation with the plasmid expressing the C10A MiaB mutant did not result in the production of ms^{2}i^{6}A (Figure 5A). The purified and reconstituted C150/154/157A MiaB was also found to be inactive under in vitro conditions (data not shown). In contrast, the WT MiaB enzyme was active in both assays (Figure 5B shows the in vitro activity).

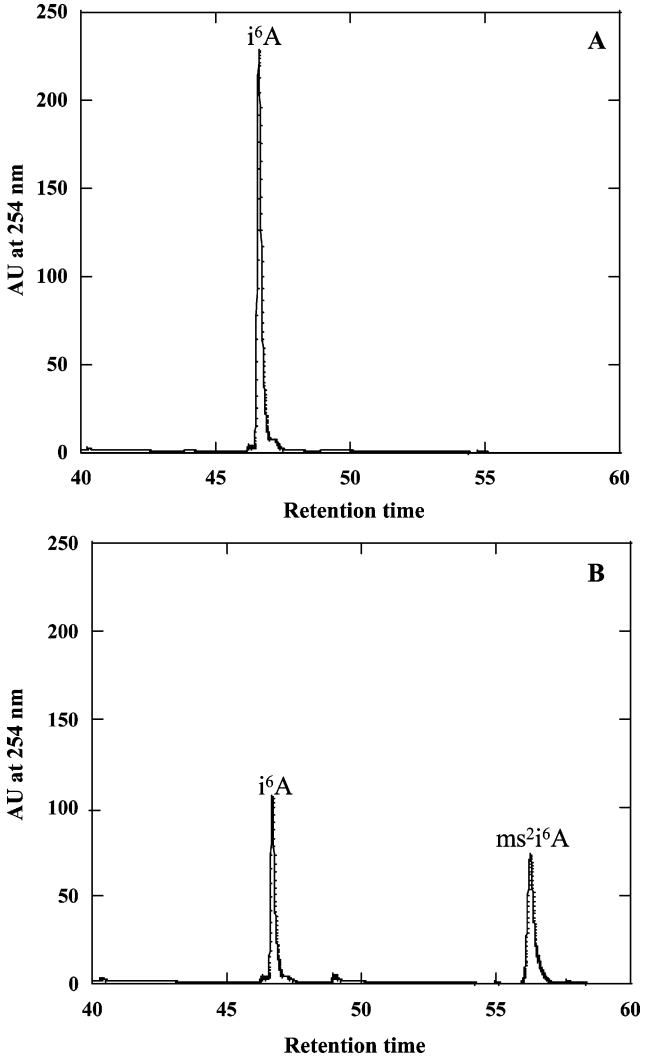


FIGURE 5: HPLC chromatograms of tRNA hydrolysates. Panel A: from an in vivo complementation of *miaB*$^-$ *E. coli* strain transformed with pT_7-MiaBC10A or pT_7-miaBC150/154/157A. Panel B: from an in vitro assay of reconstituted WT MiaB containing two [4Fe-4S] clusters. The identification of i^6A and ms^2i^6A is based on UV−visible spectra (data not shown) and retention times (i^6A elutes at ~47 min and ms^2i^6A at ~57 min).

The in vitro production of ms^2i^6A was monitored as a function of reaction time. In Figure 6, it is shown that there is a time-dependent production of ms^2i^6A during the first 10−20 min and then a plateau is observed after about 20−30 min reaction. Quantification revealed that the reaction stopped after production of approximately 1 ms^2i^6A per MiaB monomer. Thus the enzyme does not make more than one turnover. When the assay was carried out with a WT MiaB preparation containing only 4 Fe and 4 acid-labile S atoms, no more than 0.2−0.3 ms^2i^6A per MiaB monomer could be produced as previously reported (*10*). The results shown in Figure 6 and the effect of the mutation at the conserved cysteine 10 on the activity strongly support the concept that the N-terminal cluster plays a functional role.

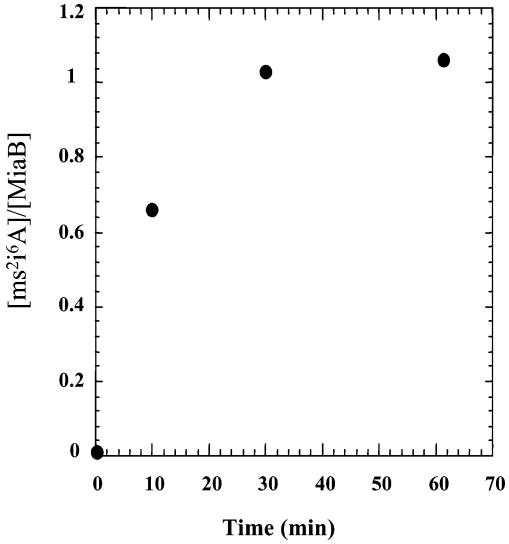


FIGURE 6: $[ms^2i^6A]/[MiaB]$ as a function of reaction time. The assay mixture (50 μL) contained 100−150 μg of bulk tRNAs and 50 μM reconstituted MiaB protein in 100 mM Tris-HCl buffer, pH 7.5, with 1 mM AdoMet and 2 mM dithionite. The reaction was carried out at 50 °C under anaerobic conditions.

DISCUSSION

There are very few redox reactions of tRNA nucleoside modification. Formation of ms^2i^6A, which consists in the introduction of a methylthio group at position 2 of adenosine, is one of the rare examples. Accordingly, the MiaB enzyme which catalyzes this reaction is a redox protein and is unique in terms of tRNA-modifying enzyme in that it requires iron−sulfur clusters for activity (*10*, *19*). In the original characterization of MiaB protein from *T. maritima* we have reported that MiaB contained an average of 4 Fe and 4 acid-labile S atoms per monomer, which we proposed to form one [4Fe-4S] cluster (*20*). In the same study we have speculated that this [4Fe-4S] is chelated by the CysXXXCysXXCys motif and thus MiaB is a radical-AdoMet enzyme.

The spectroscopic and analytical results reported here unambiguously demonstrate the presence of two, and not one, $[4Fe\text{-}4S]^{2+,1+}$ clusters in MiaB, and the activity studies indicate that both are important for methylthiolation of *N*-6-isopentenyladenosine in tRNAs in a single turnover reaction. While the role of the radical-AdoMet [4Fe-4S] cluster clearly lies in generating a 5′-deoxyadenosyl radical and thereby activating the substrate for methylthiolation via H-abstraction (*10*), the role of the N-terminal [4Fe-4S] cluster remains to be established. By analogy with MoaA/MOCS1A (*36*), one possibility is that the N-terminal cluster plays a role in positioning the tRNA substrate for effective H-abstraction at position 2 of i^6A-37. To investigate this possibility, the properties of the oxidized and reduced N-terminal $[4Fe\text{-}4S]^{2+,1+}$ clusters in C150/154/157A MiaB variant were investigated by resonance Raman and EPR, respectively, in the presence and absence of an excess of the i^6A-37-tRNA substrate. The results (data not shown) clearly demonstrate that the presence of the substrate has no significant effect on the resonance Raman spectrum of the $[4Fe\text{-}4S]^{2+}$ cluster or the EPR spectrum of the $[4Fe\text{-}4S]^{1+}$ cluster and hence argue against a role for the N-terminal cluster in interacting with the substrate.

MiaB has many similarities to lipoate synthase (LipA) and biotin synthase (BioB). First, they are all radical-AdoMet enzymes that also catalyze the insertion of the sulfur into their respective substrates (*9*). Second, the three proteins, MiaB, BioB, and LipA, contain three conserved cysteine residues, in addition to the three cysteines in the CysXXXCysXXCys motif that is common to all members of the

radical-AdoMet superfamily. In MiaB from *T. maritima* a ^{10}Cys-Xaa$_{35}$-^{46}Cys-Xaa$_{32}$-^{79}Cys motif, which is conserved in all MiaB proteins, is localized upstream of the ^{150}CysXXX^{154}-CysXX^{157}Cys sequence in the N-terminal half of the enzyme (*20*). In the LipA protein the three additional conserved cysteines are also in the N-terminal region, but with a CysXaa$_4$CysXaa$_5$Cys motif (*30*). In the BioB enzyme, the three cysteine residues that are not part of the radical-AdoMet motif are found downstream of the CysXXXCysXXCys sequence in the C-terminal domain of the enzyme (*31*). Third, the available evidence does suggest that the N-terminal [4Fe-4S] cluster of MiaB may function as a sacrificial S-donor during single turnover experiments; a similar role has been suggested for the [2Fe-2S] cluster in BioB (*31*, *48*) and the additional [4Fe-4S] cluster in LipA (*30*). Prior to the discovery of the N-terminal [4Fe-4S] in MiaB, wild-type MiaB was reconstituted with selenide in place of sulfide, and the presence of approximately one [4Fe-4Se] cluster was inferred on the basis of Fe analyses and UV−visible absorption data (*10*). This protein was nevertheless functional in activity assays, suggesting that, at least under the in vitro assay conditions, the 4Fe and 4Se were shared by the two binding sites. As a consequence the two sites were incompletely filled, which explains why only 0.2−0.3 turnover could be achieved. In contrast, reconstituted WT MiaB containing two [4Fe-4S] clusters is capable of 1 turnover as shown herein. Furthermore, the product was found to contain mse^{2}i^{6}A rather than ms^{2}i^{6}A, indicating that the S/Se atom in ms^{2}i^{6}A/ mse^{2}i^{6}A derives from the MiaB protein itself and is introduced into MiaB during reconstitution of the Fe-S clusters with iron and sulfide/selenide (*10*). Since the radical-AdoMet cluster is expected to function exclusively for AdoMet and substrate activation, it is thus reasonable to suggest that the N-terminal cluster serves as a S-donor during the reaction. Still we are stuck with the problem that MiaB, like BioB and LipA, does not turn over in the presence of an excess of sulfur atoms in the assay. The reason for this remains unclear. Since we exclude the possibility that these proteins are reactant rather than enzymes, we reason that either these enzymes are inactivated after 1 turnover or the correct assay conditions for multiple turnovers have yet to be determined.

In conclusion, we have unambiguously demonstrated that the competent form of MiaB chelates two distinct [4Fe-4S] clusters per monomer and both clusters are essential for converting i^{6}A substrate to ms^{2}i^{6}A. There is a pressing need for additional spectroscopic and mechanistic studies to clarify the role of the N-terminal [4Fe-4S] cluster and to define its fate during catalysis. These important questions are currently under investigation in our laboratories.

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