Supporting Information

9-Mercaptodethiobiotin is Generated as a Ligand to the [2Fe-2S]$^{+}$ Cluster During the Reaction Catalyzed by Biotin Synthase from *Escherichia coli*

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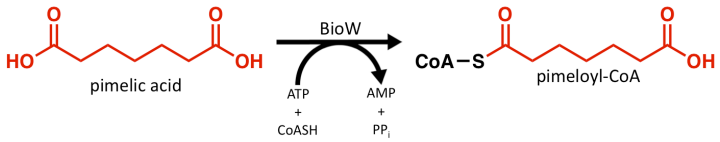
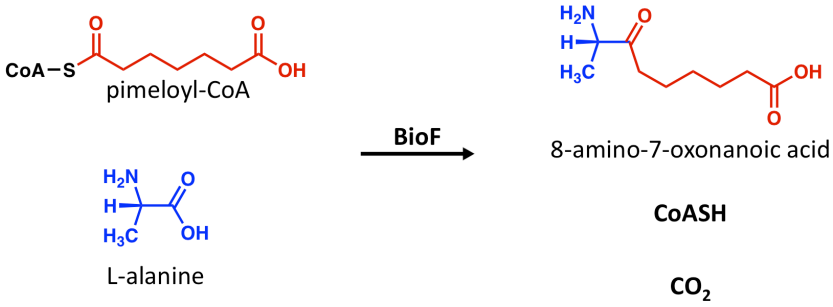
1. Production of ^{13}C-(9-*methyl*)-dethiobiotin

^{13}C-(*9-methyl*)-dethiobiotin ($^{13}C_9$-DTB) was produced in a two-step process utilizing the biotin biosynthetic enzymes from *B. sphaericus* and *E. coli* (expression and purification described in the next section). Although the last 3 reactions were run together in one reaction vessel, the individual biosynthetic reactions are depicted separately in Figure S1. The first step involved the production and purification of pimeloyl coenzyme A (Pim-CoA). A reaction containing 50 mM pimelic acid, 50 mM adenosine 5'-triphosphate, and 12 mM coenzyme A in 50 mM tris HCl, 10 mM $MgCl_2$, 200 mM NaCl, pH 8, was initiated by the addition of 1 mg of BioW.[1] The reaction was incubated at 37 °C for 1 h and was then quenched by the addition of 400 mM sodium acetate, pH 4.5. The quenched reaction mixture was allowed to sit on ice for >10 min and the precipitate removed by centrifugation. Purification was performed on a Waters Atlantis dC18 reversed-phase HPLC column (10x150 mm, 10 μm) equilibrated with 2% acetonitrile/H_2O (0.1% formic acid) at a flow rate of 4 mL/min. Pim-CoA was eluted using a linear gradient from 2 – 30 % acetonitrile over 9 min in the same buffer and detected by absorbance at 260 nm. Fractions containing Pim-CoA were pooled and lyophilized to dryness. The resulting powder was dissolved in water and the concentration was determined by UV absorbance at 230 nm (ε_{230} = 4500 M^{-1} cm^{-1}).[1]

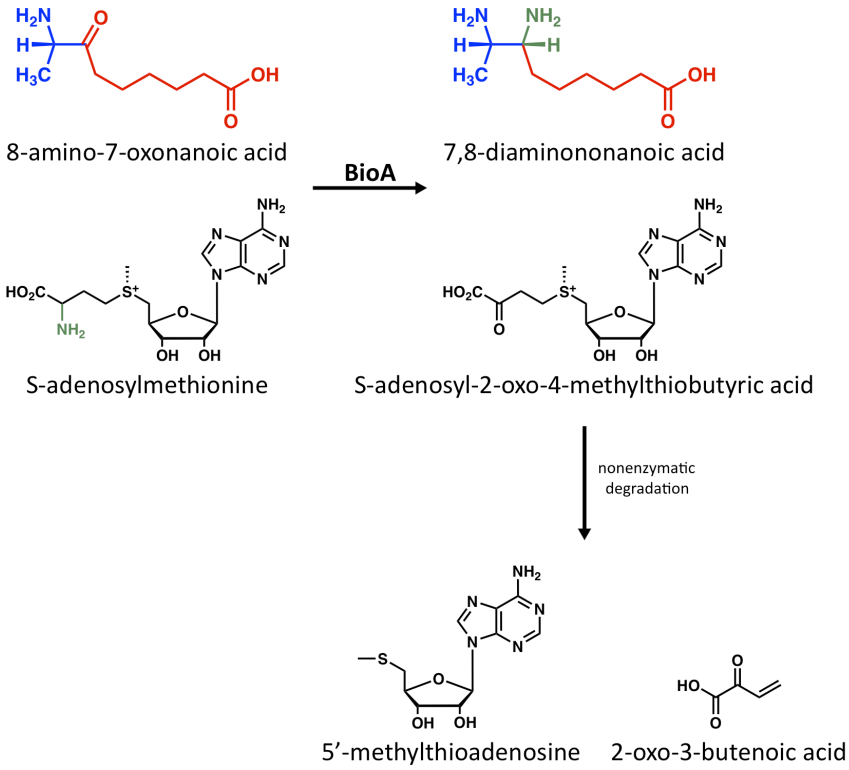
^{13}C-(*9-methyl*)-DTB was then produced from Pim-CoA and ^{13}C-(*3-methyl*)-L-alanine (Cambridge Isotope Laboratories) using BioF, BioA, and BioD from *E. coli* together with the appropriate substrates. A reaction mixture was generated containing 10 mM $NaHCO_3$, 20 mM *S*-adenosyl-L-methionine,[2] 20 mM adenosine 5'-triphosphate, 8 mM ^{13}C-(*3-methyl*)-L-alanine, and 8 mM pim-



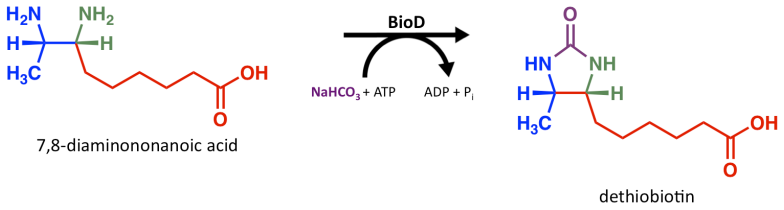
BioW (pimeloyl-CoA synthetase): Reaction containing 50 mM adenosine 5'-triphosphate, and 12 mM coenzyme A, 10 mM $MgCl_2$, 200 mM NaCl, pH 8, and 1 mg of BioW in 50 mM trisHCl pH 8.



BioF (8-amino-7-oxonanoic acid synthase): Reaction containing 8 mM pimeloyl-CoA, 8 mM L-alanine, and 1 mg BioF in 50 mM bis-tris propane HCl pH 7.5.



BioA (7,8-diaminononanoic acid synthase): Reaction containing 8 mM 8-amino-7-oxonanoic acid, 20 mM *S*-adenosyl-L-methionine, and 1 mg BioA in 50 mM bis-tris propane HCl pH 7.5.



BioD (dethiobiotin synthetase): Reaction containing 8 mM 7,8-diaminononanoic acid, 20 mM adenosine 5'-triphosphate, 10 mM $MgCl_2$, 10% glycerol, 10 mM $NaHCO_3$, and 1 mg BioD in 50 mM bis-tris propane HCl pH 7.5.

Figure S1. Biosynthetic reactions used to construct (^{13}C-9-*methyl*)-dethiobiotin from (^{13}C-3-methyl)-L-alanine. Pimeloyl CoA was synthesized from pimelic acid and CoA using BioW and ATP as depicted and purified by HPLC. Dethiobiotin was constructed by mixing BioF, BioA, and BioD, labeled L-alanine and the appropriate reagents in a single reaction vessel and incubating overnite. Dethiobiotin was also purified by HPLC and lyophilized prior to use in enzyme assays.

CoA in a buffer containing 50 mM bis-tris propane HCl, 10 mM $MgCl_2$, 10% glycerol, pH 7.5, and the reaction initiated by the addition of 1 mg each of BioF, BioA, and BioD. The reaction was incubated at room temperature for 24 h and was then quenched by the addition of 400 mM sodium acetate, pH 4.5. The quenched reaction was allowed to sit on ice for >10 min and the precipitate were removed by centrifugation. The reaction was passed through a C_{18} solid phase extraction column (100 mg, Alltech) equilibrated with 0.1% formic acid. The flow-through was loaded onto a Waters Atlantis dC18 reversed-phase HPLC column (10 x 150 mm, 10 μm) equilibrated with 2 % acetonitrile/H_2O (10 mM HCl) at a flow rate of 4 mL/min. The column was washed with the initial buffer for 30 minutes to insure the complete removal of glycerol. $^{13}C_9$-DTB was eluted using a linear gradient from 18-25% acetonitrile over 9 minutes in the same buffer and detected by absorbance at 210 nm. Fractions containing $^{13}C_9$-DTB were pooled, frozen at -80 °C, and lyophilized to dryness. The resulting powder was dissolved in 10 mM sodium hydroxide and the concentration was determined by analytical HPLC using a commercial dethiobiotin standard.[2]

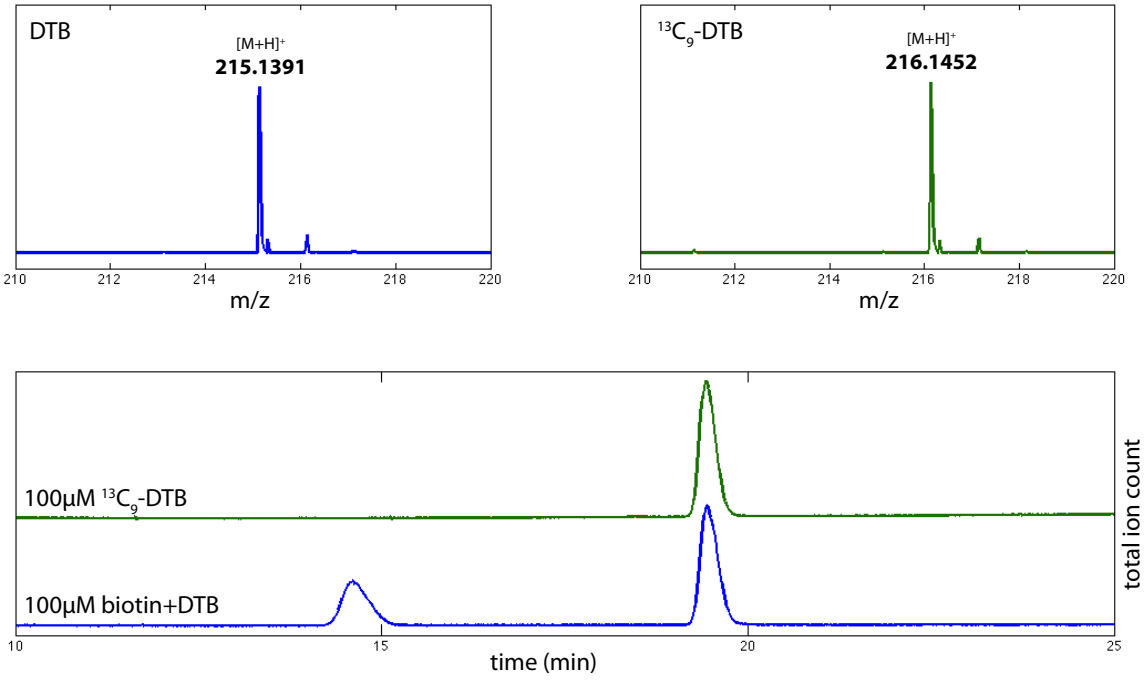


Figure S2. HPLC electrospray mass spectrometry analysis of $^{13}C_9$-DTB. (top left) Mass spectrum of natural abundance dethiobiotin. (top right) Mass spectrum of $^{13}C_9$-DTB. (bottom) TIC chromatogram of biosynthetic $^{13}C_9$-DTB (green) and commercial biotin and DTB standards (blue). LCMS analysis was carried out using a Waters Atlantis dC18 reversed-phase HPLC column (1.0 x 150 mm, 3 µm) equilibrated with 5 % acetonitrile/H_2O (0.1% formic acid) at a flow rate of 0.7 mL/min. The column was washed with the initial buffer for 10 minutes. Biotin and DTB were eluted using a linear gradient from 7-25% acetonitrile over 18 minutes in the same buffer.

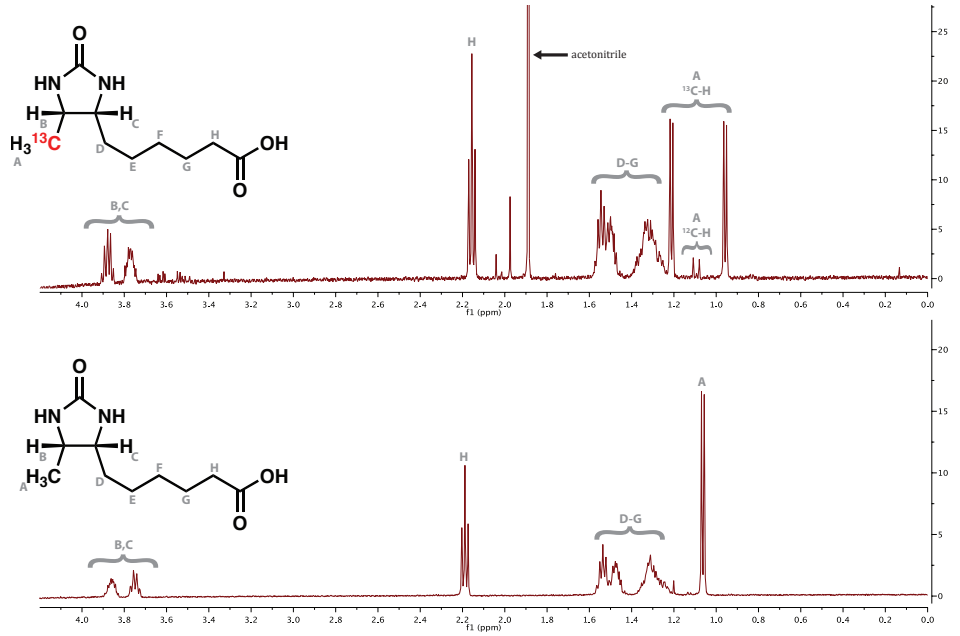


Figure S3. ^{1}H NMR spectra of $^{13}C_9$-DTB (top) and natural abundance DTB (bottom) at 500 MHz in 5% D_2O/H_2O. Top: δ: 3.73-3.92 (m, 1.1H, B, C), 2.15 (t, 2.0H, H), 1.23-1.59 (m, 7.7H, D–G), 1.1 (dd, 3.3H; A). Bottom: δ: 3.72-3. 90 (m, 0.4H, B, C), 2.2 (t, 2.0H, H), 1.19-1.57 (m, 7.9H, D–G), 1.06 (d, 2.8H, A).

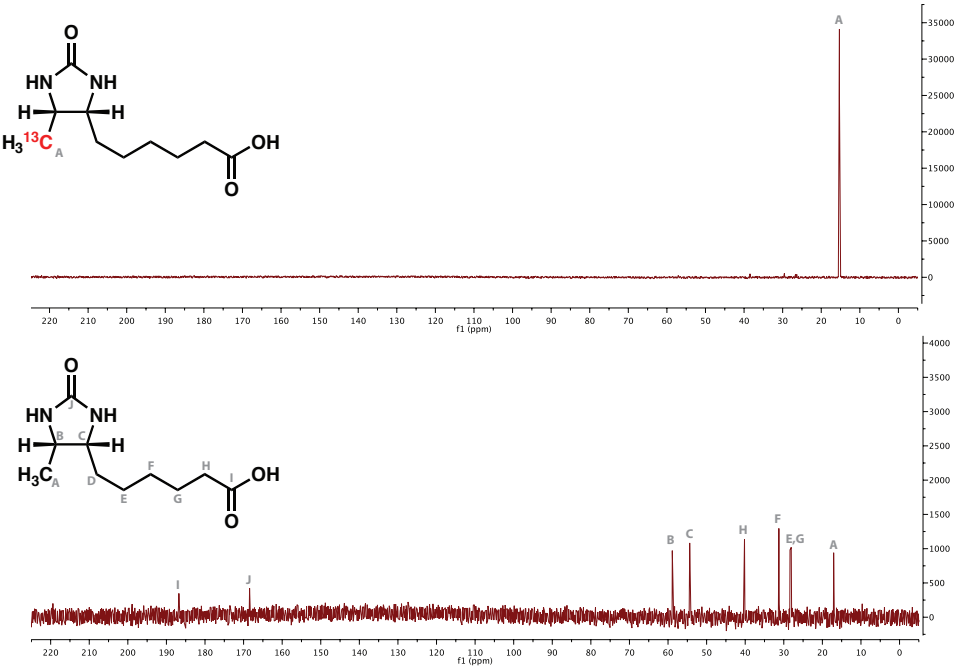


Figure S4. ^{13}C NMR spectra of $^{13}C_9$-DTB (top) and natural abundance DTB (bottom) at 125 MHz in 5% D_2O/H_2O.

2. Purification of biotin biosynthetic enzymes

Unless otherwise noted, each enzyme was expressed in Luria broth containing the appropriate antibiotic (50 mg/L ampicillin for BioF and BioD, 25 mg/L kanamycin for BioW and BioA). Cultures were grown at 37 °C to an $OD_{600} = 1$, at which point expression was induced by the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Expression proceeded for 3 h and the cells were collected by centrifugation and frozen at –80 °C.

BioW was expressed and partially purified using a truncated purification protocol described by Marquet.[1] A plasmid containing the *bioW* gene under control of a lactose promoter was a kind gift from A. Marquet, and was transformed into *E. coli* strain BL21(DE3)pLysS. The cell pellet was resuspended in 20 mM tris HCl, 50 mM NaCl, pH 8 (buffer A), lysed by sonication, and the cell debris was removed by centrifugation. The supernatant was loaded onto a DEAE fast-flow sepharose anion exchange column (2 x 12 cm) equilibrated with buffer A. The column was washed with 200 mL buffer A followed by a gradient to 1 M NaCl over 300 mL. BioW containing fractions were identified by SDS polyacrylamide gel electrophoresis, and were pooled and desalted using a Bio-Gel P2 size-exclusion chromatography resin (2 x 20 cm, BioRad). The protein solution was loaded on a Reactive Red-Agarose column (1 x 4 cm) equilibrated with buffer A. The column was washed with 50 mL buffer A and the protein eluted with 20 mM tris HCl, 2 M NaCl, pH 8. BioW containing fractions were pooled and desalted on a Bio-Gel P2 column into 50 mM tris HCl, pH 8.

The *bioF* gene was amplified using forward amplification primer (5'-GGCAGCCATATGAGCTGGCAGGAGAAAATCG-3') contained a *Nde*I restriction site (underlined), and reverse amplification primer (5'-GCTTGTCTCGAGACCGTTGCCATG CAGCACC-3') contained a *Xho*I restriction site (underlined). The *bioF* gene and the expression

vector pET-23b(+) were digested, gel purified, and ligated using standard procedures. The sequence of resulting plasmid was verified by DNA sequencing. The plasmid was transformed into BL21(DE3)pLysS, and protein expression was carried out as described above. The resulting cell pellet was resuspended in 50 mM bis-tris propane, 0.5 M NaCl, 10% glycerol, pH 7.5 (buffer A), lysed by sonication, and the cell debris removed by centrifugation. The supernatant was loaded onto a Ni-NTA agarose column (2 x 4 cm, Qiagen), washed with buffer A until the absorbance reached baseline, and the protein was eluted with buffer A + 300 mM imidazole. Imidazole was removed by desalting through a Bio-Gel P2 column into 50 mM bis-tris propane, 10% glycerol, pH 7.5.

The BioA containing cell pellet was resuspended in 50 mM HEPES, 0.1 mM EDTA, 10 mM β-mercaptoethanol, pH 8 (HEB buffer), lysed by sonication, and the cell debris was removed by centrifugation. The supernatant was diluted with HEB buffer to 6 mg protein/mL solution. Pyridoxal phosphate (PLP) and streptomycin sulfate were added to a final concentration of 2 mM each. The solution was rapidly heated to 55 °C in boiling water, held at 55 °C for 15 min, and cooled to 10 °C in an ice bath. The precipitated protein was removed by centrifugation. The solution was adjusted to pH 5 with acetic acid, allowed to sit on ice for 20 min, and then adjusted to pH 7 using sodium hydroxide. The solution sat on ice overnight and the precipitated was again removed by centrifugation. The supernatant was loaded onto a Q-sepharose column (2 x 12 cm, Sigma) equilibrated with 20 mM tris HCl, 50mM NaCl, pH 8 (buffer A). The column was washed with 200 mL of buffer A followed by a gradient to 1 M NaCl over 600 mL. Fractions containing BioA were identified by the UV/visible spectrum of the PLP cofactor and by SDS polyacrylamide gel electrophoresis, and were pooled and desalted using a Bio-Gel P2 column into 50 mM tris HCl, pH 8.

The *bioD* gene was amplified using forward amplification primer (5'-TGATTGGCTAGCAGTAAACGTTATTTTGTC -3') contained a *Nhe*I restriction site (underlined) and reverse amplification primer (5'-GAATGGGAATTCCCCAAGGCAAG GTTTATG -3') contained a *Xho*I restriction site (underlined). The *bioD* gene and the expression vector pET-23b(+) were digested, gel purified, and ligated using standard procedures. The sequence of resulting plasmid was verified by DNA sequencing. The plasmid was transformed into BL21(DE3)pLysS, and protein expression was carried out as described above. The resulting cell pellet was resuspended in 50 mM bis-tris propane, 0.5 M NaCl, 10% glycerol, pH 7.5 (buffer A), lysed by sonication, and the cell debris was removed by centrifugation. Ni-NTA agarose column (2 x 4 cm, Qiagen), washed with buffer A until the absorbance reached baseline, and the protein was eluted with buffer A + 300 mM imidazole. Imidazole was removed using a Bio-Gel P2 column into 50 mM bis-tris propane, 10% glycerol, pH 7.5.

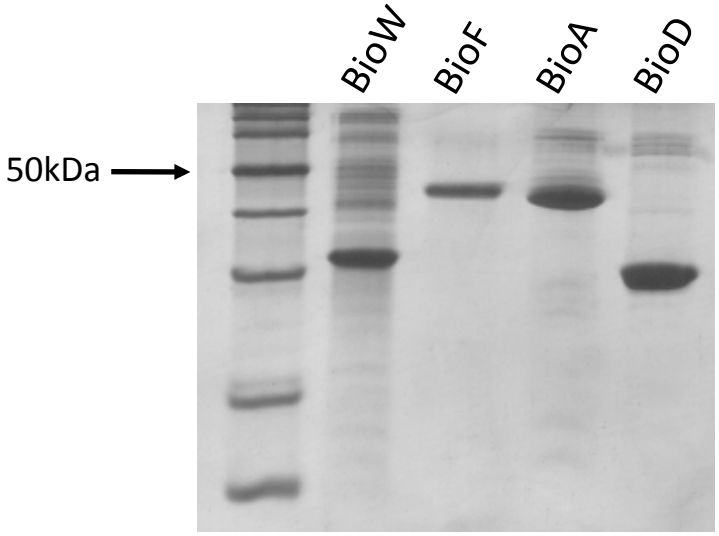


Figure S5. SDS polyacrylamide gel electrophoresis showing the purity of enzymes used for production of labeled dethiobiotin. Each lane contains ~5 μg total protein.

3. Production of (*guanidino*-$^{15}N_2$)-arginine biotin synthase

(*guanidino*-$^{15}N_2$)-Arginine biotin synthase was produced using a Δ*arg*H strain of *E. coli*, (strain JW3932 from the Keio collection[3] obtained from the Coli Genetic Stock Center, Yale University). This strain contains a kanamycin insertion into *argH*, which encodes the enzyme arginosuccinate lyase from the *E. coli* arginine biosynthesis pathway. This strain cannot make endogenous arginine, and only grows significantly in the presence of exogenously supplied arginine in the medium (Figure S6). Using labeled arginine, nearly 100% isotopic incorporation can be achieved (see below).

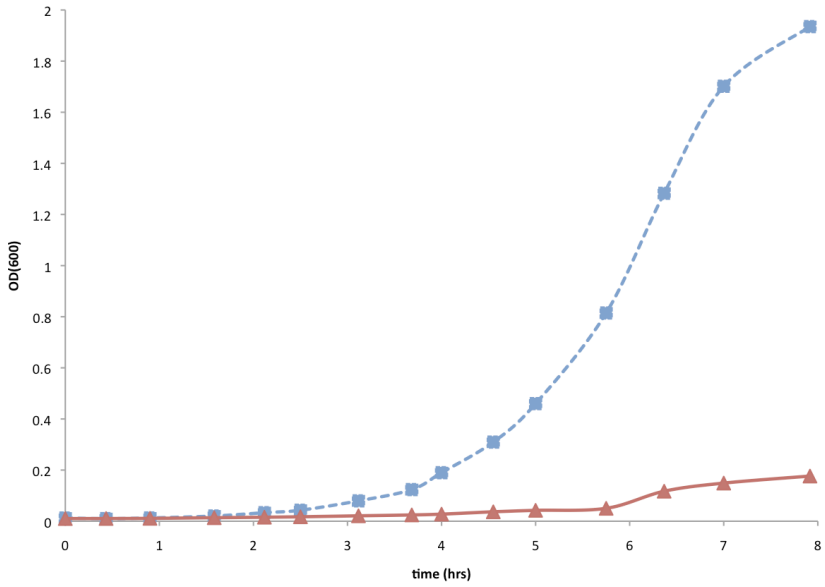


Figure S6. Growth of Δ*arg*H strain (JW3932) in a defined M9-glucose media with arginine (squares, dashed curve) or without arginine (triangles, solid curve). The medium also included additional amino acids and inorganic micronutrients as previously described.[4]

For protein expression, a Δ*argH*(DE3) lysogen was produced using a λDE3 lysogenization kit (EMD Biosciences). A defined M9-glucose-amino acid media was prepared that was modified from that described previously described for production of B_{12}-dependent MetH.[4] The hydroxocobalamin added to this media for production of a B_{12}-dependent enzyme

was not added, and instead we added filter-sterilized ferric citrate directly to the final media (50 μM final conc.). In addition, unlabeled arginine in the amino acid supplement was replaced by (*guanidino*-$^{15}N_2$)-arginine (Cambridge Isotope Labs). Cultures were grown at 37 °C to an OD_{600} = 1, at which point the temperature was dropped to 25 °C and protein expression was induced by the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside. Expression proceeded for 3 h at 25 °C and the cells were collected by centrifugation and frozen at -80 °C. (*guanidino*-$^{15}N_2$)-Arginine biotin synthase was purified as previously described.[5] The degree of labeling at Arg260 was determined by tryptic digestion followed by MALDI mass spectrometric analysis, following the protocol described by Reyda, et al.[6]

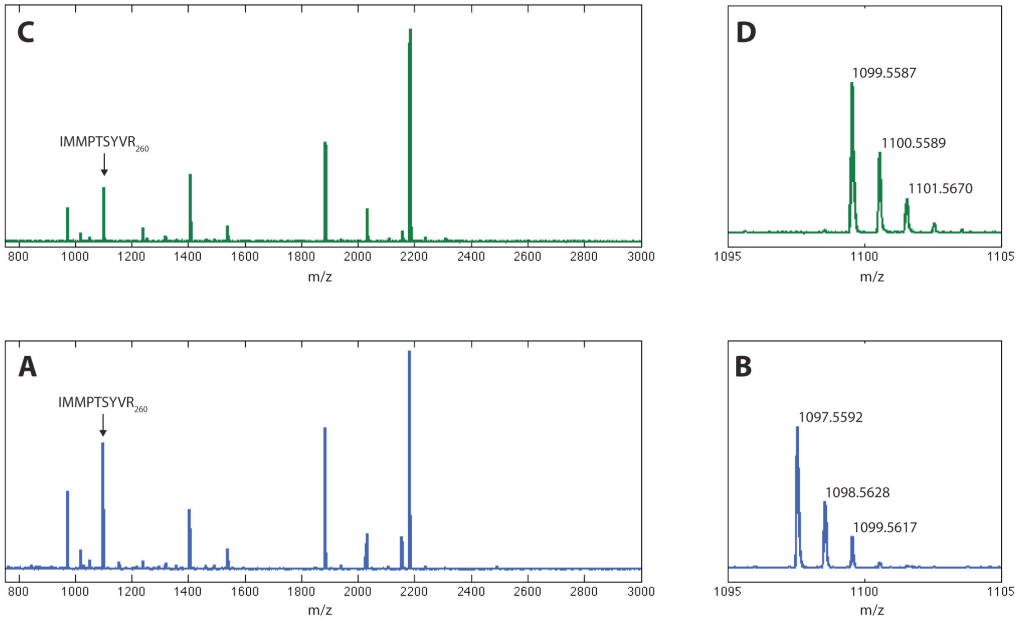


Figure S7. MALDI-MS analysis of a tryptic digest of unlabeled biotin synthase (A-B) and (*guanidino*-$^{15}N_2$)-arginine biotin synthase (C-D). Tryptic digestion is predicted to produce the peptide $I_{252}MMPTSYVR_{260}$ with a calculated $[M_{mono}+H]_{calc}$ = 1097.5. (A) MALDI-MS of the complete tryptic digest of unlabeled biotin synthase. (B) Expanded view of the mass window corresponding to the peptide containing Arg260 ($[M+H]_{exper}$ = 1097.6). (C) MALDI-MS of the complete tryptic digest of (*guanidino*-$^{15}N_2$)-arginine biotin synthase. (D) Expanded view of the mass window corresponding to the peptide containing (*guanidino*-$^{15}N_2$)-Arg260 ($[M+H]_{exper}$ = 1099.6).

4. Production of biotin synthase intermediate state

Samples of biotin synthase in the reaction intermediate state were prepared in a glove box under anaerobic conditions at approximately 27-30 °C. A solution was prepared containing 300 μM biotin synthase (monomer conc.), 10 mM dithiothreitol, 1 mM Na_2S, 1 mM $FeCl_3$, 10 μM flavodoxin, 5 μM ferredoxin(flavodoxin):$NADP^+$ oxidoreductase, 2 mM NADPH, and 300 μM dethiobiotin in 100 mM tris HCl, 10 mM KCl, pH 8, was incubated for 15 min. The reaction was initiated by the addition of 300 μM AdoMet and was allowed to incubate for 30 – 60 min. After this incubation time, a small fraction was quenched with acid for HPLC analysis[7] and the remainder was transferred to an EPR tube, removed from the glove box, and frozen in liquid N_2.

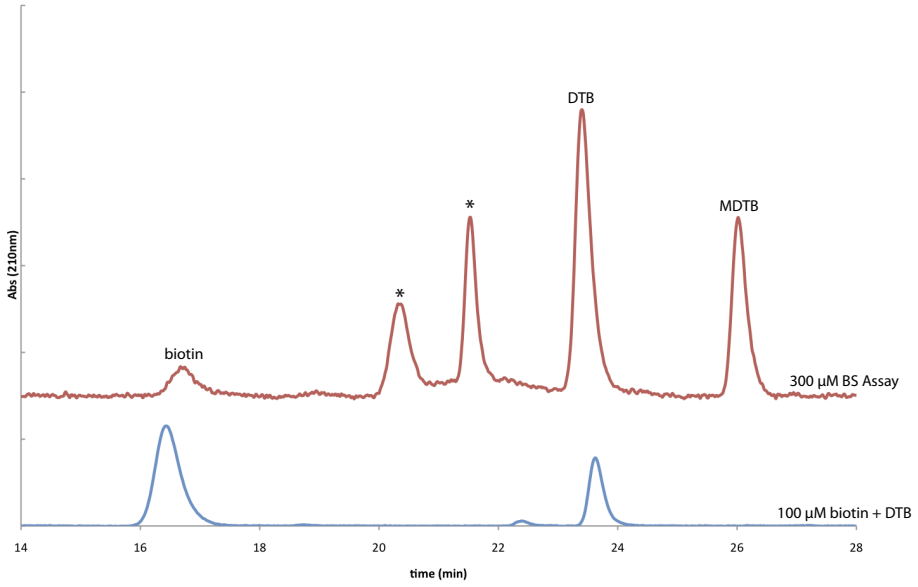


Figure S8. HPLC analysis of biotin, dethiobiotin, and MDTB. Bottom trace (blue): 100 μM biotin and 100 μM dethiobiotin standard. Top trace (red): BS assay mixture as described above after 30 min incubation. Biotin and MDTB peak assignments were confirmed by LCMS analysis. Peaks marked with * correspond to FAD and FMN from the biological reducing system employed in the assay.

5. Electron paramagnetic resonance spectroscopy

All EPR spectra were measured at the CalEPR Center at the University of California, Davis. Continuous-wave (CW) X-band spectra were acquired with an ECS106 spectrometer (Bruker, Billerica, MA) using a Super-High Q resonator (ER 4122SHQE). Cryogenic temperatures were achieved and maintained using an Oxford Instruments ESR900 liquid helium cryostat in conjunction with an Oxford Instruments ITC503 temperature and gas flow controller. Hyperfine sublevel correlation (HYSCORE) spectra were measured using an Elexsys E580 spectrometer (Bruker) with either a dielectric (MD4) or split-ring (MS5) resonator using the pulse sequence $\pi/2$-τ-$\pi/2$-t_1-π-t_2-$\pi/2$-τ-echo wherein both the inversion pulse length (t_π) and the $\pi/2$ pulse ($t_{\pi/2}$) are identical (16 ns). Four-step phase cycling was employed. Time-domain spectra were baseline-corrected (third-order polynomial), apodized with a Hamming window, zero-filled to eight-fold points, and fast Fourier-transformed to yield the frequency-domain. Spectral simulations were performed with Matlab using the EasySpin 4.0 toolbox.[8]

We note that all ^{13}C-derived spectral features are well-simulated using a single ^{13}C hyperfine tensor (i.e. we see no evidence of a second class of ^{13}C-coupling that could be attributed to a carbon hyperfine-coupled to a form of the [FeS] that gives rise to the second CW EPR signal). Nonetheless, using the four distinct *g*-tensors determined in Taylor *et al*. (Biochemistry, 2011) we have found the optimal ^{13}C hyperfine tensors that reproduce the experimental spectra (cf. Table S1 and Figure S10). As can be seen from comparing the values given in Table S1, the magnitude of the ^{13}C hyperfine interaction does not change by much (less than 15%) as a function of the *g*-tensor that is used. Despite these small differences, the main conclusion derived from our reported ^{13}C *A*-tensor remains valid; namely that the isotopically labeled substrate is covalently attached to the [2Fe-2S] cluster.

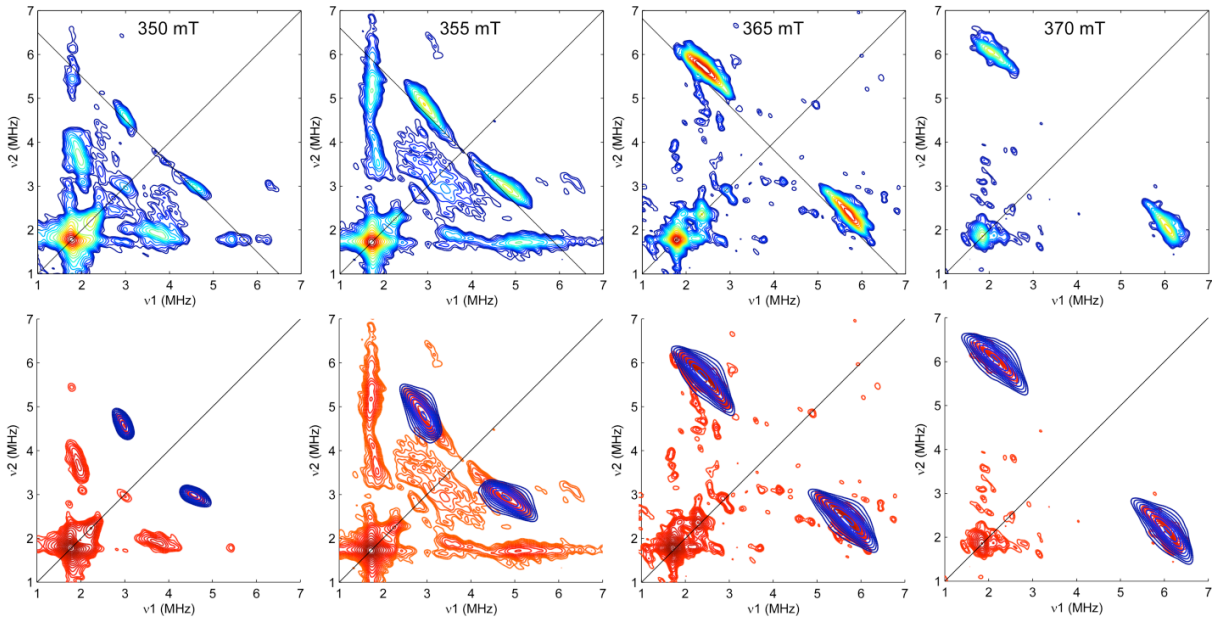


Figure S9. Field-dependent HYSCORE spectra of the BS paramagnetic intermediate prepared with DTB labeled with (*9-methyl*-^{13}C)-DTB (top). In the bottom half of the figure, these data (red contours) are compared to spectral simulations (blue contours) for a single hyperfine coupled ^{13}C nucleus (simulation parameters: g = [1.9947, 1.9410, 1.8458]; $A(^{13}C)$ = [−1.2 −1.2 +5.7] MHz; Euler angles = [0 0 0]°; excitation bandwidth = 125 MHz. Spectrometer settings (from left to right): B_0 = 350.0 mT, 355.0 mT, 365.0 mT, and 370.0 mT. All other settings were identical to those given in the caption of Figure S9.

Table S1. Optimized ^{13}C Hyperfine Tensors for All Possible g-Tensors

[1]	g	$A(^{13}C)$ (MHz)
A	[1.9947, 1.9410, 1.8458]	[−1.2 −1.2 +5.7]
B	[2.0079, 1.9590, 1.8787]	[−1.1 −1.1 +5.0]
C	[2.0037, 1.9533, 1.8469]	[−1.2 −1.2 +5.6]
D	[1.9906, 1.9375, 1.8796]	[−1.1 −1.1 +5.2]

[1] Corresponding simulations are shown in Figure S9.

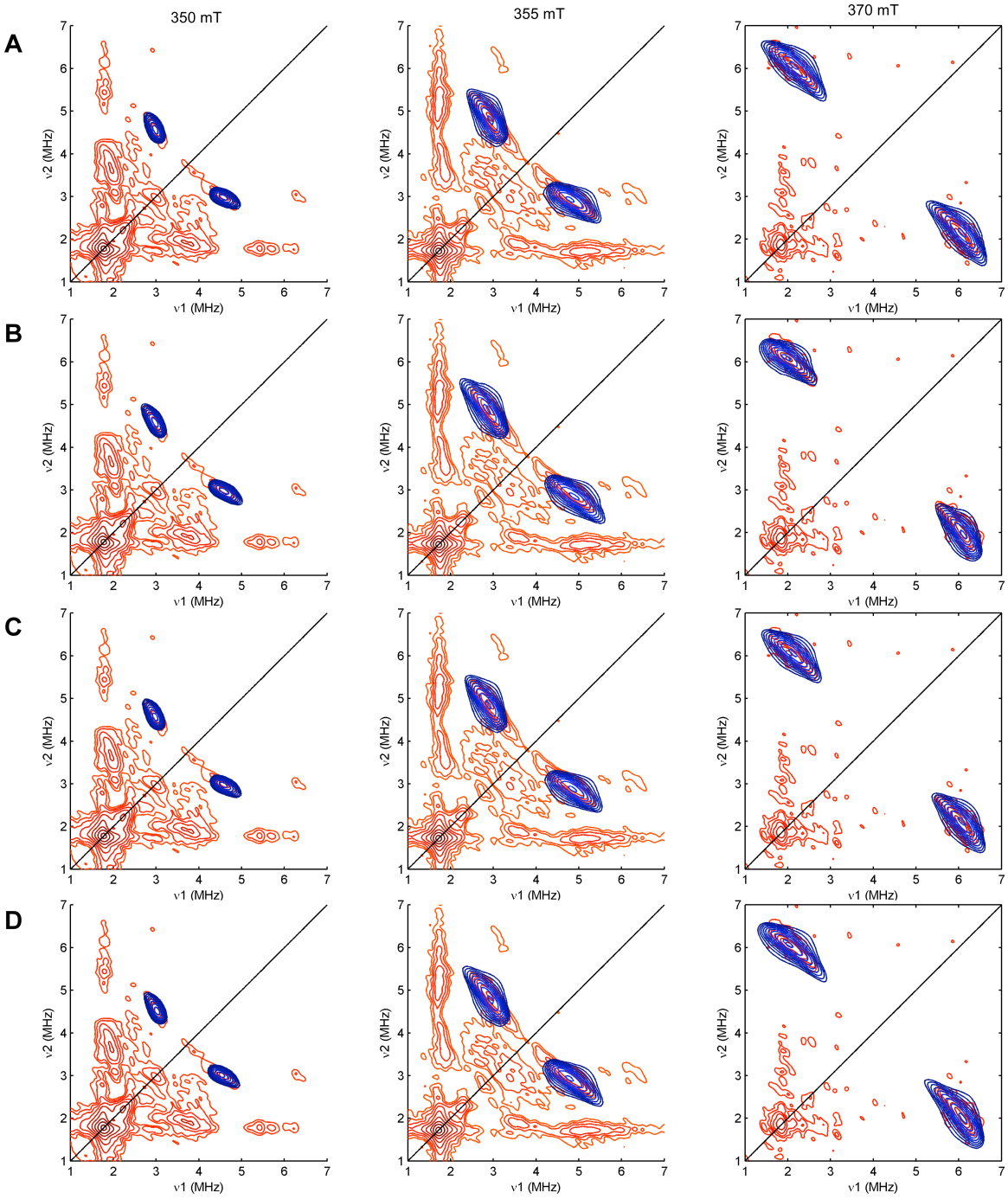


Figure S10. Comparison of field-dependent HYSCORE spectra of the BS paramagnetic intermediate prepared with DTB labeled with (*9-methyl*-^{13}C)-DTB (red) with spectral simulations (blue) obtained using parameters in Table S1.

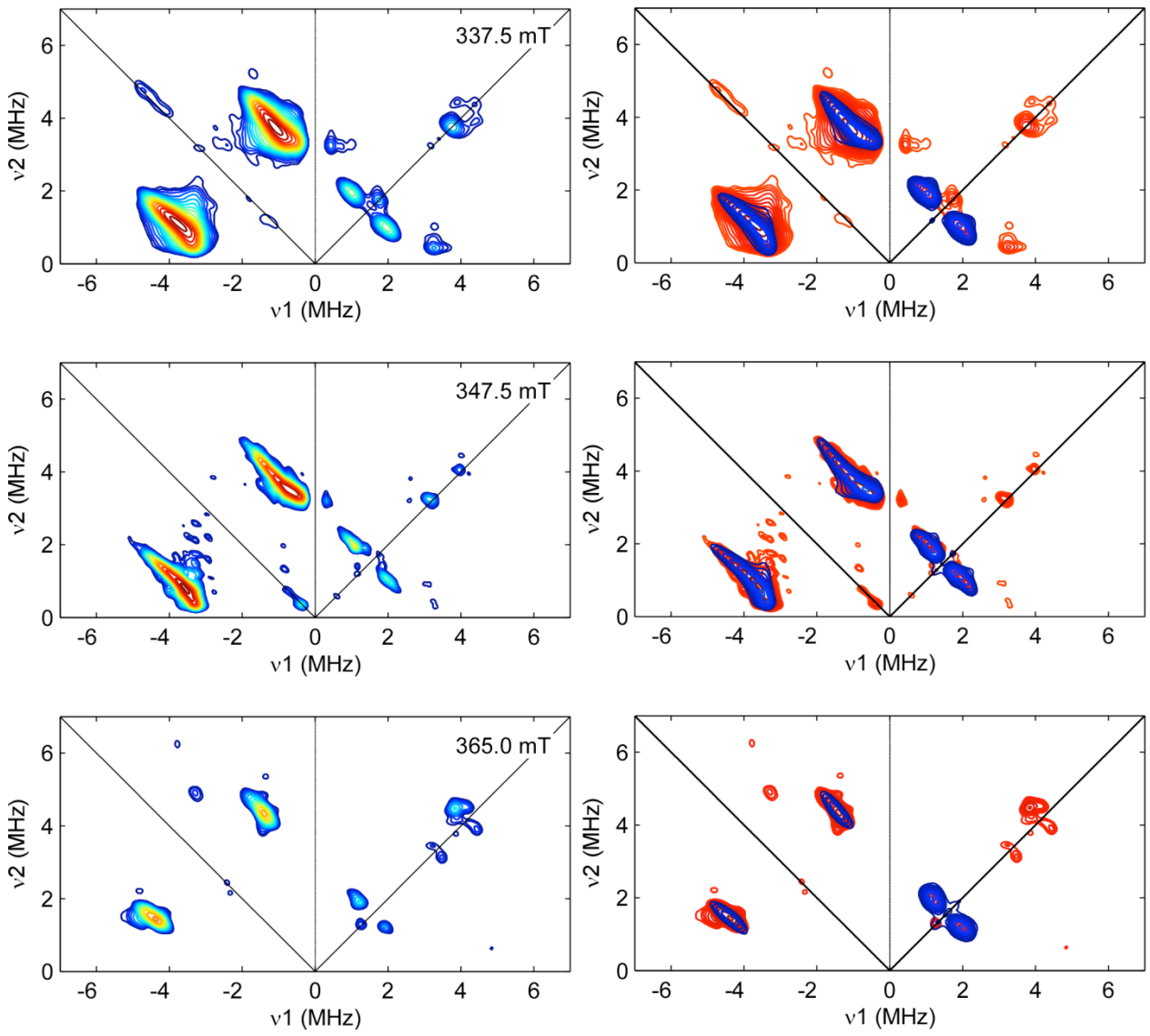


Figure S11. Field-dependent HYSCORE spectra of the BS paramagnetic intermediate prepared BS grown with (*guanidino*-$^{15}N_2$)-L-arginine with natural abundance DTB. In the right half of the figure, these data (red contours) are compared to spectral simulations (blue contours) for a two distinct classes of hyperfine coupled ^{15}N nuclei $A(^{15}N1)$ = [3.65 3.65 7.43] MHz and $A(^{15}N2)$ = [0.29 0.41 1.68] MHz. Spectrometer settings (from top to bottom): B_0 = 337.5 mT, 347.5 mT, and 365.0 mT; excitation frequency = 9.317 GHz, 9.419 GHz, 9.445 GHz ; τ = 136 ns, 136 ns, and 180 ns. Other settings: temperature = 10 K; $t\pi_{/2} = t\pi$ 16 ns; $t_1 = t_2$ = 100 ns; Δt = 20 ns.

The HYSCORE spectrum of ^{15}N-arginine labeled enzyme exhibits 2 sets of cross peaks associated with strongly coupled (N1) and weakly coupled (N2) ^{15}N nuclei. We have tentatively assigned these two classes of ^{15}N nuclei to the two structurally distinct guanidino nitrogen atoms on Arg260, a ligand to the [2Fe-2S] cluster, but also suggest that the nearby Arg95 residue could also contribute to the HYSCORE spectrum. The anisotropic portion of the effective hyperfine tensor for both classes of nitrogen nuclei is found to be nearly axial and rather large (A_{aniso} = [−1.26 −1.26 3.52] MHz for N1 and [–0.50 –0.39 +0.89] MHz for N2). Using the point-dipole approximation, and assuming that each iron carries 100% of its native spin density and that the Arg-coordinated iron is ferrous (and thus has a projection factor of $c = -4/3$; $c = +7/3$ for the ferric ion), we can compute the dipolar part of the hyperfine anisotropy (see Equations A.1–A.4 from Randall *et al.*[9]). Based on the 3.4 Å resolution x-ray structure coordinates of biotin synthase (PDB 1R30), the two guanidino nitrogens of Arg95 are too distant to account for the observed hyperfine anisotropy (see Table S2), let alone the non-zero isotropic hyperfine coupling, without invoking a significant rearrangement of amino acid residues coordinating the [2Fe-2S] cluster upon formation of the paramagnetic intermediate. Table S2 also contains A_{dip} computed using the alternative oxidation state assignment and correspondingly swapped projection factors for the iron ions.

Table S2. Internuclear Fe—N Distances from 1R30 and Corresponding Computed Dipolar Hyperfine Interaction.

internuclear distance for chain A/B[3] (Å)	N1(Arg260)	N2(Arg260)	N1(Arg95)	N2(Arg95)
Fe1	2.35/2.40	3.15/3.07	5.37/5.23	7.55/7.40
Fe2	3.98/4.04	4.64/4.71	5.63/5.67	7.49/7.52
A_{dip} (MHz) chain A[1]	[–1.18 –0.53 1.71]	[–0.50 –0.15 0.65]	[–0.17 0.04 0.13]	[–0.06 0.02 0.04]
A_{dip} (MHz) chain B[1]	[–1.11 –0.49 1.60]	[–0.50 –0.15 0.65]	[–0.17 0.03 0.14]	[–0.06 0.02 0.04]
A_{dip} (MHz) chain A[2]	[–2.89 1.27 1.62]	[–1.15 0.49 0.66]	[–0.20 0.06 0.14]	[–0.06 0.02 0.04]
A_{dip} (MHz) chain B[2]	[–2.72 1.19 1.52]	[–1.15 0.49 0.66]	[–0.22 0.07 0.15]	[–0.06 0.02 0.04]

[1] For this computation, the ferrous ion was assumed to be coordinated by Arg260.
[2] For this computation, the ferric ion was assumed to be coordinated by Arg260.
[3] Chain A and B refer to opposing monomers in the BS dimer, with slightly different structures in PDB 1R30.

The total hyperfine anisotropy is the sum of the dipolar couplings summarized in Table S1 and the hyperfine coupling due to asymmetric distribution of unpaired spin density about the three nitrogen 2p orbitals. Such asymmetry is introduced by effects of covalency, in this case involving the paramagnetic iron-sulfur cluster. Again, the guanidino nitrogen atoms of Arg95 are too far away from the spin-bearing iron ions to contribute significantly, indicating that the weakly coupled class of nitrogen atoms (N2) is not due to Arg95. Thus the weakly coupled nitrogen atom is most likely one of the guanidino nitrogen atoms on Arg260, favoring structural model B in which the guanidino group is bound in approximately monodentate fashion.

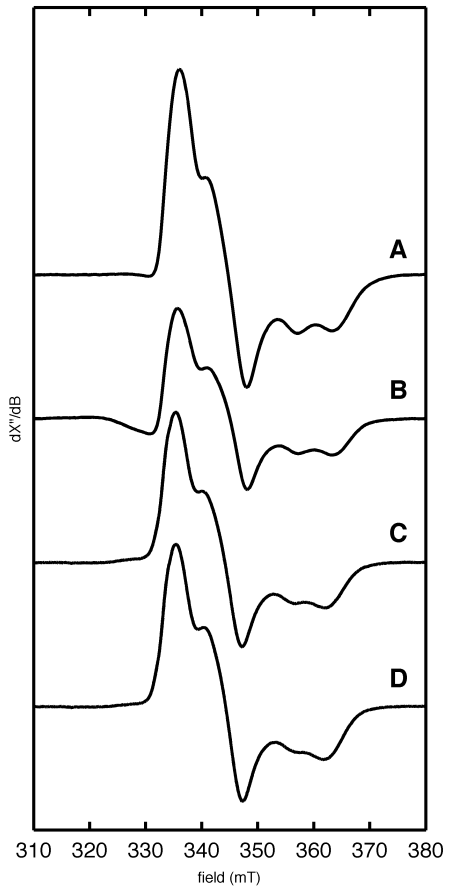


Figure S12. Continuous-wave (CW) EPR spectra of (**A**) natural abundance BS + DTB allowed to react for 60 min; (**B**) (*guanidino*-$^{15}N_2$)-L-arginine BS + DTB allowed to react for 60 min; (**C**) natural abundance BS + DTB allowed to react for 30 min; and (**D**) natural abundance BS + (*9-methyl*-^{13}C)-DTB allowed to react for 30 min. Spectrometer settings: temperature = 40 K; excitation frequency = 9.375 GHz; power = 100 mW.

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