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COMMUNICATIONS

Published on Web 11/05/2002

The [4Fe−4S]$^{2+}$ Cluster in Reconstituted Biotin Synthase Binds *S*-Adenosyl-L-methionine

Michele Mader Cosper,[§] Guy N. L. Jameson,[†] Roman Davydov,[‡] Marly K. Eidsness,[§] Brian M. Hoffman,[‡] Boi Hanh Huynh,[†] and Michael K. Johnson*[,§]

Department of Chemistry and Center for Metalloenzyme Studies, University of Georgia, Athens, Georgia 30602, Department of Physics, Emory University, Atlanta, Georgia 30322, and Department of Chemistry, Northwestern University, Evanston, Illinois 60208

Received August 27, 2002

The final step of the biotin biosynthetic pathway is catalyzed by biotin synthase (BioB) and involves the insertion of sulfur into dethiobiotin. The discovery that [2Fe-2S]$^{2+}$ clusters in as-isolated BioB can be converted into [4Fe−4S]$^{2+}$ clusters under reducing conditions,[1] coupled with the requirement of *S*-adenosyl-L-methionine (SAM) for activity in vitro and the conserved C−X_3−C−X_2−C cluster-binding motif, led to speculation that BioB was a member of the radical SAM family of enzymes,[2] that utilize a [4Fe−4S]$^{2+,+}$ cluster to initiate a radical reaction by mediating reductive cleavage of SAM to yield methionine and a 5′-deoxyadenosyl radical.[3] Subsequent spectroscopic, analytical and mutagenesis studies have demonstrated that BioB can accommodate one [4Fe−4S]$^{2+,+}$ cluster per monomer,[4] and provided direct evidence that the [4Fe−4S]$^{+}$ cluster is responsible for reductive cleavage of SAM and is ligated by the cysteines in the C−X_3−C−X_2−C motif.[5] In this study, the combination of resonance Raman (RR), Mössbauer, and EPR have been used to investigate the effect of SAM on the spectroscopic properties of the [4Fe−4S]$^{2+}$ cluster in BioB. The results indicate that SAM interacts directly at a unique Fe site of the [4Fe−4S]$^{2+}$ cluster in BioB, mimicking the interaction between SAM and the [4Fe−4S]$^{2+}$ cluster established in pyruvate−formate lyase activating enzyme (PFL-AE).[6] This suggests a common mechanism of reductive cleavage of SAM for all enzymes in the radical SAM family.[1]

The resonance Raman (RR) spectra of [4Fe−4S]$^{2+}$ BioB[7] in the absence and presence of SAM are shown in Figure 1. Both spectra are characteristic of [4Fe−4S]$^{2+}$ clusters, and the changes in the frequencies of the Fe−S stretching modes induced by SAM are characteristic of binding a nonthiolate ligand at a unique Fe site. This is best illustrated by the symmetric stretching mode of the [4Fe−4S] core which shifts from 338 to 342 cm^{-1} on addition of SAM, outside the range established for [4Fe−4S]$^{2+}$ cluster with complete cysteinyl coordination [333−339 cm^{-1}].[8] For example, analogous spectral changes to those associated with SAM addition to [4Fe−4S]$^{2+}$ BioB are observed in *Pyrococcus furiosus* ferredoxin (*Pf* Fd) when one cysteine ligand of the all-cysteinyl ligated [4Fe−4S]$^{2+}$ cluster in the D14C variant is replaced by an aspartate in the WT protein.[8]

The 4.2 K Mössbauer spectrum of the [4Fe−4S]$^{2+}$ BioB shows a quadrupole doublet (hatched marks, Figure 2a)[9] with parameters (ΔE_Q = 1.16 mm/s, δ = 0.45 mm/s) similar to values reported previously for the [4Fe−4S]$^{2+}$ cluster in BioB.[4,5,10] In the presence of SAM, the doublet broadened substantially and changed shape

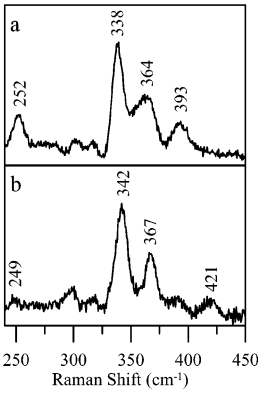

Figure 1. RR spectra of [4Fe−4S]$^{2+}$ BioB in the absence (a) and presence of SAM (b).[7] The RR spectra were recorded with 457-nm excitation using a 10-μL frozen droplet at 16 K with 220 mW laser power at the sample. Each scan involved photon counting for 1 s at 0.5 cm^{-1} increments with 8 cm^{-1} spectral resolution, and the spectra are the sum of ∼100 scans. A linear ramp fluorescence baseline has been subtracted.

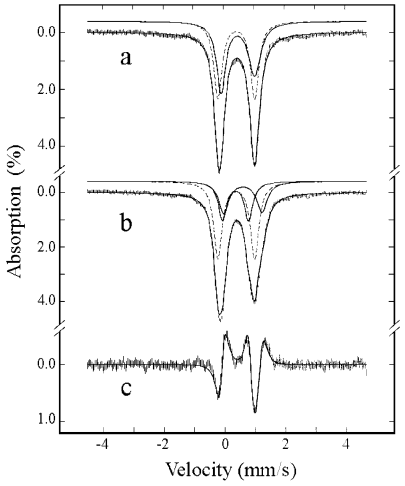

Figure 2. Mössbauer spectra of ^{57}Fe-enriched [4Fe−4S]$^{2+}$ BioB in the absence (a) and presence of SAM (b).[7] The spectra (hatched marks) were recorded at 4.2 K in a field of 50 mT applied parallel to the γ beam. A difference spectrum of a − b is shown in c. The solid lines overlaid with the experimental spectra are theoretical simulations based on the analysis described in text. The solid and dashed lines shown above the experimental spectra are the deconvoluted spectra that resulted from the analysis (see text).

(hatched marks, Figure 2b). A difference spectrum of a − b (hatched marks, Figure 2c) reveals in detail the spectral changes caused by the addition of SAM; a quadrupole doublet contained in spectrum a (seen as two troughs in c) has converted into two quadrupole

* To whom correspondence should be addressed. Telephone: 706-542-9378. Fax: 706-542-2353. E-mail: johnson@chem.uga.edu.
† Emory University.
‡ Northwestern University.
§ University of Georgia.

10.1021/ja0283044 CCC: $22.00

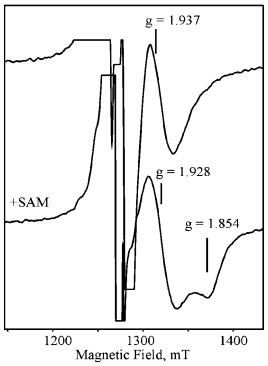

Figure 3. Q-band EPR spectra of $[4Fe-4S]^{2+}$ BioB in the absence and presence of SAM,[7] after radiolytic reduction at 77 K. EPR conditions: frequency, 35.64 GHz; microwave power, 1 mW; modulation amplitude 0.2 mT; temperature, 2 K. The intense signals centered at $g = 2$ originate from matrix radicals induced by irradiation.

doublets (seen as three upward pointing peaks in c). Detailed analysis of the difference spectrum indicates that this spectral change accounts for 50% of the $[4Fe-4S]^{2+}$ cluster absorption (i.e., two Fe sites). Using the difference spectrum as a constraint, the Mössbauer data can be analyzed as follows. The $[4Fe-4S]^{2+}$ BioB spectrum (Figure 2a) is the sum of two equal intensity quadrupole doublets corresponding to the two mixed-valence $Fe^{II}Fe^{III}$ pairs of the $[4Fe-4S]^{2+}$ cluster (pair **1**, dashed line shown above spectrum a, $\Delta E_Q = 1.21$ mm/s, $\delta = 0.42$ mm/s; pair **2**, solid line above spectrum a, $\Delta E_Q = 1.10$ mm/s, $\delta = 0.47$ mm/s). In the presence of SAM, pair **1** remains unchanged, while pair **2** splits into two equal intensity doublets (solid lines above spectrum b) with the following parameters: $\Delta E_Q = 0.86$ mm/s, $\delta = 0.40$ mm/s for doublet 1; $\Delta E_Q = 1.26$ mm/s, $\delta = 0.64$ mm/s for doublet 2. The parameters of doublet 1, although different from that of pair **2**, are within the range of values observed for Fe sites in $[4Fe-4S]^{2+}$ clusters. The isomer shift of 0.64 mm/s determined for doublet 2, however, is large for $[4Fe-4S]^{2+}$ clusters and suggests an increase in coordination number or binding of noncysteinyl ligands.[11] The above analysis yielded theoretical simulations (solid lines overlaid with the experimental data in Figure 2) that compare very well with the experimental spectra. Thus, the Mössbauer data indicate SAM binds to a unique Fe site in the $[4Fe-4S]^{2+}$ cluster of BioB and affects the electronic properties of another Fe site that pairs with the unique Fe. Binding of SAM to a unique Fe of a $[4Fe-4S]^{2+}$ cluster has also been observed in PFL-AE.[6] However, a larger increase in the isomer shift (0.42 to 0.72 mm/s) was detected for the unique Fe site in PFL-AE.[6a]

EPR spectra of $[4Fe-4S]^{2+}$ BioB in the absence and presence of SAM after radiolytic reduction at 77 K are shown in Figure 3.[7] In the cryoreduced protein, the paramagnetic $[4Fe-4S]^{+}$ site retains the geometry of the oxidized precursor, and EPR is effectively being used to study the diamagnetic oxidized state.[12] The cryoreduced BioB shows a nearly axial $S = {}^1/_2$ EPR resonance with g-values of $\sim$2, 1.937, 1.937, which are analogous to those observed for chemically reduced $[4Fe-4S]^{+}$ clusters in BioB ($g = 2.042$, 1.937, 1.937).[1,4] In the presence of SAM, a new rhombic $S = {}^1/_2$ EPR resonance, with g-values of $\sim$2, 1.928, 1.854, is observed on cryoreduction. The changes in the EPR spectrum of cryoreduced BioB are consistent with changes in ligation at a specific Fe site of an $S = {}^1/_2$ $[4Fe-4S]^{+}$ cluster. For example, the binding of citrate to the $[4Fe-4S]^{+}$ cluster in aconitase,[13] or replacing a cysteine ligand to the $[4Fe-4S]^{+}$ cluster in D14C *Pf* Fd with an asparate ligand,[8] result in similar changes in the g-tensors. Moreover, the EPR spectra of $[4Fe-4S]^{+}$ clusters in other radical SAM enzymes have been shown to be affected by the presence of SAM,[3a,6b,14] and recent ENDOR studies of $[4Fe-4S]^{2+,+}$ PFL-AE have revealed that the EPR spectral changes are caused by SAM coordination to a unique iron site via the amino and carboxylato groups of the methionine fragment.[6b,c]

Taken together, the RR, Mössbauer and EPR results provide convincing evidence for SAM binding at a unique Fe site of the $[4Fe-4S]^{2+}$ cluster in BioB. Hence SAM is shown to bind to the $[4Fe-4S]^{2+}$ clusters in both BioB and PFL-AE[6] prior to injection of the electron required for reductive cleavage of SAM to yield methionine and the 5′-deoxyadenosyl radical. This strongly supports the hypothesis of a common inner-sphere mechanism for the reductive cleavage of SAM in the radical SAM family of Fe−S enzymes.

Acknowledgment. This work was supported by grants from the National Institutes of Health (GM62542 to M.K.J., GM47295 to B.H.H., HL13531 to B.M.H., and National Research Service Postdoctoral Fellowship, DK 59730 to M.M.C.). We thank Dr. Katherine Gibson for the plasmid containing the *bioB* gene.

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(7) *Escherichia coli* $[2Fe-2S]^{2+}$ his_6-BioB was overexpressed in *E. coli* BL21-[DE3] cells. His_6-BioB was purified by passing the cell-free extract through a Ni-chelating affinity column and eluting his_6-BioB with an increasing gradient of imidazole. Overexpression and purification of WT *E. coli* $[2Fe-2S]^{2+}$ BioB were performed using standard protocols, and the preparation of $[4Fe-4S]^{2+}$ BioB followed the procedure developed by Fontecave and co-workers.[4] Samples of $[4Fe-4S]^{2+}$ BioB in the absence or presence of 10 × SAM were prepared in 50 mM Tris-HCl buffer, pH 8.5, with 200 mM NaCl and 1 mM DTT, and 3 mM, 0.25 mM, and 1 mM $[4Fe-4S]^{2+}$ his_6-BioB were used for RR, Mössbauer, and EPR spectroscopies, respectively. RR spectra of $[4Fe-4S]^{2+}$ BioB in the absence and presence of SAM were collected on both polyhistidine-tagged and WT protein, and the spectra showed no differences.
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JA0283044