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Evidence from Mössbauer Spectroscopy for Distinct [2Fe-2S]²⁺ and [4Fe-4S]²⁺ Cluster Binding Sites in Biotin Synthase from *Escherichia coli*

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Biotin synthase (BS) catalyzes the substitution of a sulfur atom for hydrogen at two saturated carbon atoms in the substrate dethiobiotin; this is an unprecedented reaction in biochemistry.

¹ In vitro biotin synthesis is catalyzed by BS in the presence of *S*-adenosylmethionine (AdoMet) and reduced flavodoxin² and generates methionine and 5'-deoxyadenosine,³ indicating that biotin synthase is an AdoMet-dependent radical enzyme. In general, AdoMet-dependent radical enzymes⁴ are active containing a [4Fe-4S]^{2+/+} cluster that is intimately involved in AdoMet cleavage and radical generation.⁵ This cluster is bound within a conserved CxxxCxxC sequence motif common to all AdoMet-dependent radical enzymes.⁶ However, BS from *E. coli* is purified containing only a stable [2Fe-2S]²⁺ cluster,⁷ and isotope labeling suggests that the role of this cluster is to provide sulfur for the biotin thioether ring.⁸ Thus two potentially conflicting roles for FeS clusters have been proposed in biotin synthase.

The FeS clusters in BS are remarkably pliable. While the aerobically purified enzyme is a dimer that contains two [2Fe-2S]²⁺ clusters,^{7,9} the protein can be chemically converted to contain primarily [4Fe-4S]²⁺ or [4Fe-4S]⁺ clusters by strong chemical reduction with dithionite in the presence or absence, respectively, of 60% ethylene glycol.^{7,9–11} Reconstitution under milder conditions yields protein that appears to contain ~1:1 [2Fe-2S]²⁺: [4Fe-4S]²⁺ clusters, as judged by UV/visible spectra and chemical analysis.¹² Comparison of the catalytic activity of various cluster reconstitution states of BS suggests that this latter state, with two different clusters present, is the most active for a single turnover in the absence of additional iron and sulfide.¹³

However, the precise cluster state of this active enzyme preparation remains ambiguous. Since BS is a homodimer,¹⁴ this enzyme could contain one [2Fe-2S]²⁺ and one [4Fe-4S]²⁺ cluster per dimer, both bound within the conserved cluster binding site in opposing monomers. Alternatively, this enzyme could contain one [2Fe-2S]²⁺ and one [4Fe-4S]²⁺ cluster per monomer, for a total of 4 clusters per dimer bound in two types of sites. To distinguish these potential cluster configurations, we turned to Mössbauer spectroscopy using differentially substituted ⁵⁷Fe protein.

When BS is expressed in *E. coli* grown in minimal media supplemented with ⁵⁷FeCl₃,¹⁵ the purified brown-red protein (⁵⁷Fe-BS, **B**) contains 1.9 Fe/monomer¹⁷ and exhibits a UV/visible absorption band at 452 nm consistent with the presence of [2Fe-2S]²⁺ clusters.⁷ The Mössbauer spectrum¹⁶ (Figure 1) indicates the protein contains ≥98% [2Fe-2S]²⁺ clusters (Table 1), with

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no evidence of $[4\text{Fe-4S}]^{2+}$ clusters. The isomer shift (0.27 mm/s) and quadrupole splitting (0.47 mm/s) are similar to values previously reported for BS apoprotein chemically reconstituted with $[2\text{Fe-2S}]^{2+}$ clusters. When this protein is reduced with dithionite in a buffer containing 60% ethylene glycol,⁷ the reduced $[2\text{Fe-2S}]$ clusters dissociate from the protein and reassemble as $[4\text{Fe-4S}]^{2+}$ clusters.¹¹ The Mössbauer spectrum of undesalted protein prepared according to this method¹⁷ (**D**) contains 92% of cluster-associated Fe in a single species with parameters ($\delta = 0.42$ mm/s, $\Delta E_Q = 1.00$ mm/s) that are consistent with a $[4\text{Fe-4S}]^{2+}$ cluster. A small amount of $[2\text{Fe-2S}]^{2+}$ cluster remains (~8% of total cluster Fe) in addition to Fe^{2+} in both S and O/N coordination environments, presumably present as a buffer contaminant due to only partial cluster reassembly. Similar results have been observed by Trautwein and coworkers examining the conversion of $[2\text{Fe-2S}]^{2+}$ to $[4\text{Fe-4S}]^{2+}$ clusters following dithionite reduction of reconstituted BS apoprotein.⁹

Although it was originally postulated that this $[4\text{Fe-4S}]^{2+}$ cluster occupied the same site as the original $[2\text{Fe-2S}]^{2+}$ cluster, under milder reducing conditions the protein can be reconstituted with a $[4\text{Fe-4S}]^{2+}$ cluster without disturbing the original $[2\text{Fe-2S}]^{2+}$ cluster.¹² When ^{57}Fe -BS is reconstituted with $^{57}\text{FeCl}_3$ and Na_2S using dithiothreitol (DTT) as reductant, the Mössbauer spectrum (**A**) shows the presence of both cluster types with a ratio of ~1:0.8 $[2\text{Fe-2S}]^{2+}$: $[4\text{Fe-4S}]^{2+}$ clusters. This sample also contains significant quantities of mononuclear Fe, as judged by both the Mössbauer spectrum and Fe analyses;¹⁷ we have had difficulty consistently removing excess Fe by gel filtration chromatography following chemical reconstitution. Similar mixed cluster spectra were previously obtained for ^{57}Fe -reconstituted BS following reduction and partial air oxidation, although the authors attribute the varying mixtures of clusters to conversion of $[4\text{Fe-4S}]^{2+}$ to $[2\text{Fe-2S}]^{2+}$ clusters upon exposure to air.⁹

Chemical analysis of the Fe and S^{2-} content of protein containing both $[2\text{Fe-2S}]^{2+}$ and $[4\text{Fe-4S}]^{2+}$ clusters suggested that there was one of each cluster type per monomer.¹² This would only be possible if there are two distinct cluster binding sites in each BS monomer. Since mild reduction leaves the original $[2\text{Fe-2S}]^{2+}$ cluster untouched, we used differential substitution with ^{57}Fe to test for separation of the $[2\text{Fe-2S}]^{2+}$ and $[4\text{Fe-4S}]^{2+}$ cluster sites. When ^{57}Fe -BS was reconstituted with unlabeled FeCl_3 , Na_2S , and DTT, the chemical analysis¹⁷ and the UV/visible spectrum of the resulting protein (not shown) indicated the presence of both $[2\text{Fe-2S}]^{2+}$ and $[4\text{Fe-4S}]^{2+}$ clusters, while the Mössbauer spectrum (**C**) showed that only the $[2\text{Fe-2S}]^{2+}$ cluster contains ^{57}Fe . If the $[4\text{Fe-4S}]^{2+}$ cluster were built up by addition of unlabeled Fe to the ^{57}Fe -labeled $[2\text{Fe-2S}]^{2+}$ cluster site, then the Mössbauer spectrum should show half of the ^{57}Fe present in a $[4\text{Fe-4S}]^{2+}$ environment. Since this was not observed, the $[4\text{Fe-4S}]^{2+}$ cluster must be assembled at a separate site from the original $[2\text{Fe-2S}]^{2+}$ cluster. In a complementary experiment, unlabeled BS was reconstituted with $^{57}\text{FeCl}_3$, Na_2S , and DTT. Again, the UV/visible spectrum showed both cluster types, but the Mössbauer spectrum (**E**) shows >90% of the total cluster-associated ^{57}Fe was present in $[4\text{Fe-4S}]^{2+}$ clusters. This demonstrates the stability of the original $[2\text{Fe-2S}]^{2+}$ cluster to exchange with free ^{57}Fe during the reconstitution process and again indicates that the $[4\text{Fe-4S}]^{2+}$ cluster is bound at a unique site.

BS contains six conserved cysteine residues. Resonance Raman spectra of BS purified with $[2\text{Fe-2S}]^{2+}$ clusters show a strong B_{3u}^t vibrational mode at 301 cm^{-1} that indicates a 3 S and 1 O/N coordination environment,⁷ while spectra of reduced BS containing only $[4\text{Fe-4S}]^{2+}$ clusters are more ambiguous, with an A_1^b breathing mode at 338 cm^{-1} that lies between typical values for a 4 S coordination environment ($\sim 335\text{ cm}^{-1}$) vs. a 3 S and 1 O/N coordination environment ($\sim 340\text{--}342\text{ cm}^{-1}$).^{7,18} Mutagenesis data implicates the conserved CxxxCxxC motif consisting of Cys53, Cys57, and Cys60 as the binding site for the $[4\text{Fe-4S}]^{2+}$ cluster.^{19,20} Mutation of any of these residues to alanine prevents reconstitution of a $[4\text{Fe-4S}]^{2+}$ cluster and abolishes activity,¹⁹ but leaves the initial $[2\text{Fe-2S}]^{2+}$ cluster intact.²⁰ In contrast,

mutation of the other conserved cysteine residues, Cys97, Cys128, and Cys188, to alanine results in proteins that can be reconstituted with a $[4\text{Fe-4S}]^{2+}$ cluster but are still inactive for biotin synthesis.¹⁹ We propose that these three cysteine residues are ligands to the $[2\text{Fe-2S}]^{2+}$ cluster. The ability of BS to simultaneously accommodate both $[4\text{Fe-4S}]^{2+}$ and $[2\text{Fe-2S}]^{2+}$ clusters is consistent with unique roles for these clusters in facilitating AdoMet reductive cleavage and biotin sulfur insertion.¹³

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15. ⁵⁷Fe labeled HT-BioB was expressed and purified as previously described¹¹ except that bacteria were grown in M9 media (Miller, J. H. *Experiments in Molecular Genetics*; Cold Spring Harbor Press: Cold Spring Harbor, New York, 1972) supplemented with 10 μM ⁵⁷FeCl₃, 0.4% glucose, 0.4% casamino acids, and trace elements (NeidhardtFCBlochPLSmithDFJ *Bacteriol*1974119736747 [PubMed: 4604283])
16. Mössbauer spectra were recorded at 4.2 K with 450 Gauss external field on a constant acceleration spectrometer, model MS-1200D from Ranger Scientific, using a Janis SuperVaritemp cryostat (model 8DT), a Lakeshore temperature controller (model 340), and a ⁵⁷Co source from Web Research Co.
17. Reconstituted samples were prepared as previously described,¹² excess Fe removed by gel filtration chromatography (except sample D), and the samples concentrated to ~500 μM . All steps were

performed under an argon or nitrogen atmosphere. Metal analyses (Fe/monomer): (A) 7.8, (B) 1.9, (C) 7.6, (D) 2.5, (E) 6.4.

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Figure 1.

Mössbauer spectra of biotin synthase: (A) (^{57}Fe)-BS reconstituted with $^{57}\text{FeCl}_3$, Na_2S , and DTT,¹² (B) initial (^{57}Fe)-BS containing only $[\text{2Fe-2S}]^{2+}$ clusters, (C) (^{57}Fe)-BS reconstituted with unlabeled FeCl_3 , Na_2S , and DTT, (D) (^{57}Fe)-BS containing primarily $[\text{4Fe-4S}]^{2+}$ clusters, generated by reduction with $\text{Na}_2\text{S}_2\text{O}_4$ ⁷ in the presence of $^{57}\text{FeCl}_3$ and Na_2S in 60% ethylene glycol, (E) unlabeled BS reconstituted with $^{57}\text{FeCl}_3$, Na_2S , and DTT. Model spectra used to fit spectrum A are shown at the top.

Biotin synthase is an AdoMet-dependent radical enzyme that catalyzes the insertion of an FeS cluster-derived sulfur atom into dethiobiotin. The dimeric enzyme is purified containing one [2Fe-2S]²⁺ cluster per monomer, but is most active when reconstituted with an additional [4Fe-4S]²⁺ cluster per monomer. Using Mössbauer spectroscopy coupled with differential reconstitution of each cluster with ⁵⁷Fe, we show that the reconstituted enzyme has ~1:1 [2Fe-2S]²⁺ and [4Fe-4S]²⁺ clusters, and that the [4Fe-4S]²⁺ cluster is assembled at an alternate site not previously occupied by the [2Fe-2S]²⁺ cluster. These data suggest that biotin synthase is evolved to simultaneously accommodate two different clusters with unique roles in catalysis.

Table 1
Sample Composition and Mössbauer Parameters

Sample	$[2\text{Fe-2S}]^{2+}$	^{57}Fe present in each environment (% total) ^a		$\text{Fe}^{2+}(\text{O/N})_{5/6}$
		$[4\text{Fe-4S}]^{2+}$	Fe^{2+}S_4	
A	24	40 (0.83) ^b	17	19
B	98	0	0	2
C	100	0	0	0
D	5	70	3	22
E	8	85	0	7
$\delta(\text{mm/s})$	0.27	0.42	0.63	1.08
$\Delta E_Q(\text{mm/s})$	0.49	1.00	2.80	2.60

^aCalculated from spectra in Figure 1.

^b $[4\text{Fe-4S}]^{2+}/[2\text{Fe-2S}]^{2+}$ ratio.