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Evidence from Mössbauer Spectroscopy for Distinct $[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ 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 flavodoxin[2] and generates methionine and 5′-deoxyadenosine,[3] indicating that biotin synthase is an AdoMet-dependent radical enzyme. In general, AdoMet-dependent radical enzymes[4] are active containing a $[4Fe\text{-}4S]^{2+/+}$ 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 $[2Fe\text{-}2S]^{2+}$ 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 $[2Fe\text{-}2S]^{2+}$ clusters,[7,9] the protein can be chemically converted to contain primarily $[4Fe\text{-}4S]^{2+}$ or $[4Fe\text{-}4S]^{+}$ 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 $[2Fe\text{-}2S]^{2+}$:$[4Fe\text{-}4S]^{2+}$ 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 $[2Fe\text{-}2S]^{2+}$ and one $[4Fe\text{-}4S]^{2+}$ cluster per dimer, both bound within the conserved cluster binding site in opposing monomers. Alternatively, this enzyme could contain one $[2Fe\text{-}2S]^{2+}$ and one $[4Fe\text{-}4S]^{2+}$ cluster per monomer, for a total of four clusters per dimer bound in two types of sites. To distinguish these potential cluster configurations, we turned to Mössbauer spectroscopy using differentially substituted ^{57}Fe protein.

When BS is expressed in *E. coli* grown in minimal media supplemented with $^{57}FeCl_3$,[15] the purified brown-red protein (^{57}Fe-BS, **B**) contains 1.9 Fe/monomer[17] and exhibits a UV/visible absorption band at 452 nm, consistent with the presence of $[2Fe\text{-}2S]^{2+}$ clusters.[7] The Mössbauer spectrum[16] (Figure 1) indicates that the protein contains ≥98% $[2Fe\text{-}2S]^{2+}$ clusters (Table 1), with no evidence of $[4Fe\text{-}4S]^{2+}$ clusters. The isomer shift (0.27 mm/s) and quadropole splitting (0.47 mm/s) are similar to values previously reported for BS apoprotein chemically reconstituted with $[2Fe\text{-}2S]^{2+}$ clusters.[9] When this protein is reduced with dithionite in a buffer containing 60% ethylene glycol,[7] the reduced [2Fe-2S] clusters dissociate from the protein and reassemble as $[4Fe\text{-}4S]^{2+}$ clusters.[11]

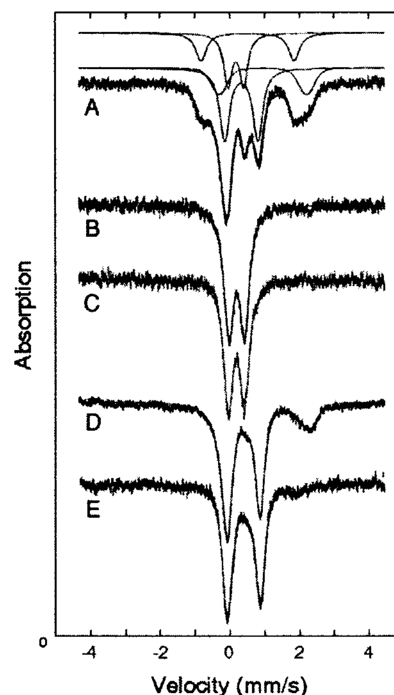

Figure 1. Mössbauer spectra of biotin synthase: (A) (^{57}Fe)-BS reconstituted with $^{57}FeCl_3$, Na_2S, and DTT;[12] (B) initial (^{57}Fe)-BS containing only $[2Fe\text{-}2S]^{2+}$ clusters; (C) (^{57}Fe)-BS reconstituted with unlabeled $FeCl_3$, Na_2S, and DTT; (D) (^{57}Fe)-BS containing primarily $[4Fe\text{-}4S]^{2+}$ clusters, generated by reduction with $Na_2S_2O_4$[7] in 60% ethylene glycol; (E) unlabeled BS reconstituted with $^{57}FeCl_3$, Na_2S, and DTT. Model spectra used to fit spectrum A are shown at the top.

Table 1. Sample Compostion and Mössbauer Parameters

sample	^{57}Fe present in each environment (% total)[a]			
	$[2Fe\text{-}2S]^{2+}$	$[4Fe\text{-}4S]^{2+}$	$Fe^{2+}S_4$	$Fe^{2+}(O/N)_{5/6}$
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
δ (mm/s)	0.27	0.42	0.63	1.08
ΔE_Q (mm/s)	0.49	1.00	2.80	2.60

[a] Calculated from spectra in Figure 1. [b] $[4Fe\text{-}4S]^{2+}$/$[2Fe\text{-}2S]^{2+}$ ratio.

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The Mössbauer spectrum of undesalted protein prepared according to this method[17] (**D**) contains 92% of cluster-associated Fe in a single species with parameters ($d = 0.42$ mm/s, $\Delta E_Q = 1.00$ mm/s) that are consistent with a $[4Fe\text{-}4S]^{2+}$ cluster. A small amount of $[2Fe\text{-}2S]^{2+}$ 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 co-workers on examining the conversion of $[2Fe\text{-}2S]^{2+}$ to $[4Fe\text{-}4S]^{2+}$ clusters following dithionite reduction of reconstituted BS apoprotein.[9]

Although it was originally postulated that this $[4Fe\text{-}4S]^{2+}$ cluster occupied the same site as the original $[2Fe\text{-}2S]^{2+}$ cluster, under milder reducing conditions the protein can be reconstituted with a $[4Fe\text{-}4S]^{2+}$ cluster without disturbing the original $[2Fe\text{-}2S]^{2+}$ cluster.[12] When ^{57}Fe-BS is reconstituted with $^{57}FeCl_3$ 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 $[2Fe\text{-}2S]^{2+}$:$[4Fe\text{-}4S]^{2+}$ clusters. This sample also contains significant quantities of mononuclear Fe, as judged by both the Mossbauer 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 $[4Fe\text{-}4S]^{2+}$ to $[2Fe\text{-}2S]^{2+}$ clusters upon exposure to air.[9]

Chemical analysis of the Fe and S^{2-} content of protein containing both $[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ 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 $[2Fe\text{-}2S]^{2+}$ cluster untouched, we used differential substitution with ^{57}Fe to test for separation of the $[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ cluster sites. When ^{57}Fe-BS was reconstituted with unlabeled $FeCl_3$, Na_2S, and DTT, the chemical analysis[17] and the UV/visible spectrum of the resulting protein (not shown) indicated the presence of both $[2Fe\text{-}2S]^{2+}$ and $[4Fe\text{-}4S]^{2+}$ clusters, while the Mössbauer spectrum (**C**) showed that only the $[2Fe\text{-}2S]^{2+}$ cluster contains ^{57}Fe. If the $[4Fe\text{-}4S]^{2+}$ cluster were built up by addition of unlabeled Fe to the ^{57}Fe-labeled $[2Fe\text{-}2S]^{2+}$ cluster site, then the Mössbauer spectrum should show half of the ^{57}Fe present in a $[4Fe\text{-}4S]^{2+}$ environment. Since this was not observed, the $[4Fe\text{-}4S]^{2+}$ cluster must be assembled at a separate site from the original $[2Fe\text{-}2S]^{2+}$ cluster. In a complementary experiment, unlabeled BS was reconstituted with $^{57}FeCl_3$, Na_2S, and DTT. Again, the UV/visible spectrum showed both cluster types, but the Mössbauer spectrum (**E**) shows that >90% of the total cluster-associated ^{57}Fe was present in $[4Fe\text{-}4S]^{2+}$ clusters. This demonstrates the stability of the original $[2Fe\text{-}2S]^{2+}$ cluster to exchange with free ^{57}Fe during the reconstitution process and again indicates that the $[4Fe\text{-}4S]^{2+}$ cluster is bound at a unique site.

BS contains six conserved cysteine residues. Resonance Raman spectra of BS purified with $[2Fe\text{-}2S]^{2+}$ 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 $[4Fe\text{-}4S]^{2+}$ clusters are more amibiguous, with an A_1^{b} breathing mode at 338 cm^{-1} that lies between typical values for a 4 S coordination environment (~335 cm^{-1}) vs a 3 S and 1 O/N coordination environment (~340−342 cm^{-1}).[7,18] Mutagenesis data implicate the conserved CxxxCxxC motif consisting of Cys53, Cys57, and Cys60 as the binding site for the $[4Fe\text{-}4S]^{2+}$ cluster.[19,20] Mutation of any of these residues to alanine prevents reconstitution of a $[4Fe\text{-}4S]^{2+}$ cluster and abolishes activity[19] but leaves the initial $[2Fe\text{-}2S]^{2+}$ 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 $[4Fe\text{-}4S]^{2+}$ cluster but are still inactive for biotin synthesis.[19] We propose that these three cysteine residues are ligands to the $[2Fe\text{-}2S]^{2+}$ cluster. The ability of BS to simultaneously accommodate both $[4Fe\text{-}4S]^{2+}$ and $[2Fe\text{-}2S]^{2+}$ clusters is consistent with unique roles for these clusters in facilitating AdoMet reductive cleavage and biotin sulfur insertion.[13]

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(16) Mössbauer spectra were recorded at 4.2 K with 450 G 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 ^{57}Co source from Web Research Co.

(17) Reconstituted samples were prepared as previously described,[12] excess Fe was removed by gel filtration chromatography (except sample D), and the samples were concentrated to ~500 mM. 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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