Recombinant *Escherichia coli* biotin synthase is a $[2Fe–2S]^{2+}$ protein in whole cells

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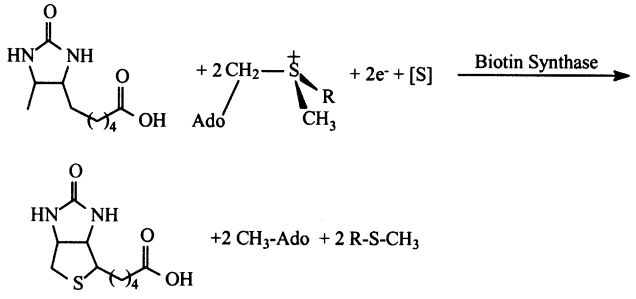
Abstract **EPR and Mössbauer spectroscopies have been used to determine the type and properties of the iron–sulfur clusters present in homologously expressed recombinant *Escherichia coli* BioB in whole cells prior to purification. Difference EPR spectra of samples of whole cells from a strain over-expressing *E. coli* BioB and a strain containing the same plasmid but without the *bioB* insertion showed an axial $S = 1/2$ resonance that was attributed to the $[2Fe–2S]^{+}$ cluster of the *E. coli* iron–sulfur cluster assembly 2Fe ferredoxin, based on principal *g*-values, linewidths and relaxation behavior. Comparison of the Mössbauer spectra of whole cells with and without the *bioB* insertion revealed that the *E. coli* cells with over-expressed BioB contain an additional species that exhibits a spectrum identical to that of the $[2Fe–2S]^{2+}$ cluster in purified recombinant BioB. The concentration of this $[2Fe–2S]^{2+}$ species in the whole cell sample was quantified using a Mössbauer standard and found to be approximately 260 μM, which was comparable to the BioB protein concentration estimated for the cell paste. The results demonstrate that the $[2Fe–2S]^{2+}$ cluster found in purified samples of recombinant BioB is not an artifact of the protein purification procedure, and indicate that recombinant BioB is over-expressed in an inactive form during aerobic growth.**

Key words: Biotin synthase; BioB; Iron–sulfur cluster; Mössbauer whole cell; EPR whole cell

1. Introduction

Biotin is an essential vitamin that is synthesized by microorganisms [1] and plants [2]. In all organisms, biotin is a necessary cofactor in enzymes which are involved in the transfer of CO_2 during carboxylation, decarboxylation, and transcarboxylation reactions [3]. The final step of the biotin biosynthetic pathway is catalyzed by the *bioB* gene product, termed BioB or biotin synthase, and involves the insertion of sulfur into dethiobiotin at the C6 and C9 positions in a *S*-adenosyl-L-methionine (SAM)-dependent reaction:



Biotin synthases from *Escherichia coli* [4], *Bacillus sphaericus* [5] and *Arabidopsis thaliana* [6] have thus far been purified to homogeneity from recombinant strains of *E. coli* or *B. sphaericus*. Biotin synthase from *E. coli* is the best characterized, and aerobically and anaerobically purified samples have been found to be 78-kDa homodimers containing one $[2Fe–2S]^{2+}$ cluster per monomer [4,7–10]. The presence of $[2Fe–2S]^{2+}$ clusters was also reported in as-purified samples of *B. sphaericus* [5] and *A. thaliana* [6] biotin synthases.

Although there is general consensus concerning the presence of $[2Fe–2S]^{2+}$ clusters in as-purified samples of recombinant biotin synthases, the number and type of the Fe–S clusters present in the functional wild-type enzyme in vivo still remains to be established. The discovery that the $[2Fe–2S]^{2+}$ clusters in as-prepared BioB can be converted into $[4Fe–4S]^{2+,+}$ clusters under reducing conditions [7], coupled with the requirement of SAM for catalytic 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 [11] that utilize a $[4Fe–4S]^{2+,+}$ cluster to initiate a radical reaction by mediating reductive cleavage of SAM to give a 5′-deoxyadenosyl radical [12–14]. Spectroscopic and mutagenesis studies have subsequently demonstrated the presence of one $[4Fe–4S]^{2+,+}$ cluster per monomer in samples of BioB anaerobically reconstituted from apo-protein with iron and sulfide [15]. In addition, the studies of Fontecave and coworkers [8] have provided direct evidence that the reduced $[4Fe–4S]^{+}$ cluster is responsible for reductive cleavage of SAM and is ligated by the cysteines in the $C–X_3–C–X_2–C$ motif. Jarrett and coworkers [16] have recently proposed that the functional form of BioB contains both one $[2Fe–2S]^{2+}$ and one $[4Fe–4S]^{2+,+}$ cluster per monomer. In this model, the $[2Fe–2S]^{2+}$ cluster functions as the immediate S donor for

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Abbreviations: BioB, gene product of *bioB* commonly referred to a biotin synthase; SAM, *S*-adenosyl-L-methionine; Fd, ferredoxin

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biotin biosynthesis and hence is degraded during each turnover, while the $[4Fe–4S]^{2+,+}$ is responsible for initiating the radical reaction by the reductive cleavage of SAM [17].

In this work we report on EPR and Mössbauer studies of over-expressed recombinant *E. coli* BioB in whole cells. The objectives were to define the cluster composition of BioB in the cellular environment prior to purification and thereby address the possibility that the $[2Fe–2S]^{2+}$ cluster which is present in all purified samples of BioB is an artifact of O_2 degradation during purification. The results indicate that recombinant BioB in whole cells contains only $[2Fe–2S]^{2+}$ clusters and hence must correspond to an inactive form of BioB.

2. Materials and methods

2.1. Materials

The experiments were performed using *E. coli* C41[DE3] pT7-7ecbioB-1, which over-expresses *E. coli* biotin synthase [10], and *E. coli* C41[DE3] pT7-7, which contains the same plasmid without the *bioB* insert and hence does not over-express *E. coli* biotin synthase. Estimates of the BioB concentration in whole cells of C41[DE3] pT7-7ecbioB-1 were based on total protein determinations of cell-free extracts multiplied by the fraction of BioB as assessed by gel densitometer measurements. For cells grown for the Mössbauer measurements, 9 mg/l of ^{57}Fe, as ferric ammonium citrate, was added to the growth media at induction. Purification of $[2Fe–2S]^{2+}$ BioB from *Ec* C41[DE3] pT7-7ecbioB-1 grown on ^{57}Fe-enriched media and preparation of $[4Fe–4S]^{2+}$ BioB was performed as described elsewhere [10]. $[4Fe–4S]^{+}$ BioB was prepared by anaerobic reduction of $[4Fe–4S]^{2+}$ BioB with a 10-fold excess of $Na_2S_2O_4$. Purified samples of *Thermococcus litoralis* 4Fe ferredoxin (Fd) were supplied by Dr. Michael Adams (University of Georgia). Recombinant samples of the iron–sulfur cluster assembly 2Fe Fd from *E. coli* were purified from an over-expressing strain of *E. coli* as previously described [18].

2.2. Preparation of ^{57}Fe-enriched ferric ammonium citrate

^{57}Fe (10.2 mg; 0.179 mmol; >95% isotopic enrichment) was converted to $^{57}Fe^{II}SO_4$ by dissolving it in H_2SO_4 (0.3 M, 10 ml) at 25°C. After the ^{57}Fe had completely dissolved, sodium citrate (0.052 mg; 0.177 mmol) was added, and the pH of the solution was adjusted to 6.8 with NH_4OH (10%). The solution was diluted with water to yield a final concentration of 13 mM ^{57}Fe. The solution was either used immediately or stored at −80°C until further use.

2.3. Spectroscopic studies

X-band (~9.5 GHz) EPR spectra were recorded on a Bruker ESP-300E EPR spectrometer equipped with an ER-4116 dual-mode cavity and an Oxford Instrument ESR-9 flow cryostat. Resonances were quantified under non-saturating conditions using a 1-mM CuEDTA standard. The EPR spectrum of *E. coli* 2Fe Fd was simulated using Bruker WinEPR software.

Mössbauer spectra were recorded in a weak-field spectrometer described elsewhere [19] operating in a constant acceleration mode. The zero velocity of the spectra refers to the centroid of the room temperature spectrum of a metallic iron foil. Analysis of the Mössbauer data was performed with the program WMOSS (Web Research). Quantifications of Mössbauer resonances in the BioB whole-cell sample were performed by using a standard containing 3.0 mg of sodium nitroprusside ($Na_2Fe(CN)_5NO{\cdot}2H_2O$; 220 nmol of ^{57}Fe) in mineral oil. The standard was placed together with the whole-cell sample and a spectrum of the standard and the sample was recorded as one. The amount of the Fe species that gives rise to the Mössbauer resonances can then be estimated from the absorption ratio of the component and the standard, and the known quantity of ^{57}Fe in the standard.

3. Results

3.1. Mössbauer spectroscopy

In accord with the previous finding that aerobically and anaerobically purified BioB is a homodimer with one $[2Fe–2S]^{2+}$ cluster per monomer [4,10], the Mössbauer spectrum (Fig. 1a) of the purified samples of BioB comprises a quadrupole doublet with parameters (quadrupole splitting $\Delta E_Q = 0.52$ mm/s, and isomer shift $\delta = 0.27$ mm/s) that are indicative of $[2Fe–2S]^{2+}$ clusters. Similar spectra have been reported for the $[2Fe–2S]^{2+}$ clusters in BioB generated by O_2 exposure of $[4Fe–4S]^{2+}$ BioB [15] or by reconstitution of apo-BioB under semi-anaerobic conditions [20].

The 4.2 K Mössbauer spectrum of *E. coli* C41[DE3] pT7-7 control cells, which have the same plasmid used for expression of BioB but without the *bioB* insert, is shown in Fig. 1b (hatched marks). These control cells were cultivated using the same growth conditions for obtaining whole cells over-expressing BioB. The spectrum consists of two broad quadrupole doublets (shown as a solid and a dotted line above the experimental spectrum in Fig. 1b) arising from Fe either in the

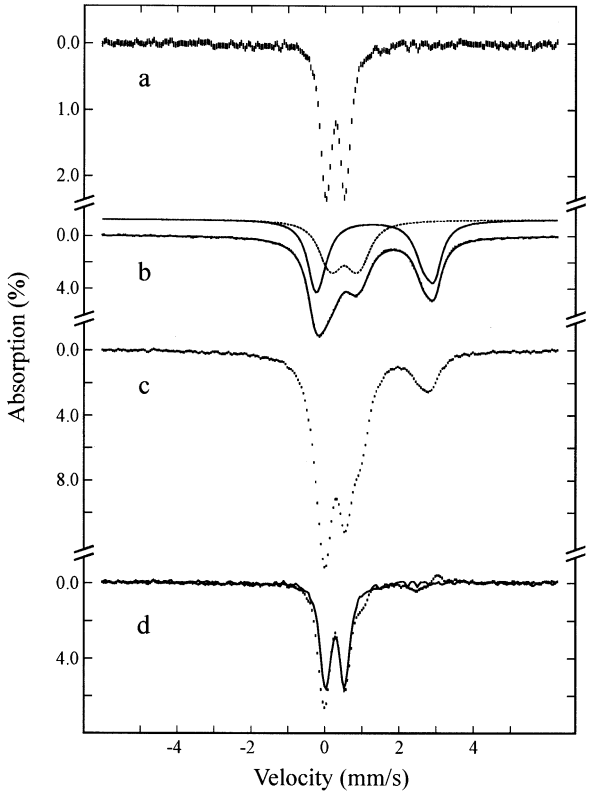

Fig. 1. Mössbauer spectra (hatched marks) of: as-purified $[2Fe–2S]^{2+}$ BioB (a); ^{57}Fe-enriched *E. coli* C41[DE3] pT7-7 control whole cells, which contain the same plasmid used for expression of BioB but without the *bioB* insert (b); and ^{57}Fe-enriched *E. coli* C41[DE3] pT7-7ecbioB-1 whole cells with over-expression of BioB (c). The data were recorded at 4.2 K in a field of 50 mT applied parallel to the γ-beam. The solid and dotted lines plotted above the experimental spectrum in b are theoretical simulations of the Fe^{II} and Fe^{III} components, respectively. Each component is simulated with two equal-intensity quadrupole doublets using the following parameters: (Fe^{II}: $\Delta E_Q = 3.24$ mm/s, $\delta = 1.33$ mm/s, linewidth (Γ) = 0.49 mm/s for doublet 1, and $\Delta E_Q = 2.80$ mm/s, $\delta = 1.26$ mm/s, $\Gamma = 0.61$ mm/s for doublet 2; Fe^{III}: $\Delta E_Q = 0.97$ mm/s, $\delta = 0.52$ mm/s, $\Gamma = 0.62$ mm/s for doublet 1, and $\Delta E_Q = 0.50$ mm/s, $\delta = 0.52$ mm/s, $\Gamma = 0.62$ mm/s for doublet 2). The solid line overlaid with the spectrum in b is an addition of the two theoretical simulations. Removal of the contributions of the Fe^{II} component (20%) and Fe^{III} component (55%) from spectrum c yields the spectrum (hatched marks) shown in d. The solid line in d is the same spectrum of purified BioB shown in a, scaled to 25% of the total absorption of the spectrum shown in c.

growth media or stored in the cells. The major component, which accounts for 57% of the Fe absorption, exhibits parameters ($\Delta E_Q = 3.02$ mm/s and $\delta = 1.30$ mm/s) that are typical for high-spin Fe^{II} species ligated by O, N ligands. The minor component, which accounts for 43% of the Fe absorption, shows parameters ($\Delta E_Q = 0.74$ mm/s and $\delta = 0.52$ mm/s) that are characteristic of high-spin Fe^{III} species. The line shapes of both components are very broad, indicating distributions in the Fe environments. Similar Fe^{II} and Fe^{III} components have been observed previously in the whole cells of *E. coli* [21], as well as in the whole cells of *Neurospora crassa* [22]. In our analysis, to better reproduce the shape of the spectrum, each component (i.e. each broad quadrupole doublet) is simulated with two overlapping quadrupole doublets, the parameters of which are given in the caption of Fig. 1. The solid and dotted lines, shown above the experimental spectrum in Fig. 1b, are simulations of the Fe^{II} and Fe^{III} components, respectively, using these parameters. The solid line overlaid with the experimental spectrum is the superposition of the two simulated spectral components.

Three major spectral components are observed in the 4.2 K Mössbauer spectrum (Fig. 1c) of *E. coli* C41[DE3] pT7-7ecbioB-1 cells (referred to as $BioB^+$ cells), with over-expressed BioB. The well-resolved absorption peak at ~2.8 mm/s and the shoulder seen at ~0.9 mm/s indicate that the Fe^{II} and Fe^{III} components, respectively, observed in the control cells are also present in the $BioB^+$ cells. In addition, a third component with absorption peaks identical to those of the $[2Fe–2S]^{2+}$ BioB spectrum is clearly detected. This is best illustrated by removing the contributions of the Fe^{II} (22%) and Fe^{III} (50%) components from the spectrum of the $BioB^+$ cells. The resulting spectrum (Fig. 1d, hatched marks) is very similar to that of the purified $[2Fe–2S]^{2+}$-containing BioB shown in Fig. 1a. For comparison, the spectrum of the purified BioB is also plotted in Fig. 1d as a solid line, and is scaled to 25% of the total absorption of the raw spectrum of the $BioB^+$ cells. The small shoulder observed at the ~1.0 mm/s region could be an indication of the presence of small amounts of $[4Fe–4S]^{2+}$ clusters (approximately 5% of the total Fe) in the $BioB^+$ cells or the result of imperfect removal of contributions of the Fe^{III} component, because the Fe^{III} components in the control and $BioB^+$ cells may have slightly different spectra. Current evidence does not allow us to distinguish these two situations. In any event, the major additional Fe component detected in the $BioB^+$ cells is consistent with a $[2Fe–2S]^{2+}$ cluster.

To estimate the amount of $[2Fe–2S]^{2+}$ clusters in the $BioB^+$ cells, we recorded a spectrum of the $BioB^+$ cells together with a standard containing 3.0 mg of sodium nitroprusside (see Materials and methods). Analysis of the spectrum shows an absorption ratio of 1.67 for the spectral component arising from the $[2Fe–2S]^{2+}$ cluster and the standard, yielding an estimation of ~260 μM of $[2Fe–2S]^{2+}$ clusters in the $BioB^+$ cells. Gel densitometer traces indicated that approximately 15% of the soluble cellular protein was BioB, which translates into a BioB concentration of ~300 μM in the $BioB^+$ cell paste on the basis of a total protein concentration of ~2.0 mM. If all the over-expressed BioB molecules in the cells contain $[2Fe–2S]^{2+}$ clusters, the data suggest a ^{57}Fe enrichment of 87%, which is a reasonable number considering that the BioB protein estimation contains a large uncertainty and that the ^{57}Fe enrichment should be below 95%. Consequently, taken together, the data indicate that most, if not all, of the over-expressed recombinant BioB contain $[2Fe–2S]^{2+}$ clusters in whole cells prior to purification.

3.2. EPR spectroscopy

The only fully characterized EPR signal that has been reported for BioB is a near-axial resonance, $g_{\parallel} = 2.042$ and $g_{\perp} = 1.937$ (Fig. 2a), which originates from the $S = 1/2$ $[4Fe–4S]^+$ cluster that is formed during anaerobic reduction of as-purified $[2Fe–2S]^{2+}$-containing BioB [7] or by reduction of $[4Fe–4S]^{2+}$-containing BioB that is prepared from apo-BioB by anaerobic cluster reconstitution [10,15]. The spin relaxation of the $S = 1/2$ $[4Fe–4S]^+$ cluster in BioB is slightly slower than that of $S = 1/2$ $[4Fe–4S]^+$ clusters in typical 4Fe Fds, but much faster than that of the $[2Fe–2S]^+$ clusters in simple 2Fe Fds [7]. This is best illustrated by the half-saturation powers, $P_{1/2}$ values, which increase with increasing rates of relaxation. For example, at 12 K the $P_{1/2}$ value for the $S = 1/2$ $[4Fe–4S]^+$ resonance in reconstituted BioB was determined to be 12 mW, compared to values of 15 mW for the $S = 1/2$ $[4Fe–4S]^+$ resonance observed for reduced *T. litoralis* Fd ($g = 2.07$, 1.93, 1.89) and 0.7 mW for the $S = 1/2$ $[2Fe–2S]^+$ resonance observed for the reduced *E. coli* iron–sulfur cluster assembly 2Fe Fd ($g_{\parallel} = 2.021$ and $g_{\perp} = 1.937$), determined under identical conditions.

The EPR spectrum of *E. coli* C41[DE3] pT7-7 cells, which

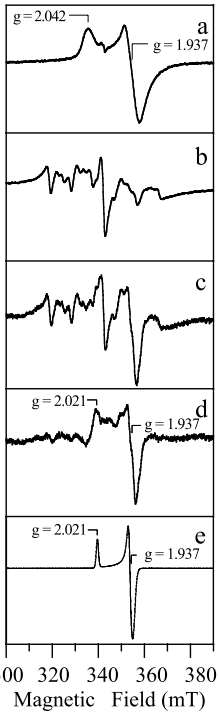

Fig. 2. Comparison of the X-band EPR spectra of reconstituted $[4Fe–4S]^+$ BioB, whole cells of *E. coli* with and without over-expression of BioB, and the $[2Fe–2S]^+$ center in the *E. coli* iron–sulfur cluster assembly 2Fe Fd. a: $[4Fe–4S]^{2+}$ BioB, 320 μM, in 50 mM HEPES buffer, pH 7.5, anaerobically reduced with 3.2 mM sodium dithionite. b: *E. coli* C41[DE3] pT7-7 control whole cells. c: *E. coli* C41[DE3] pT7-7ecbioB-1 whole cells. d: Subtraction of c−b to yield the contribution of the resonance from paramagnetic Fe–S clusters in over-expressing BioB whole cells. e: Dithionite-reduced *E. coli* iron–sulfur cluster assembly 2Fe Fd. The indicated g values were determined by spectral simulation. Conditions of measurement: temperature, 35 K; microwave power, 10 mW; frequency, 9.604 GHz; modulation amplitude, 0.63 mT.

lack over-expressed BioB, is shown in Fig. 2b. The spectrum is dominated by a six-line resonance arising from high-spin Mn^{2+} and an isotropic radical signal close to the free electron g-value. The EPR spectrum of the $BioB^+$ cells has the same features plus an intense derivative-shaped component centered near $g = 1.94$, see Fig. 2c. Upon subtraction of the background signals from the whole cells lacking over-expressed BioB, an axial resonance with $g_{\parallel} = 2.021$ and $g_{\perp} = 1.937$ ($g = 2.021$ resonance) is observed (Fig. 2d). Analogous axial $g = 2.021$ resonances with identical principal g-values were observed for equivalent difference spectra obtained at 12 K, 20 K, and 35 K with microwave powers in the range 1–10 mW. Power saturation studies of the $g = 2.021$ resonance at 12 K indicate a $P_{1/2}$ value of $\sim$1 mW, after correction for the contribution from the underlying resonance seen in the spectrum of the whole cells.

The $g = 2.021$ resonance that is present in the EPR spectrum of the $BioB^+$ cells is distinct from that observed for the $S = 1/2$ $[4Fe–4S]^+$ clusters in reconstituted BioB in having a lower $g_{\parallel}$ value, narrower linewidths and much slower relaxation, as evidenced by the $P_{1/2}$ value. However, it is identical within experimental error to the $S = 1/2$ $[2Fe–2S]^+$ resonances observed for reduced iron–sulfur cluster assembly 2Fe Fds. This is illustrated by Fig. 2e, which shows the EPR spectrum of the reduced iron–sulfur cluster assembly 2Fe Fd from *E. coli*. Hence this resonance is attributed to enhanced expression of the iron–sulfur-cluster (*isc*) assembly genes in *E. coli* in response to the need to bolster Fe–S cluster production for insertion into over-expressed BioB. The $g = 2.021$ resonance in whole cells corresponds to a spin concentration of 60 μM. Since Mössbauer quantification of the total ^{57}Fe concentration in samples of analogous $BioB^+$ cells is $\sim$2 mM, the EPR spin concentration indicates that the $[2Fe–2S]^+$ clusters observed by EPR will not contribute significantly to the whole-cell Mössbauer spectrum.

4. Discussion

BioB has never been purified to homogeneity, anaerobically or aerobically, as a wild-type non-recombinant protein. Thus, the nature of the Fe–S clusters in BioB that function in cellular processes has not been assessed directly. The EPR and Mössbauer studies reported herein constitute the first attempt to assess Fe–S cluster composition of over-expressed, recombinant BioB in whole cells prior to purification. EPR studies of whole cells containing over-expressed BioB reveal enhanced expression of the 2Fe Fd that is encoded by the *fdx* gene in the IscR-regulated *isc* operon [23,24]. This is readily rationalized in terms of the need to bolster Fe–S cluster biosynthesis in response to over-expression of BioB, which was estimated to constitute at least 15% of the total cellular protein. To our knowledge this is the first direct evidence for up regulation of an Isc protein in response to the need to over-express an Fe–S protein. No EPR signals that could be interpreted in terms of a paramagnetic Fe–S cluster in over-expressed BioB were apparent in whole cells. This is in accord with parallel Mössbauer studies which indicate that over-expressed, recombinant BioB contains diamagnetic $[2Fe–2S]^{2+}$ clusters in whole cells. Since recombinant *E. coli* BioB is purified as a homodimer with one $[2Fe–2S]^{2+}$ cluster per monomer [4,7,10], the Mössbauer results indicate that the $[2Fe–2S]^{2+}$ clusters which are invariably and exclusively present in purified samples of recombinant plant and bacterial BioBs [4–6] are not artifacts of oxidative degradation during purification. The implications of this result for understanding the Fe–S cluster composition of catalytically competent BioB are discussed below.

Although the SAM-dependent family of Fe–S enzymes catalyze an extremely diverse range of reactions [11], their unifying attribute is an oxygen-sensitive, redox-active $[4Fe–4S]^{2+,+}$ cluster, ligated by three cysteines in a $C–X_3–C–X_2–C$ arrangement, that initiates radical chemistry by effecting reductive cleavage of SAM to yield methionine and the 5′-deoxyadenosyl radical [12–14]. While the detailed mechanism of the reaction is still to be determined, recent Mössbauer [25] and ENDOR [26] studies of pyruvate formate-lyase activating enzyme have shown that the reductive cleavage involves direct binding of SAM to a unique Fe site of the $[4Fe–4S]^{2+,+}$ cluster. Moreover, the recent work of Fontecave and coworkers [8] has shown that the $[4Fe–4S]^+$ cluster in anaerobically reconstituted BioB is capable of reductive cleavage of SAM to yield methionine and a $[4Fe–4S]^{2+}$ cluster and that each of the three cysteines in the $C–X_3–C–X_2–C$ arrangement is required for this reaction to occur. Consequently, an active form of BioB must contain a $[4Fe–4S]^{2+,+}$ cluster. The absence of significant amounts of $[4Fe–4S]^{2+,+}$ clusters in recombinant BioB prior to purification therefore demonstrates that over-expression of BioB under aerobic growth conditions leads predominantly to inactive recombinant enzyme containing only $[2Fe–2S]^{2+}$ clusters.

We have considered two explanations for the observation that recombinant BioB contains only $[2Fe–2S]^{2+}$ clusters, and both implicate O_2 as the causative factor that prevents assembly of $[4Fe–4S]^{2+,+}$ clusters. The first is based on the model proposed by Jarrett and coworkers [16,17] in which each monomer of catalytically competent BioB contains one $[2Fe–2S]^{2+}$ and one $[4Fe–4S]^{2+,+}$ cluster. The former functions as the sacrificial S donor and is degraded during each turnover and the latter functions to generate a 5′-deoxyadenosyl radical by reductive cleavage of SAM. In addition, the $[4Fe–4S]^{2+,+}$ clusters that can be assembled in BioB under rigorously anaerobic conditions are extremely O_2 sensitive, being rapidly degraded via a transient $[2Fe–2S]^{2+}$ cluster intermediate that is spectroscopically distinct from the $[2Fe–2S]^{2+}$ cluster in as-isolated BioB [10,27]. Hence $[4Fe–4S]^{2+,+}$ clusters would not be expected to be assembled in BioB under aerobic growth conditions unless BioB is rigorously protected from O_2 in the cell.

The attraction of the Jarrett proposal is that it provides a rationalization of the observation that S from the $[2Fe–2S]^{2+}$ cluster in BioB has been shown to be incorporated in biotin in single turnover experiments [28,29]. However, the interpretation of this observation in terms of a sacrificial $[2Fe–2S]^{2+}$ cluster acting as the S donor during catalytic turnover is open to question, since the $[2Fe–2S]^{2+}$ clusters in isolated BioB are known to undergo degradation followed by reassembly as $[4Fe–4S]^{2+,+}$ clusters under reducing conditions [7,30]. Consequently, S from the breakdown of [2Fe–2S] clusters may be utilized for biotin production in a single turnover experiment in the absence of the true S donor.

The second explanation for the whole-cell results presented herein is that each BioB monomer contains only one Fe–S cluster and that the type of cluster assembled depends on the extent of O_2 exposure during the in vivo cluster assembly

process. Support for this proposal comes from the observation that the type of cluster assembled by in vitro reconstitution of apo-BioB is critically dependent on the presence of O_2. Reconstitutions with ferric chloride and sulfide under semi-anaerobic conditions result in BioB containing one $[2Fe–2S]^{2+}$ cluster per monomer that is indistinguishable from as-isolated BioB [10,20]. In contrast, reconstitutions with ferrous ammonium sulfate and sulfide under rigorously anaerobic conditions result in BioB containing one $[4Fe–4S]^{2+,+}$ per monomer [10,15]. Consequently, this rationalization of the whole-cell spectroscopy leads to the conclusion that the $[2Fe–2S]^{2+}$ cluster in recombinant BioB is an artifact of over-expression of BioB under aerobic growth conditions.

Both rationalizations of the whole-cell spectroscopic results discussed above lead to the conclusion that wild-type, non-recombinant BioB is protected from O_2 exposure during aerobic growth and that the O_2-protected form of BioB is responsible for activity in cell-free extracts. In accord with this hypothesis, Flint and coworkers [4] found that addition of purified $[2Fe–2S]^{2+}$ BioB to cell-free extracts from BioB over-expressing cells does not increase biotin production. This conclusion has important implications for ongoing attempts to achieve large-scale microbiological conversion of dethiobiotin to biotin. Since the radical chemistry catalyzing all SAM-dependent Fe–S enzymes is likely to be exquisitely O_2 sensitive, mechanisms must exist in vivo to scavenge O_2 or protect BioB from O_2 exposure during aerobic growth of wild-type *E. coli*. In order to generate functional over-expressed BioB in vivo, it will clearly be necessary to understand and over-express the proteins associated with the O_2 protection mechanism or develop an over-expressing strain of *E. coli* for anaerobic growth conditions.

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