Spectroscopic Changes during a Single Turnover of Biotin Synthase: Destruction of a [2Fe-2S] Cluster Accompanies Sulfur Insertion†

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ABSTRACT: Biotin synthase catalyzes the insertion of a sulfur atom between the saturated C6 and C9 carbons of dethiobiotin. Catalysis requires AdoMet and flavodoxin and generates 5′-deoxyadenosine and methionine, suggesting that biotin synthase is an AdoMet-dependent radical enzyme. Biotin synthase (BioB) is aerobically purified as a dimer of 38.4 kDa monomers that contains 1 – 1.5 [2Fe-2S]2+ clusters per monomer and can be reconstituted with exogenous iron, sulfide, and reductants to contain up to two [4Fe-4S] clusters per monomer. The iron–sulfur clusters may play a dual role in biotin synthase: a reduced iron–sulfur cluster is probably involved in radical generation by mediating the reductive cleavage of AdoMet, while recent in vitro labeling studies suggest that an iron–sulfur cluster also serves as the immediate source of sulfur for the biotin thioether ring. Consistent with this dual role for iron–sulfur clusters in biotin synthase, we have found that the protein is stable, containing one [2Fe-2S]2+ cluster and one [4Fe-4S]2+ cluster per monomer. In the present study, we demonstrate that this mixed cluster state is essential for optimal activity. We follow changes in the Fe and S content and UV/visible and EPR spectra of the enzyme during a single turnover and conclude that during catalysis the [4Fe-4S]2+ cluster is preserved while the [2Fe-2S]2+ cluster is destroyed. We propose a mechanism for incorporation of sulfur into dethiobiotin in which a sulfur atom is oxidatively extracted from the [2Fe-2S]2+ cluster.

Biotin synthase (BioB) catalyzes the insertion of a sulfur atom between the C6 and C9 positions of dethiobiotin (Scheme 1). Aerobically purified recombinant BioB is a homodimeric [2Fe-2S]2+ protein that requires additional iron and reductants for maximal activity (1, 2). In addition, biotin synthesis requires AdoMet, flavodoxin, flavodoxin reductase, and NADPH (2 – 4), suggesting that biotin synthase is an AdoMet-dependent radical enzyme (2, 3, 5). Consistent with this proposal, BioB contains an essential iron–sulfur cluster binding motif, CxxxxCxxC, shared with known radical enzymes that include lysine 2,3-aminomutase (6), class III ribonucleotide reductase (7), and pyruvate formate-lyase activase (8). Further, BioB-catalyzed biotin synthesis is accompanied by the formation of 5′-deoxyadenosine (9, 10), an intermediate or product characteristic of AdoMet-dependent radical enzymes (11).

The inability to absolutely identify the sulfur-containing substrate for biotin synthase has hampered previous investigations. Cysteine is identified as the most likely source of sulfur in vivo (12) and in crude cell extracts (2, 5) but will not serve as sulfur donor with the purified enzyme (4, 13). Recent in vitro labeling studies suggest that sulfide is the immediate sulfur substrate (14 – 16). Marquet and co-workers reconstituted apoprotein with iron and [35S]sulfide and showed that the biotin formed retains about 70% of the label (14). Gibson and co-workers expressed and purified labeled BioB from medium containing [35S]cysteine and demonstrated partial incorporation of radiolabel into biotin (15). The most straightforward conclusion from these studies is that the iron–sulfur clusters serve as an immediate sulfur donor for thioether ring formation, although sulfide could also be tightly bound to another site on the protein.

The proposed role for iron–sulfur clusters as sulfur donor in biotin synthase distinguishes this enzyme from other AdoMet-dependent radical enzymes. In those enzymes, the iron–sulfur cluster has been proposed to play an essential role in generating an organic radical (17). For example, in lysine 2,3-aminomutase, enzyme containing a [4Fe-4S]+ cluster and AdoMet will catalyze the rapid isomerization of lysine and β-lysine in the absence of additional iron or reductants (18). This suggests a mechanism in which the reaction between a [4Fe-4S]+ cluster and AdoMet (17) results in the reductive cleavage of AdoMet, generating a transient 5′-deoxyadenosynol radical (19). The 5′-deoxyadenosynol radical then abstracts a hydrogen from the substrate, generating 5′- deoxyadenosine and a substrate radical. Since the reaction

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* Abbreviations: AdoMet, S-adenosyl-L-methionine; BioB, biotin synthase; DTT, dithiothreitol; EPR, electron paramagnetic resonance; Fd, flavodoxin; FNR, ferredoxin (flavodoxin); NADP+ oxidoreductase; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; NADP+, nicotinamide adenine dinucleotide phosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

Scheme 1: Conversion of Dethiobiotin to Biotin Catalyzed by Biotin Synthase
catalyzed by biotin synthase also requires AdoMet and generates 5'-deoxyadenosine, it has been postulated that biotin synthase catalyzes the reductive cleavage of AdoMet and the formation of a substrate radical (9, 10) in a reaction requiring a [4Fe-4S]$^{2+/3+}$ cluster (20–23).

Thus two very different roles have been proposed for the iron–sulfur clusters in biotin synthase. Since BioB is a homodimer, it is difficult to envision how these opposing functions could be accommodated within a single protein. However, we have found that although BioB is aerobically purified with ca. one [2Fe-2S]$^{2+}$ cluster per monomer, the protein contains an additional iron–sulfur cluster binding site (24). At the physiologic redox potential of aerobic Escherichia coli [ca. $-330$ mV (25)], BioB contains two stable clusters per monomer: one [2Fe-2S]$^{2+}$ cluster and one [4Fe-4S]$^{2+}$ cluster (24). The presence of two clusters may allow each to play a different role in the overall reaction mechanism. In the present study, we first examine the relative activity of enzyme containing either a 1:1 mixture of [2Fe-2S]$^{2+}$ and [4Fe-4S]$^{2+}$ clusters or entirely [2Fe-2S]$^{2+}$, [4Fe-4S]$^{2+}$, or [4Fe-4S]$^{3+}$ clusters. We conclude that the 1:1 mixed cluster state is the active enzyme. We then follow changes in these clusters over a single turnover using Fe and S analysis along with UV/visible and EPR spectroscopy and conclude that biotin synthesis is accompanied by selective destruction of the [2Fe-2S]$^{2+}$ cluster. In contrast, the [4Fe-4S]$^{2+}$ cluster is either preserved or transformed to a new unidentified cluster, depending on whether excess iron is present in the assay buffer. We propose a mechanism for radical-mediated sulfur insertion in which the [2Fe-2S]$^{2+}$ cluster is the immediate sulfur donor for biotin biosynthesis.

**MATERIALS AND METHODS**

**Materials.** All reagents were obtained from commercial sources and used without further purification. Wild-type BioB was purified as previously described (20) and was used for all of the results described in this paper. Unless otherwise stated, all protein purification steps were performed under aerobic conditions and all protein reduction, reconstitution, and analyses were performed under oxygen-free argon or nitrogen atmosphere. Protein concentration was determined using the Bradford protein assay (Bio-Rad) with BSA as a standard after standardization by quantitative amino acid analysis. Sulfide was determined by a modification of the Beinert method (26) described by Broderick (27). Iron was determined using bathophenanthroline under reductive conditions after digestion of the protein in 0.8% KMnO$_4$/0.2 N HCl as described by Fish (28).

**Reconstitution of [4Fe-4S] Clusters in BioB.** BioB containing two [4Fe-4S]$^{2+}$ clusters per monomer was obtained by incubation with excess FeCl$_3$, Na$_2$S, and dithionite in 100 mM Tris-HCl, pH 8, as previously described (20). Again, the buffer components were removed by passing the sample through a Sephadex G25 desalting column equilibrated with anaerobic 50 mM Tris-HCl and 10 mM KCl, pH 8, in a nitrogen glovebox. The greenish gray protein had no distinguishable UV/visible bands, but EPR spectra showed the presence of ~1.9 [4Fe-4S]$^{2+}$ clusters per monomer. Fe and S analyses were consistent with ca. 1.7–2.0 [4Fe-4S]$^{2+}$ clusters per BioB monomer. EPR spectra for protein containing two [4Fe-4S]$^{2+}$ clusters per monomer are essentially identical to those previously reported (see Figure 6 in ref 20), although the previous report wrongly attributed this spectrum to ~1.9 clusters per dimer. The cluster content reported here for chemically reconstituted [4Fe-4S]$^{2+/3+}$ protein is approximately twice that of our previous report (20), largely due to an overestimation of the protein content of EPR samples in that report resulting from our use of an erroneous extinction coefficient. We have now determined a more accurate extinction coefficient (24) and also routinely use Bradford assays to corroborate protein concentrations.

BioB containing one [2Fe-2S]$^{2+}$ and one [4Fe-4S]$^{2+}$ cluster per monomer was prepared by incubation of an anaerobic solution of BioB (100 μM) with DTT (10 mM), FeCl$_3$ (500 μM), and Na$_2$S (500 μM) in 100 mM Tris-HCl, pH 8, at room temperature for 30 min. The protein was passed through a Sephadex G25 desalting column equilibrated with anaerobic 50 mM Tris-HCl and 10 mM KCl, pH 8, in a nitrogen glovebox. The UV/visible spectrum has shoulders at ca. 410 and 460 nm, suggesting the presence of both [4Fe-4S]$^{2+}$ and [2Fe-2S]$^{2+}$ clusters, respectively (see Figure 2). Fe and S analyses indicate the presence of 5.8 ± 0.3 Fe and 6.2 ± 0.2 S$^{2-}$ per BioB monomer.

**Biotin Synthase Assay.** Assays were carried out under argon, and all solutions were purged with argon prior to addition to the assay with a gastight syringe. In a typical assay, BioB (40 μM monomer) was incubated in 50 mM Tris-HCl and 10 mM KCl, pH 8 (0.2–2.0 mL final assay volume), in a septum-covered glass vial and purged with argon. DTT (10 mM final concentration), FeCl$_3$ (200 μM), and Na$_2$S (200 μM) were added, and the solution was incubated for 10 min at room temperature. AdoMet (500 μM), flavodoxin (20 μM), flavodoxin reductase (4 μM), and NADPH (1 mM) were added, and the solution was incubated 10 min. Turnover was initiated by the addition of dethiobiotin (200 μM) and the solution incubated at 37 °C. At various intervals, a 100 μL sample was withdrawn and added to saturated sodium acetate, pH 4 (5 μL), resulting in rapid precipitation of the enzyme. The sample was chilled on ice for 10 min and then the protein removed by centrifugation for 10 min at 18000g. The supernatant (25 μL) was injected onto a C18 column ( Waters Novapak 2.5 × 150 mm) equilibrated with 97:3 H$_2$O:CH$_3$CN (10 mM H$_3$PO$_4$) at 30 °C and biotin eluted with a linear gradient from 3% to 6% CH$_3$CN over 25 min. Biotin and dethiobiotin were readily resolved and detected by their UV absorbance at 200 nm. Blanks were prepared that contained all assay components except dethiobiotin or BioB and after 4 h incubation at 37 °C contained less than 50 pmol of biotin. Standards were prepared by addition of varying concentrations of biotin to the blank. Under these conditions, 0.005–10 nmol of biotin was readily detected, corresponding to 0.05–100 μM biotin in the final assay mixture.
min⁻¹. The observed yield of biotin after 4 h is 1.8 ± 0.1 equiv per BioB dimer, and longer incubation never produces more than 2 equiv of biotin. At 25 °C (open squares), approximately 0.6 equiv of biotin is formed with an apparent rate constant of 0.03 min⁻¹, while additional biotin is formed much slower at ~0.001 min⁻¹. At this rate, we project that formation of 2 equiv of biotin would require incubation for >30 h at 25 °C, although in general this is not observed, probably due to the instability of the enzyme over this prolonged incubation time. Note that we do not add cysteine (as a sulfur source), nor do we add asparagine and MioC (2, 30) or fructose 1,6-bisphosphate (4), factors that other authors have used to improve activity; we see no rate enhancements upon addition of these potential effectors.

While our selection of components is based in part upon a rationalization of the order of enzymatic events in vivo, it is possible that certain components would not be required in vitro. We eliminated each component from the assay (Table 1) and found that only dethiobi, AdoMet, and flavodoxin are absolutely essential. Although FeCl₃, Na₂S, DTT, flavodoxin reductase, NADPH are not absolutely essential, activity is dramatically decreased if any of these components are left out of the assay. The accurate measurement of activity in the absence of sulfide was particularly difficult; DTT was prepared fresh to eliminate possible contamination due to hydrolysis of the stock solution, and assays in the absence of sulfide were performed on a separate argon manifold to prevent the diffusion of volatile hydrogen sulfide through the argon purge line. Only when these precautions were followed was decreased activity observed. The apparent requirement for FeCl₃, Na₂S, and DTT suggests that iron–sulfur cluster assembly or reconstitution is a prerequisite for catalysis, although partial activity may be possible if dissociation of clusters from one protein molecule allows reconstitution of clusters in another protein molecule.

BioB Containing a 1:1 Mixture of [2Fe-2S] and [4Fe-4S] Clusters Is Maximally Active in the Absence of Exogenous Iron. Although BioB is aerobically purified containing air-stable [2Fe-2S]²⁺ clusters (1), we have shown that chemical reduction in the presence of iron and sulfide can produce enzyme that contains either two [4Fe-4S]²⁺ clusters or two [4Fe-4S]⁺ clusters per monomer (20, 24). More recently, we have also discovered that incubation of the enzyme with Fe³⁺, S²⁻, and DTT produces a stable protein whose UV/visible spectrum suggests a mixture of cluster states (Figure 2, solid curve). These clusters are not EPR

Table 1: BioB Activity in the Absence of Each Assay Component

<table>
<thead>
<tr>
<th>missing assay component</th>
<th>activity [pmol of biotin min⁻¹ (nmol of BioB)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>complete assay</td>
<td>19.0 (100%)</td>
</tr>
<tr>
<td>−BioB</td>
<td>0</td>
</tr>
<tr>
<td>−dethiobi</td>
<td>0</td>
</tr>
<tr>
<td>−AdoMet</td>
<td>0</td>
</tr>
<tr>
<td>−flavodoxin</td>
<td>0</td>
</tr>
<tr>
<td>−flavodoxin reductase</td>
<td>0.3 (1.5%)</td>
</tr>
<tr>
<td>−NADPH</td>
<td>0.6 (3.2%)</td>
</tr>
<tr>
<td>−FeCl₃</td>
<td>1.2 (6.3%)</td>
</tr>
<tr>
<td>−Na₂S</td>
<td>1.5 (7.9%)</td>
</tr>
<tr>
<td>−DTT</td>
<td>0.3 (1.5%)</td>
</tr>
</tbody>
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* Activity detected following 20 min incubation at 37 °C. Our lower detection limit is 5 pmol of biotin; therefore, zero activity is less than 0.25 pmol min⁻¹ (nmol of BioB)⁻¹ (1.3%).

## RESULTS

BioB Dimer Catalyzes Two Turnovers of Biotin Synthesis. Biotin synthase (BioB) purified from *E. coli* or *Bacillus sphaericus* catalyzes the synthesis of trace amounts of biotin in vitro (4, 13, 29), but the yield is dramatically improved in the presence of several additional components. Ifuku and co-workers first identified the requirement for iron (29), and Sanyal later showed that inclusion of Fe³⁺ and DTT in assays generated optimal activity (1). Ifuku and others showed that AdoMet stimulates biotin synthesis (2, 5, 29), and more recently flavodoxin, flavodoxin reductase, and NADPH have been employed as the presumed physiologic reducing system for radical generation (2–4). We have divided our assay system into three steps. First, FeCl₃, Na₂S, and DTT are added to anaerobic enzyme to reconstitute the active iron–sulfur clusters. After several minutes, AdoMet, flavodoxin, flavodoxin reductase, and NADPH are added to set up the putative radical generation machinery. Finally, dethiobi is added to initiate catalysis. The assay components and final assay mix are purged and maintained under an O₂-free argon atmosphere. Biotin production begins immediately, with no distinguishable lag period, and proceeds with two exponential phases over 2 h at 37 °C and >4 h at 25 °C (Figure 1). At 37 °C (open circles), approximately 1 equiv of biotin per BioB dimer is formed with an apparent first-order rate constant of 0.07 min⁻¹, while an additional 0.8–1 equiv is formed with an apparent first-order rate constant of 0.006...
active, and we can fit the UV/visible spectrum quite well to a 1:1 mixture of [2Fe-2S]$^{2+}$ and [4Fe-4S]$^{2+}$ clusters. The presence of the [2Fe-2S]$^{2+}$ cluster is distinguishable due to the broad shoulders in the UV/visible spectrum at 320, 460, and 550 nm, while the presence of a [4Fe-4S]$^{2+}$ cluster is suggested by the band at 410 nm. Further, chemical analysis indicates that the Fe content is increased from 2.5 ± 0.2 Fe atoms in the air-purified BioB monomer to 5.8 ± 0.3 Fe atoms in the reconstituted BioB monomer, and the S$^{2-}$ content is increased from 3.8 ± 0.2 S$^{2-}$ ions in the reconstituted BioB monomer to 6.2 ± 0.2 S$^{2-}$ ions in the reconstituted BioB monomer. A 1:1 mixture of [2Fe-2S]$^{2+}$ and [4Fe-4S]$^{2+}$ clusters is also consistent with electrochemical analysis which demonstrates that BioB is stable in an asymmetric state containing one [2Fe-2S]$^{2+}$ cluster and one [4Fe-4S]$^{2+}$ cluster per monomer over a potential range from −140 to −430 mV (24).

Since BioB could be prepared in a number of cluster forms and these clusters are reasonably stable under strictly anaerobic conditions, we expected that only one of these cluster states would be maximally active. BioB was reductively reconstituted to each cluster state as described in Materials and Methods, and the protein was repurified by gel filtration chromatography, all under anaerobic conditions. The protein was then added to anaerobic assays, with FeCl$_3$, Na$_2$S, and DTT intentionally omitted from the assays to prevent further cluster assembly. Note that omission of any one of these reagents from our standard assay results in a 92—98% decrease in activity (Table 1), probably due to the inability to reconstitute the active cluster state in situ. However, we find that the mixed cluster state, with a 1:1 ratio of [2Fe-2S]$^{2+}$ and [4Fe-4S]$^{2+}$ clusters, is active in the absence of any additional iron (Figure 3, solid circles), producing 0.9 ± 0.1 equiv of biotin per BioB monomer with an apparent first-order rate constant of 0.07 min$^{-1}$. Note that this rate of biotin formation is identical to the initial rate of biotin production in our standard in vitro assay (Figure 1), suggesting that this mixed cluster state is the active species in these assays. Addition of sulfide or iron and sulfide did not result in a further increase in activity. BioB that contained two [4Fe-4S] clusters was much less active, regardless of the redox state of the clusters. BioB containing two [4Fe-4S]$^{2+}$ clusters produced biotin with an initial rate constant of 0.001 min$^{-1}$ (closed squares), while BioB containing two [4Fe-4S]$^{2+}$ clusters produced biotin with an initial rate constant of 0.0005 min$^{-1}$ (open circles). These enzyme species are more sensitive to degradation by oxygen, and we suspect that the activity detected is actually due to a contamination of these reconstituted [4Fe-4S] proteins with some enzyme that has been oxidized back to the active mixed cluster state. Finally, as has been previously demonstrated (1, 29), oxidized [2Fe-2S]$^{2+}$ enzyme is completely inactive in the absence of additional iron and reductants.

**Biotin Synthesis Is Accompanied by the Loss of the [2Fe-2S]$^{2+}$ Cluster.** The source of sulfur for the biotin thioether ring has recently been identified as enzyme bound sulfide, probably derived from the iron−sulfur clusters (14−16). If sulfide were extracted from either the [2Fe-2S]$^{2+}$ or [4Fe-4S]$^{2+}$ cluster, then we would predict that this would result in characteristic changes in the UV/visible spectrum of BioB. Starting with reconstituted BioB containing 1:1 [2Fe-2S]$^{2+}$ and [4Fe-4S]$^{2+}$ clusters, we scaled up our typical assay to ~2 mL and conducted the assay in an anaerobic cuvette to allow observation of the UV/visible spectrum. We observed a slow time-dependent decrease in the UV/visible absorbance over the range from 400 to 600 nm (Figure 4); the difference spectrum (Figure 4 inset) shows a maximal absorbance loss at 460 nm, characteristic of a [2Fe-2S]$^{2+}$ cluster. The total absorbance change after 2 h is ΔA$_{460}$ = −0.18, consistent with the loss of 30 μM [2Fe-2S]$^{2+}$ clusters (~0.85 equiv per monomer assuming Δε$_{460}$ ≈ 6000 M$^{-1}$ cm$^{-1}$ per cluster (24)). No EPR-detectable iron−sulfur clusters are observed during or following turnover. Chemical analysis of the amount of iron and sulfide bound to the protein indicates a decrease in the level of iron from 5.8 ± 0.3 Fe atoms to 3.7 ± 0.3 Fe atoms and a decrease in the level of sulfide from 6.2 ± 0.2 S$^{2-}$ ions to 3.4 ± 0.4 S$^{2-}$ ions. These spectra and stoichiometries are consistent with loss of the [2Fe-2S]$^{2+}$ cluster and preservation of the [4Fe-4S]$^{2+}$ cluster. The spectra in Figure 4 were recorded over 2 h at room temperature (~23
The similarity in the rates of biotin formation and \([2Fe-2S]^{2+}\) (Figure 5). Although we cannot rule out more complicated ring formation.

Cluster destruction leads us to conclude that the \([2Fe-2S]^{2+}\) is indistinguishable from the rate of the spectral changes observed at 37 °C to minimize the slight protein precipitation that is observed at 37 °C. We did not observe these spectral changes if either dethiobiotin or AdoMet was left out of the assay. We also removed samples for HPLC analysis and observed the formation of biotin at a rate (0.03 min\(^{-1}\)) that is consistent with the decreased temperature.

**DISCUSSION**

Despite recent progress, the mechanism of the enzymatic formation of the biotin thioether ring by biotin synthase remains largely unsolved. Previous efforts have been hampered by the very low activity of the overexpressed enzyme, the inability to positively identify the sulfur donor, continuing questions regarding other components essential for catalysis, and the potential sensitivity of the assay to oxygen. We have defined a minimal assay system that results in 1 turnover of biotin synthase within ca. 30 min and 2 turnovers in 4 h at 37 °C. Both the yield of biotin (1 equiv per BioB monomer) and the initial rate of biotin formation (0.07 min\(^{-1}\)) are improved over previous reports. Components absolutely essential for catalysis by BioB include dethiobiotin, AdoMet, and flavodoxin (Table 1). Omission of flavodoxin reductase destruction of the \([2Fe-2S]^{2+}\) cluster in active BioB. We reasoned that addition of iron, sulfide, and DTT to the assay buffer might lead to reconstitution of this cluster and multiple turnovers of the enzyme. This was not observed. Starting with BioB containing a 1:1 mixture of \([2Fe-2S]^{2+}\) and \([4Fe-4S]^{2+}\) clusters and with Fe\(_{31}\), Na\(_2\)S, and DTT added to the buffer, we followed the UV/visible spectral changes in the enzyme and removed samples for biotin determination and EPR analysis every 15 min. The exogenous iron species present under these conditions make accurate observation of the UV/visible spectrum difficult; however, biotin was formed, and the UV/visible spectrum was altered in a manner that is consistent with the results in Figures 4 and 5. When EPR spectra were recorded over time, we observe that a new EPR detectable species is formed at a rate that is comparable to the rate of biotin formation (Figure 6A). This species is not formed in the absence of substrates or in the absence of exogenous iron, and this protein-bound cluster is stable to repurification by gel filtration chromatography. The EPR spectrum is complex, but the dominant features include two positive peaks with \(g\) values of 2.0 and 1.96 and a negative peak with a \(g\) value of 1.93, which resemble the \([3Fe-4S]^{+}\) clusters found in ferredoxin mutants from *Paracoccus furiosus* (31). An additional feature at \(g = 1.86\) is not typically observed for \([3Fe-4S]^{+}\) clusters. Consistent with this interpretation, the spectrum is partially lost upon reduction with sodium dithionite (Figure 6B), which would reduce a \([3Fe-4S]^{+}\) cluster to an EPR-silent \([3Fe-4S]^{2+}\) cluster.

A new EPR-detectable species appears upon dithionite reduction whose spectrum is consistent with partial formation of a \([4Fe-4S]^{2+}\) cluster (a spectrum of chemically reconstituted \([4Fe-4S]^{2+}\) BioB is shown for comparison). Chemical analysis indicates that this new enzyme species contains 5.2 ± 0.4 Fe atoms and 4.9 ± 0.2 S\(^{2-}\) ions per monomer. Overall, we feel that these analyses and spectral data suggest that the presence of exogenous iron during the assay probably results in formation of a \([3Fe-4S]^{2+}\) cluster. Precise spectral characterization of these species is beyond the capabilities of the methods employed in this study; experiments in progress will use Mossbauer spectroscopy to further examine this transformation. Regardless of the identity of this new cluster species, the inability to observe multiple turnovers of biotin formation either in the absence or in the presence of exogenous iron appears to result from the inability to simultaneously maintain both the \([2Fe-2S]^{2+}\) and \([4Fe-4S]^{2+}\) clusters in the correct configuration following turnover.

**Figure 4:** Changes in the UV/visible spectrum of BioB observed during a single turnover. BioB (≈35 μM) was generated with 1:1 \([2Fe-2S]^{2+}\) and \([4Fe-4S]^{2+}\) clusters and was preincubated with AdoMet (500 μM), flavodoxin (10 μM), flavodoxin reductase (4 μM), and NADPH (1 mM) in an anaerobic cuvette, and the reaction was initiated by addition of dethiobiocin (200 μM) at 23 °C. UV/visible spectra were recorded at intervals (10, 30, 60, and 90 min shown) and show a gradual decrease in absorbance from 400 to 600 nm. The broad absorbance from 550 to 700 nm is due to the presence of flavodoxin semiquinone. (Inset) The difference spectrum shown) and show a gradual decrease in absorbance from 400 to 600 nm, consistent with the loss of a \([2Fe-2S]^{2+}\) cluster.

**Figure 5:** The production of biotin correlates with the decrease in absorbance at 460 nm. Biotin formation (squares) and the decrease in absorbance at 460 nm (circles) are both fit with a single rate constant of 0.03 min\(^{-1}\) at 23 °C. Assay conditions are as described for Figure 4. The absorbance at 460 nm was monitored, samples were withdrawn at intervals and quenched in saturated sodium acetate, pH 4, and biotin was quantified by HPLC analysis. The rate of biotin production is decreased relative to Figure 3 due to the decreased temperature.

Following Biotin Synthesis in the Presence of Iron, the Enzyme Contains a New EPR-Detectable Cluster. In the absence of exogenous iron, biotin formation appears to involve the extraction of sulfide from and the subsequent
or NADPH results in a 100-fold decrease in activity, while the omission of FeCl₃, Na₂S, and DTT results in a 10–20-fold decrease in activity. We do not appear to require additional small molecule components such as asparagine (2) or fructose 1,6-bisphosphate (4), nor do we require additional stoichiometric protein components (30) for a single turnover. Of course, since our BioB preparations are not 100% pure, we cannot rule out the possibility that a trace catalytic protein contributes to the enzymatic reaction. Our assay system also requires sustained anaerobiosis for maximal activity; assays in closed vials that have not been purged with argon do not generate detectable levels of biotin.

The requirement of Fe³⁺ and DTT for maximal activity suggests that the initial [2Fe-2S]²⁺ clusters must be at least partially reconstituted to [4Fe-4S]²⁺ clusters for full activity. We have demonstrated by electrochemical analysis of cluster reduction and reconstitution that the stable cluster state in the presence of sufficient iron, sulfide, and NADPH and/or DTT as reductants (E ≈ −330 mV) is a 1:1 mixture of [2Fe-2S]²⁺ and [4Fe-4S]²⁺ clusters in the BioB monomer (24). Similar heterogeneous mixed cluster states have been consistently observed by Mössbauer spectroscopy (22). BioB containing one [2Fe-2S]²⁺ and one [4Fe-4S]²⁺ cluster per monomer can be generated by incubation with FeCl₃, Na₂S, and DTT and repurified by anaerobic gel filtration and is stable under anaerobic conditions. When this protein is added to an assay, biotin is produced in the absence of additional iron, indicating that further cluster assembly is not necessary. BioB containing either two [4Fe-4S]²⁺ clusters or two [4Fe-4S]²⁺ clusters per monomer can also be produced chemically, but these proteins are much less active. This suggests that the active form of biotin synthase contains one [2Fe-2S]²⁺ and one [4Fe-4S]²⁺ cluster per monomer.

Previous studies using labeled sulfide had indicated that the sulfur destined for biotin is derived from sulfide bound within BioB, most likely from the iron—sulfur clusters. When we follow the UV/visible spectra of the mixture of [2Fe-2S]²⁺ and [4Fe-4S]²⁺ clusters during biotin production, we observe a decrease in absorbance that is consistent with transformation of the [2Fe-2S]²⁺ cluster. In the absence of excess exogenous iron, there are no EPR-detectable species formed and Fe and S analyses suggest a loss of 2.1 Fe atoms and 2.8 S²⁻ ions, and we therefore conclude that the cluster is not simply reduced but is dissociated from the enzyme. These results are consistent with the [2Fe-2S]²⁺ cluster serving as the source of sulfide for the biotin thioether ring. Further selective labeling studies are necessary to support this conclusion.

When additional iron and sulfide are included in the assay buffer, we observe the formation of a new cluster bound to BioB following biotin production. The EPR spectrum of this cluster is highly complex, but the central features include two positive peaks at g = 2.0 and g = 1.96 and a negative peak at g = 1.93 that are similar to the EPR spectrum of the [3Fe-4S]⁺ cluster in some mutants of ferredoxin from P. furiosus (31). This cluster largely disappears upon reduction with dithionite, while a new spectrum appears that is similar to our previously reported EPR spectrum of the [4Fe-4S]⁺ cluster in BioB. Definitive assignment of these cluster states is beyond the capabilities of the spectroscopic techniques employed here, but we would suggest that the addition of iron to the assay buffer may result in the improper assembly of a [3Fe-4S]⁺ cluster, and this could cause inactivation of the enzyme following a single turnover.

We propose a scheme for sulfur insertion into dethiobiotin from the [2Fe-2S]²⁺ cluster in BioB (Scheme 2). In this general scheme, the [4Fe-4S]²⁺ cluster in the mixed cluster form of BioB accepts an electron from flavodoxin and catalyzes the reductive cleavage of AdoMet, generating a 5′-deoxyadenosyl radical. This radical abstracts a hydrogen atom from the C9 position of dethiobiotin, generating 5′-deoxyadenosine and a transient C9-dethiobiotin radical, which then is attacked by the sulfur atom of the [2Fe-2S]²⁺ cluster. C–S bond formation must also be accompanied by

![Image](68x403 to 281x744)
one-electron oxidation of the sulfur, and we propose that this occurs through reduction of Fe$^{3+}$ to Fe$^{2+}$. The reaction of a second equivalent of AdoMet at the C6 position of the 9-mercaptodethiobiotin intermediate followed by ring closure leads to biotin formation and destruction of the [2Fe-2S]$^{2+}$ cluster. This scheme is consistent with our observations of the relatively high activity of the mixed cluster state of BioB and the loss of the [2Fe-2S]$^{2+}$ cluster during biotin formation. It is also consistent with previous observations of direct transfer of the C9 and C6 hydrogens of dethiobiotin to 5'-deoxyadenosine (9) with a 5'-deoxyadenosine:bihotin product ratio of ca. 2:1 (10, 32). On the basis of this scheme, we predict that 9-mercaptodethiobiotin should be a true intermediate in biotin formation; this species has been directly observed in the Arabidopsis biotin biosynthetic pathway (33) and has been shown to be converted to biotin by BioB from B. sphaericus (5).

UV/visible spectra and quantitative Fe and S analysis indicate that destruction of the [2Fe-2S]$^{2+}$ cluster accompanies biotin formation. We suggest that the failure to observe multiple turnovers of biotin formation with BioB is due to the inability to spontaneously reassemble the [2Fe-2S]$^{2+}$ cluster under our highly reducing assay conditions, where most of the free iron is reduced Fe$^{2+}$. Correct cluster reassembly may require specific proteins that facilitate cluster assembly prior to donating these preformed clusters to BioB (34). We expect that BioB will be capable of multiple turnovers of biotin production when specific proteins are found that can facilitate correct reassembly of the [2Fe-2S]$^{2+}$ cluster.

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REFERENCES