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Biotin Synthase Is Catalytic In Vivo, but Catalysis Engenders Destruction of the Protein

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Summary

Biotin synthase is responsible for the synthesis of biotin from dethiobiotin and sulfur. Although the name of the protein implies that it functions as an enzyme, it has been consistently reported that biotin synthase produces <1 molecule of biotin per molecule of protein in vitro. Moreover, the source of the biotin sulfur atom has been reported to be the [2Fe-2S] center of the protein. Biotin synthase has therefore been designated as a substrate or reactant rather than an enzyme. We report in vivo experiments demonstrating that biotin synthase is catalytic but that catalysis puts the protein at risk of proteolytic destruction.

Introduction

Escherichia coli biotin synthase (BioB), an S-adenosylmethionine-dependent radical enzyme, is required for the last step of biotin synthesis, sulfur insertion into dethiobiotin (DTB) (Figure 1). The composition of BioB and the mechanism of its reaction are the subjects of a large and often conflicting literature. A point of agreement in the literature is that the BioB reaction is not catalytic in vitro [1]. Despite extensive efforts over the 10 years since the in vitro activity was first demonstrated [2], synthesis of <1 molecule of biotin per BioB monomer has been consistently reported by many investigators [3–8]. Bio2, the BioB homolog of *Arabidopsis thaliana*, was reported to be very weakly catalytic in a crude in vitro system [9], but a later report from the same group using a purified system reported turnovers of <1 [10] consistent with the BioB values. In the case of BioB numerous and diverse justifications have been put forth for the observed lack of catalysis, including absence of an essential cofactor [11], intrinsically inactive enzyme preparations [5], and inhibition by reaction products [12], but no general agreement has emerged. The currently favored and most provocative explanation for the lack of catalysis is that the [2Fe-2S] cluster of the protein provides the biotin sulfur atom [6, 7, 13–16]. In this view BioB would be a reactant or substrate rather than an enzyme and in the absence of repair of the [2Fe-2S] center the protein would be sacrificed. A serious limitation in evaluating this and other proposals is that it has not been proven that BioB is (or is not) catalytic in vivo; i.e., is BioB catalysis in vitro an obtainable goal? It is not a given that BioB is an enzyme because most organisms require only miniscule amounts of biotin (perhaps as little as a few hundred molecules per cell). Hence, sacrifice of a BioB molecule for each biotin molecule synthesized is not implausible. We report that *E. coli* BioB is intrinsically catalytic in vivo. Twenty to sixty biotin molecules were synthesized per molecule of BioB protein. However, the view that BioB is sacrificed in its reaction remains germane since a significant fraction of the protein was degraded upon addition of DTB to the bacterial cultures. We discuss our results in light of the recently reported crystal structure of BioB [16].

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Results and Discussion

Experimental Approach

Our experimental approach to measuring the catalytic power of BioB in vivo is illustrated in Figure 2. A key feature was that we measured biotin synthesis by a new method, the covalent attachment of biotin to a specific protein domain catalyzed by the *E. coli* BirA biotin protein ligase (Figure 1B). This method was developed because the same detection method (antibody and/or radioactive labeling) could be used to detect and quantify both BioB and the modified (biotinylated) biotin-accepting domain. This enabled us to directly measure the ratio of biotin molecules formed per molecule of BioB and thereby test catalysis in a straightforward manner. Moreover, in *E. coli* the synthesis and covalent attachment of biotin are very tightly coupled [17–19] and thus our assay was physiologically more appropriate than the usual determination of free biotin. Our biotin assay depended on the fact that DTB is a much poorer (ca. 50,000-fold) substrate for the BirA than biotin [20]. At the concentrations used BirA was unable to attach DTB to acceptor proteins either in vivo (Figure 3) or in vitro [20]. The acceptor protein was a hexahistidine-tagged version of the 87 residue biotinoyl domain of *E. coli* AccB [21], the biotinylated subunit of acetyl-CoA carboxylase. This biotin acceptor domain and BirA ligase (which biotinylates a specific lysine residue of the domain [22–24]) were expressed from a T7 promoter plasmid (the powerful T7 promoter was used to ensure that the biotin acceptor and ligase were in excess over BioB). The host strain carried a deletion of the entire *bio* operon including *bioB* such that the sole source of BioB activity was a hexahistidine-tagged protein. The tagged BioB proteins were expressed from genes that encoded fully active variants of BioB having a hexahistidine tag on either the N terminus or the C terminus. In these constructs BioB was expressed from either an arabinose-inducible *araBAD* promoter on a compatible multicopy plasmid or from the native *bioB* promoter in its normal chromosomal location. It should be noted that both termini of BioB protrude from the structure and are highly mobile in the crystal structure [16]. Similarly, the NMR [25] and crystal structures of `AccB [26–28] show the N terminus of the protein to be highly mobile. Therefore, the hexahistidine-tagged versions of these proteins are expected to readily bind

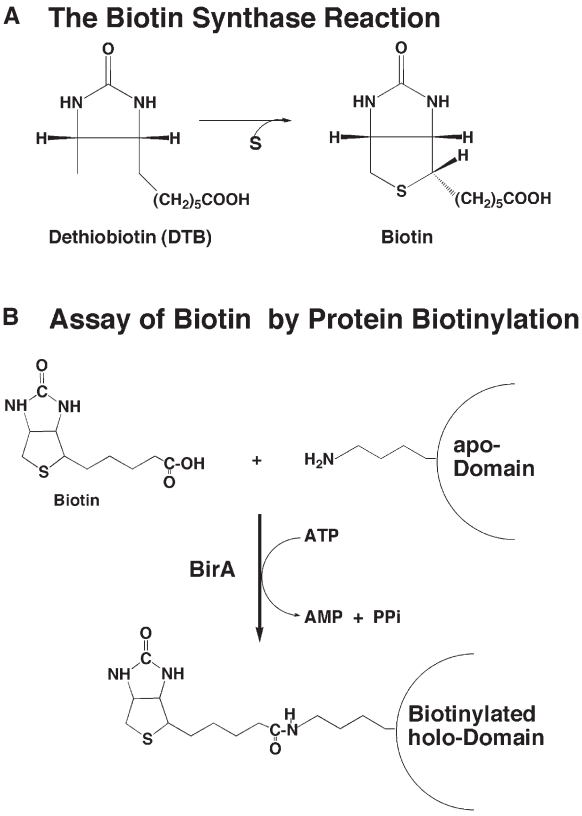

Figure 1. The Biotin Synthase and Biotin Protein Ligase Reactions

The serrated line extending from the biotin acceptor domain represents the side chain of the lysine that is modified. The biotin synthase (A) and biotin protein ligase (B) of E. coli are called BioB and BirA, respectively, based on the names of the encoding genes.

tag-specific affinity columns and antibodies (as we have observed).

Addition of DTB Results in Degradation of BioB

E. coli strains carrying the appropriate plasmid(s) were grown in the presence of near growth-limiting concentrations of biotin and then washed free of biotin (Figure 2A). The cells were then returned to growth medium lacking biotin. The synthesis of BioB, the acceptor protein and BirA were induced (despite the lack of biotin growth proceeds for at least four generations due the preexisting supplies of biotinylated AccB and membrane lipids). After induction had progressed, tetracycline was added to block further protein synthesis. The cultures were then split into three flasks. One of these cultures was left without supplementation whereas the other two received either dethiobiotin or biotin. Following incubation for various periods of time, the cells were collected, lysed, and the hexahistidine-tagged proteins were quantitatively adsorbed to a column of immobilized nickel. Following elution of unbound proteins, the tagged proteins were then eluted with imidazole and analyzed using two different gel electrophoresis systems (Figure 2B). In the first system, standard SDS-PAGE resolved BioB from the biotin acceptor domain.

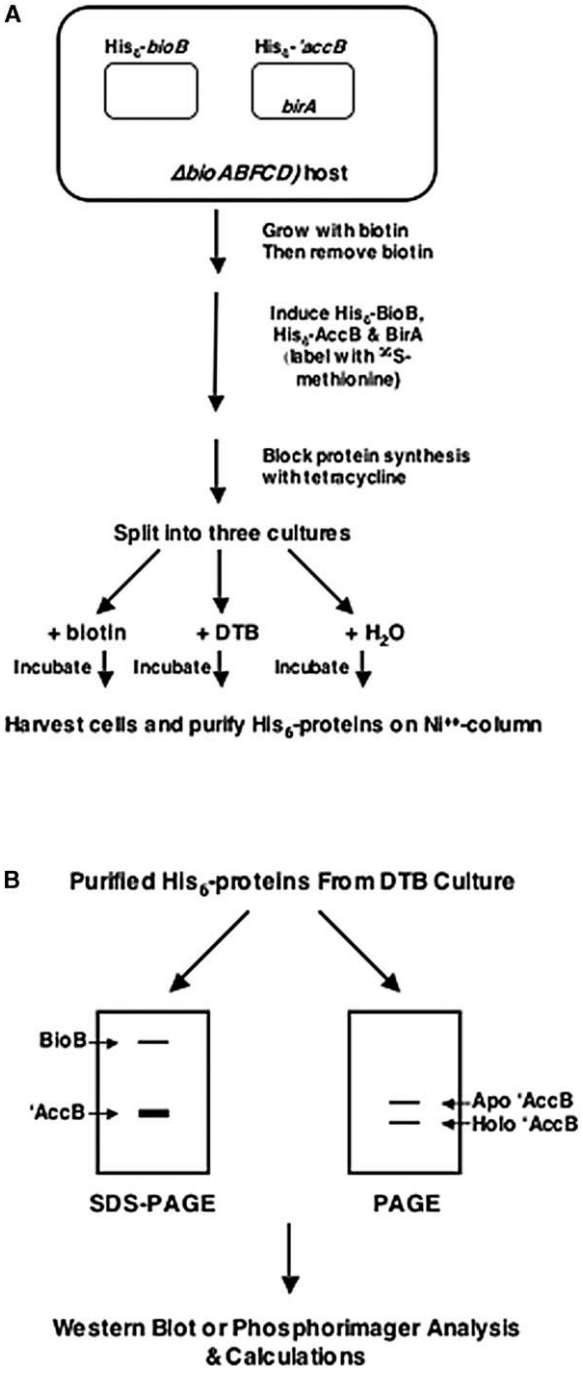

Figure 2. Experimental Design

(A) The large and small ovals represent an *E. coli* cell and its plasmids, respectively. In some experiments ^{35}S-methionine labeling was omitted and the assays were performed by Western blotting (see text).

(B) A cartoon showing the expected PAGE separations is shown.

In the second PAGE system, a nondenaturing gel resolved the modified (biotinylated) form of the biotin acceptor domain from the unmodified (apo) form. The biotinylated protein migrates more rapidly than the apo form due to loss of the charge of the modified lysine

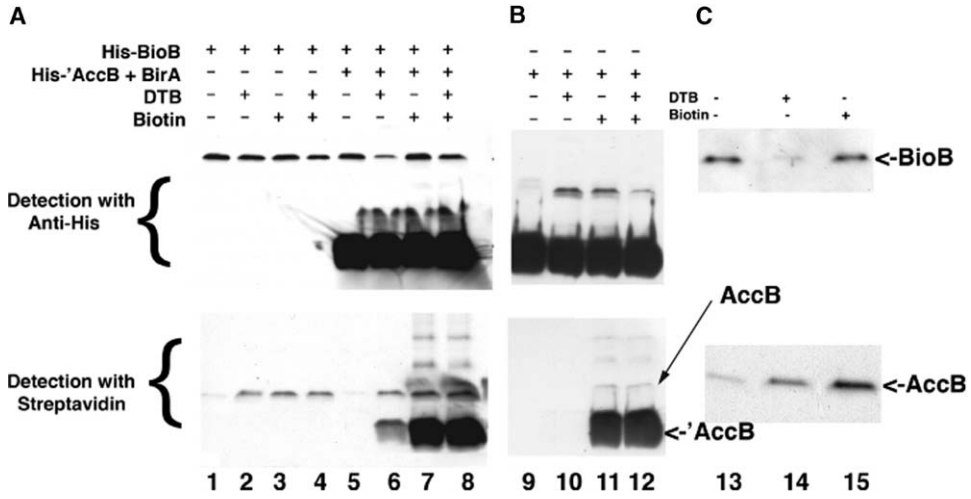

Figure 3. Instability of BioB upon Addition of DTB

(A), (B), and (C) are two different gels. In (A) and (B) two identical gels were run in parallel and the proteins were transferred to Immobilon P membranes. The proteins of one membrane (upper gel of each panel) were visualized by Western blotting with an anti-pentahistidine antibody whereas the proteins of the other membrane (lower gel of each panel) were visualized by Western blotting with a streptavidin conjugate. In (C), the proteins of the gel were transferred to an Immobilon P membrane that was cut into halves that contained the proteins from the top and bottom halves of the gel. The proteins of the top halves were visualized by Western blotting with an anti-pentahistidine antibody whereas the bottom half was exposed to the streptavidin conjugate. Note that the strain of lanes 9–12 lacked BioB and that BioB expression in lanes 13–15 was from a hexahistidine-tagged chromosomal allele of *bioB* rather than from an expression plasmid. Biotin or DTB was added as shown. The streptavidin-reactive band above 'AccB (which is the 87 residue C-terminal fragment of AccB) is the full-length AccB. Western blots of nondenaturing gels with the anti-pentahistidine antibody showed that addition of biotin resulted in quantitative conversion of 'AccB to its biotinylated form (data not shown). Thus, both BirA and 'AccB are present in large excess in the DTB-supplemented cultures.

residue upon modification [21, 24] (the modified and unmodified species comigrate on SDS-PAGE gels). The BioB protein plus the biotinylated and unbiotinylated forms of the AccB biotin acceptor protein were detected by Western blotting with an anti-pentahistidine antibody. In addition, the modified form of 'AccB could also be readily detected by Western blotting with streptavidin, a protein that binds biotin and DTB [29]. Note that streptavidin blotting also detected the full-length version of AccB, an essential endogeneous *E. coli* protein.

The striking result from the SDS-PAGE gels was that the band corresponding to BioB was markedly decreased in cultures that contained both DTB and an excess of biotin acceptor protein (Figure 3, upper panels). The decreased intensity of the BioB band in the DTB supplemented cultures correlated with modification of the biotin acceptor domain (Figure 3, lower panels). Modification was due to BioB-dependent conversion of DTB to biotin because cells that lacked BioB synthesized no modified acceptor domain when supplemented with DTB (Figure 3, lower panels). Therefore, appearance of the modified form of the biotin acceptor domain in DTB-supplemented cultures denoted BioB activity. In five such experiments the intensity of the BioB band decreased by 50%–90% upon addition of DTB. The loss of the BioB band upon DTB addition required high levels of the acceptor protein. When only the endogenous biotin acceptor protein (the full-length AccB) was present, the BioB band retained much of its intensity (Figure 3). These results suggested that accumulation of free biotin due to limiting acceptor protein might inhibit the action of BioB on DTB. Consistent with this premise, addition of biotin together with DTB generally resulted in higher levels of the BioB band than those seen with addition of only DTB (Figure 3). Note that upon addition of biotin in place of DTB a large increase in modified biotin acceptor domain was seen indicating that the ligase and acceptor domain were in great excess over that needed to utilize the small amounts of biotin made by BioB (Figure 3).

A potential difficulty in interpretation of these results was the report that overproduction of BioB in aerobically grown cells results in protein preparations that lack the [4Fe-4S] center required for biotin synthesis [5]. This raised the possibility that the BioB protein remaining in the DTB-supplemented cultures might have survived because it was inactive. To test this possibility we examined the fate of BioB synthesized at a physiological level (about 20-fold lower than the levels made by the plasmid construct) by using homologous recombination to replace the chromosomal *bioB* gene with a modified version encoding a C-terminally hexahistidine-tagged BioB [30]. Thus, expression of this hexahistidine-tagged BioB protein was under control of the native *bioB* promoter and was induced by biotin limitation (part of *bioF* was removed in inserting the tag resulting in biotin auxotrophy). The *E. coli* strain was then grown as in Figure 2 except that expression of the BioB-hexahistidine protein was induced by biotin deprivation and thus addition of DTB or biotin was correspondingly delayed. The tagged BioB was then recovered in concentrated form by affinity chromatography and analyzed by Western blotting with the anti-penta-

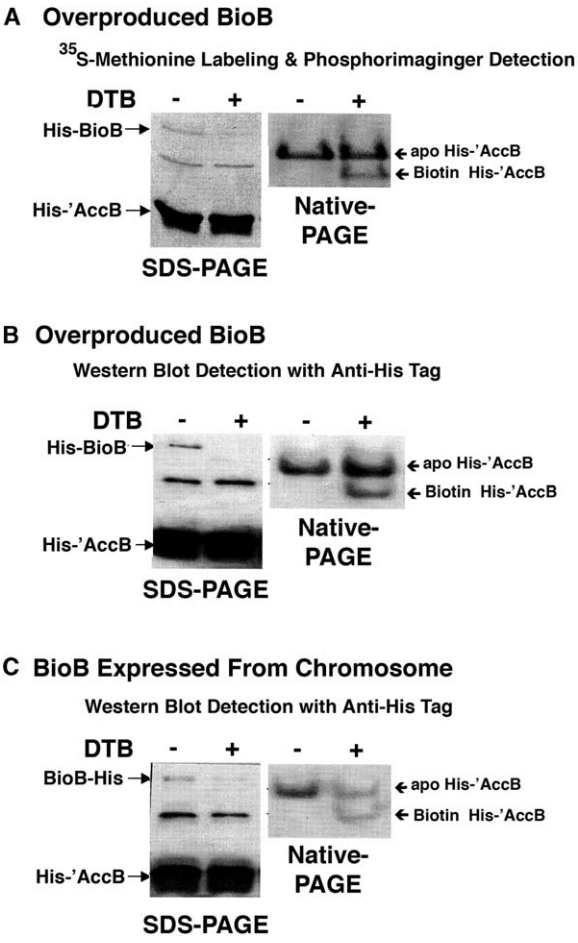

Figure 4. Assay of BioB Catalysis

In each panel, the left-hand gel is an SDS-PAGE separation of hexahistidine-tagged versions of BioB (ca. 41 kDa) and `AccB (ca. 8.2 kDa, although it migrates as a larger protein in SDS-PAGE), whereas the right-hand band is a nondenaturing PAGE separation of the biotinylated (holo) and apo forms of `AccB due to the charge difference imparted by the modification. (A) and (B) are proteins from the same ^{35}S-methionine labeled cultures in which BioB was overproduced with detection by radioactivity or by Western blotting, respectively. (C) Expression of BioB at a physiologically appropriate level with detection by Western blotting. A contaminant band that migrates between the two proteins of interest was sometimes seen. The molecular ratios of biotin synthesized per BioB monomer were 58.4, 22.2, and 39.3 in the experiments of (A), (B), and (C), respectively. The band between the BioB and `AccB that sometimes appears is believed to be a contaminating host protein. It cannot be a BioB degradation product since it was observed in experiments with both N-terminally and C-terminally tagged BioB species.

histidine antibody (Figure 3, upper panels). As previously seen when BioB was overproduced, DTB addition resulted in loss of the BioB band. As expected the decrease in the BioB band was accompanied by increased attachment of biotin to AccB as assayed by Western blotting with streptavidin (Figure 3, lower panels). (Since in wild-type cells the levels of AccB biotin acceptor protein and BirA biotin ligase are in excess over the levels required to attach the synthesized biotin to protein [17–19], the plasmid expressing `AccB and BirA was omitted from this strain.)

We believe that the loss of the BioB band indicates degradation of the protein to small peptides. It was also possible that the His-tag was clipped from the protein. However, hexahistidine tags attached to either end of BioB were examined and thus clipping seems unlikely. Moreover, in some experiments the overproduced BioB bands were serendipitously resolved from the other protein bands on SDS gels and hence visible on Coomassie Blue-stained gels. Although BioB bands were present in the biotin-treated and untreated cultures, no band was visible in the samples from DTB-treated cultures. It is possible that BioB degradation is the result of induction of a stress response by protein overexpression [31]. However, this seems unlikely since degradation is specifically triggered by DTB addition (Figures 3 and 4).

Stoichiometry of the Biotin Synthase Reaction In Vivo

Although these assays demonstrated the dependence of BioB levels upon dethiobiotin addition and quantitative densitometry of the Western blots of several experiments indicated that 20–60 molecules of biotin had been formed per molecule of BioB, we also measured this ratio by a more direct assay. Following induction of BioB, BirA, and `AccB, the cellular proteins were labeled by incorporation of ^{35}S-methionine for 3 hr (Figure 4A). Tetracycline was then added to block further incorporation and the cultures were incubated overnight. The radioactive hexahistidine-tagged proteins were quantitatively recovered from the nickel chelate column and subjected to electrophoresis in the two gel systems. The intensities of the radioactive bands of the resulting gels were then quantitated by phosphorimaging. From the numbers of BioB and `AccB methionine residues/monomeric protein plus the levels of radioactivity of the BioB and modified `AccB bands, it was straightforward to calculate the number of biotinylated acceptor domain molecules formed per molecule of BioB monomer. These experiments gave values of about 50 molecules of biotin formed per molecule of BioB monomer. The values given by the radioactive and Western blot assays were shown to be in reasonable agreement (<3-fold variation) by performing the immunological analyses on ^{35}S-methionine labeled samples (Figure 4B). We believe that the values given by radioactive labeling experiments are more accurate because of the direct nature of the assay and the fact that phosphorimaging analysis is linear over the large dynamic range of the experiment (whereas quantitation of Western blots requires a series of calibrations). The levels of BioB produced by the chromosomal *bioB* gene were too low for effective ^{35}S-methionine labeling so these experiments were assayed by Western blotting (Figure 4C) and gave BioB turnover values similar to those obtained when BioB was overproduced. Thus, it seems that overproduced BioB is fully active, although it should be noted that our levels of overproduction were lower than those used by others [3–5, 7, 8, 32, 33]. The kinetics of BioB action in vivo have not been explored in depth because of the instability of the protein. This

Table 1. Oligonucleotide Primers Used

Primer	Sequence (5′ to 3′)
birA up	GAATTCATGAAGGATAACACCGTGCCACTGAAATTGATTGC
birA down	GCGAAGCTTATTTTTCTGCACTACGCAGGGA
BioKO For	TGTTTGCAGAAAGTGTAGCCAGAAACCCTCACGCGGACTTCTCGTGTGTAGGCTGGAGCTGCTTC
BioKO Rev	CATGCGGGTGAGCGTGGTCATATTCAGCGTGACGTTTCCCGGCATATGAATATCCTCCTCCTTAG
BioB-g-C-HIS-FA	CCGGACACCGACGAATATTACAACGCGGCAGCATTACATCATCATCATCATTGATGTGTAGGCTGGAGCTGCTTCGA
BioB-g-HIS-RE	GCGATCATCCGCCACCAGCCAGCGTCCGGCTCCTTGCGCCACCGGATAACGCATATGAATATCCTCCTTAG

instability results in kinetic uncertainties such as the timing of biotin synthesis relative to that of protein degradation. Indeed, even in the defined in vitro systems there are disagreements concerning whether or not the time of [2Fe-2S] cluster disappearance correlates with that of biotin formation [6, 34]. However, although we were unable to examine early time points when BioB was not overexpressed due to the insensitivity of our assay (an appreciable fraction of the acceptor domain must become biotinylated), we did assay some earlier time points when BioB was overexpressed. For example, following a 3 hr induction of BioB synthesis, tetracycline was then added and samples were taken following 4 hr and 16 hr of further incubation. When expressed per the initial number of monomeric BioB molecules these samples showed formation of 19 and 63 molecules of biotin, respectively.

Conclusions

Our data indicate that BioB is catalytic and is a true enzyme rather than a substrate or reactant. However, BioB is an enzyme of very modest catalytic power. In our work we observed synthesis of only tens of biotin molecules per BioB molecule. On the other hand, there is no need for *E. coli* biotin synthase to be an efficient catalyst since *E. coli* (like most other organisms) requires only minuscule quantities of biotin for growth. *E. coli* can grow with only 100–200 molecules of biotin per cell [35, 36]. We envision the observed degradation of BioB to result from collapse of the enzyme [2Fe-2S] center due to donation of a sulfur atom to DTB [6, 7, 13–16]. The [2Fe-2S] center of BioB is located deep within the barrel of this α/β_8 (TIM) protein [16] and thus it seems probable that a substantial unfolding of the protein would be required to allow rebuilding of the [2Fe-2S] cluster. Such unfolding would allow restoration of the [2Fe-2S] center, but at the cost of exposure of the protein to proteolytic attack while unfolded. Therefore, in this scenario catalysis by a molecule of BioB would require the protein to run a gauntlet of proteolysis until restoration of normal folding (with concomitant resistance to proteolysis) by rebuilding of the [2Fe-2S] center expended in biotin synthesis. Thus, we view the turnover numbers we measured as the products of a stochastic process. If the [2Fe-2S] cluster of a BioB molecule is rebuilt before proteolysis occurs, that protein will perform another turnover. If not, the protein molecule perishes and must be resynthesized de novo. Hence, some BioB molecules would catalyze only one or a few turnovers in their lifetimes whereas others may complete >100 turnovers. The question of how the BioB sulfur centers are inserted and restored remains an open question. Although BioB has recently been reported to accept a [4Fe-4S] center from two *E. coli* Fe-S center scaffold proteins, SufA and IscA, no [2Fe-2S] center was formed [37].

Significance

Although biotin (vitamin H) is required for essential carboxylation and decarboxylation reactions throughout biology, the complete biotin biosynthetic pathway is not understood in any organism. The most chemically difficult reaction in the pathway is the last step, insertion of a sulfur atom into dethiobiotin to form biotin. Although biotin synthase, the protein responsible for this reaction, functions in vitro, <1 molecule of biotin is formed per molecule of protein. Moreover, a protein bound [2Fe-2S] center has been reported to be the sulfur donor. For these reasons biotin synthase has been designated as a reactant or substrate rather than an enzyme. We have asked if biotin synthase is catalytic in vivo and have found that 20–60 molecules of biotin are formed per molecule of protein. However, upon addition of dethiobiotin a significant fraction of the biotin synthase is destroyed by proteolysis. We suggest that this degradation is a consequence of unfolding of the protein to allow replacement of the [2Fe-2S] center consumed in biotin synthesis. Most organisms require only miniscule amounts of biotin (perhaps as little as a few hundred molecules per cell) and thus sacrifice of a biosynthetic protein may not be unreasonable. Since biotin synthase is catalytic in vivo, it should be possible to demonstrate catalysis by the enzyme in vitro. If, however, the [2Fe-2S] center of the enzyme donates the biotin sulfur atom, then a better understanding of the pathway for repair of the [2Fe-2S] center must be obtained before BioB catalysis in vitro will be possible. Similar considerations may apply to lipoic acid synthase, the protein that inserts the sulfur atoms of this essential aerobic metabolism cofactor.

Experimental Procedures

Plasmid Constructions

Plasmid pCY570 was digested with NcoI and treated with T4 DNA polymerase plus the four deoxynucleotide triphosphates to blunt the ends, followed by cutting with SalI to obtain a 2 Kb fragment containing the *bioB* gene plus a fragment of the downstream *bioF* gene. This fragment was ligated to pET28b (Novagen) that had been digested with NheI, treated with DNA polymerase as above, and then digested with SalI, resulting in pER29. Plasmid pER29 was digested with NcoI plus HindIII and ligated to plasmid pBAD24 cut with same enzymes to give pER30. Plasmid pER30 was cut with NsiI plus HindIII and the *bioBF′* fragment ligated to pBAD30

cut with same enzymes to give pER31 that encoded a BioB protein carrying an N-terminal hexahistidine tag under control of the *araBAD* promoter. Plasmid CY142 was cut with NcoI treated with DNA polymerase as above and then digested with SalI to obtain a 280 bp fragment encoding the C-terminal end of AccB (the 87 residue biotin domain). This fragment was ligated to pET28b that had been cut with NheI, treated with DNA polymerase as above, and then digested with SalI affording pER32. The *birA* gene was amplified from plasmid BirA WT HIS6 [38] with Pfu Turbo DNA polymerase with primers birA up and birA down (Table 1) and the product was inserted into pCR2.1-TOPO (Invitrogen) to give pER33. Plasmid pER33 was cut with EcoRI and treated with T4 DNA polymerase as above, followed by cutting with HindIII to obtain a 1 Kb fragment containing a *birA* gene that encoded a protein that lacked the hexahistidine tag. This fragment was ligated to pET28b that had been cut with NheI, treated with DNA polymerase as above, and then digested with HindIII to give pER34. Plasmid pER34 was cut with XbaI and treated with T4 DNA polymerase as above, followed by cutting with XhoI to obtain the *birA* gene fragment that was ligated to pER32 (which had been cut with SalI, treated with DNA polymerase as above, and digested with XhoI) to give pER35. Plasmid pER35 was used to express the N-terminal hexahistidine-tagged biotin acceptor domain and BirA proteins from a T7 promoter. Plasmid pER35 was cut with NcoI plus XhoI and then ligated to pBAD24 cut with the same enzymes to give pER84 that expressed the N-terminal hexahistidine-tagged biotin acceptor domain and BirA proteins from the *araBAD* promoter.

Strain NRD25 was constructed by N. Delay of this laboratory by replacing the entire *bio* operon (*bioABFCD*) with a chloramphenicol resistance cassette in strain MC1061 [39]. Plasmid pKD3 and primers BioKO For and BioKO Rev (Table 1) were used in PCR amplifications. The deletion/insertion was verified by colony PCR analysis and the growth requirement of the strain for biotin. The chloramphenicol resistance cassette was removed from NRD25 by Flp-catalyzed site-specific recombination [39] to give strain ER46. Strain ER47 was constructed by integration of the λDE3 prophage into strain ER46 using the λDE3 lysogenization kit from Novagen. Plasmids pER31 and pER35 were introduced into ER47 strain to allow expression of the N-terminally hexahistidine-tagged BioB, the N-terminally hexahistidine-tagged biotin acceptor domain, and BirA proteins in a *Δbio* strain such that there was no source of DTB or untagged BioB. Strain ER90 contains a chromosomal *bioB* gene that encodes a protein carrying a C-terminal histidine tag inserted in place of the native *bioB*. This strain was constructed using a modification [30] of the Datsenko and Warner method [39]. Plasmid pKD3 was amplified with Taq DNA polymerase using primers BioB-g-C-HIS-FA and BioB-g-HIS-RE (Table 1). BioB-g-C-HIS-FA contained a 5′ segment homologous to the 3′ end of *bioB* (lacking the termination codon) followed by sequences encoding a hexahistidine tag plus the pKD3 priming site, whereas BioB-g-HIS-RE contained sequences homologous to a sequence close to the 5′ end of *bioF* plus the pKD3 priming site. Strain ER90 was verified by colony PCR, followed by sequencing of the PCR product plus the biotin requirement due to inactivation of *bioF* by deletion of the segment encoding the first 22 residues.

Protein Expression and Analyses

Plasmid pER31 carrying a *bioB* gene that encoded a hexahistidine-tagged protein and pER35 that encoded the 87 residue C-terminal region of AccB (`AccB) having an N-terminal hexahistidine tag plus the *birA* gene were transformed into *E. coli* strain ER47. Cultures were grown in medium A (medium M9 plus 1% Vitamin Assay Casamino Acids, 0.2% glucose, 50 μg/ml kanamycin, 30 μg/ml chloramphenicol, and 4 nM biotin) overnight at 37°C. The cells of the overnight cultures were collected by centrifugation and washed with biotin-free medium A. The cells were then suspended in biotin-free medium A containing antibiotics as above to an optical density of 0.6 at 600 nm and grown for 2 hr at 37°C. The three proteins (BioB, BCCP, and BirA) were expressed by resuspending the cells in medium B (M9 plus 1% Vitamin Assay Casamino Acids, 0.2% glycerol, 1 mM IPTG, 0.25% arabinose, and antibiotics as above to an OD at 600 of 1.0). In some cases the cultures also received 3 μCi/ml of L-[^{35}S]methionine (Amersham) (final concentration of ca. 1.7 mCi/mmol calculated from the amino acid composition of Casamino Acids). These cultures were then split into three subcultures. One subculture received water, another received DTB to 5 μM, and a third received biotin to 5 μM. The subcultures were then incubated for 3 hr at 37°C at which time tetracycline HCl was added to 60 μg/ml to the three subcultures followed by incubation overnight at 30°C. The cells were then harvested by centrifugation, resuspended in lysis buffer (50 mM sodium phosphate [pH 8.0], 300 mM NaCl, 0.1 mM DTT, and 2 mM phenylmethylsulfonyl fluoride), and lysed by sonication. The crude extracts were analyzed by gel electrophoresis (see below).

Strain ER90 was grown in medium A containing 30 μg/ml chloramphenicol and 4 nM biotin overnight at 37°C. BioB protein was induced by resuspending cells from the overnight cultures in biotin-free medium A with 30 μg/ml chloramphenicol to an optical density of 0.6 at 600 nm after washing the cells with biotin-free medium A. The culture was grown for 6 hr at 37°C and then tetracycline was added as above and the culture was split into thirds as described above and incubated overnight at 37°C. The cells were harvested and processed as described above.

Plasmid pER84 (which encoded an 87 residue `AccB having an N-terminal hexahistidine tag plus the *birA* gene) was transformed into strain ER90. Cultures were grown in medium A with 30 μg/ml chloramphenicol, 100 μg/ml ampicillin, and 4 nM biotin overnight at 37°C. Expression of BioB was induced by resuspending the cells (after washing the cells with biotin-free medium) of overnight cultures in biotin-free medium A with antibiotics as above to optical density of 0.6 at 600 nm and growth of the culture for 5 hr at 37°C. Expression of the biotin acceptor and BirA proteins was then induced by resuspending the cells in medium B with antibiotics as above and growing for 2 hr at 37°C followed by addition of tetracycline to 60 μg/ml. The culture was then split into thirds as described above and the subcultures were incubated overnight at 37°C. The cells were then harvested, lysed by sonication, and subjected to Ni-chelate agarose chromatography as described previously [40]. The hexahistidine-tagged proteins bound quantitatively to the columns as shown by the lack of tagged proteins in the loading and wash fractions as assayed by Western blotting with the Penta-His antibody (see below) and the failure of the proteins of the flow-though fraction to bind to a second Ni-chelate column. The column elution conditions gave quantitative elution of BioB and `AccB as shown the finding that increased concentrations of imidazole gave no further elution. The fractions containing the hexahistidine-tagged proteins were separated by PAGE (Figure 2).

PAGE gels were 12% polyacylamide run as before [40, 41] except that the SDS was omitted from the buffers used with the nondenaturing gels. The proteins were transferred to an Immobilon-P membrane (Millipore). Western blotting with a streptavidin-enzyme conjugate was done exactly as described previously [41]. Detection of hexahistidine-tagged proteins was done by the ECL system (Amersham) after treating the membranes with the Penta-His monoclonal antibody (Qiagen) as primary antibody followed by a horseradish peroxidase-coupled sheep anti-mouse IgG (Amersham) as secondary antibody. For radioactively labeled proteins the gels were dried and clamped to a Fujifilm imaging plate. Following exposure the plate was read in an FLA-3000 scanner. This imaging system gives a linear response with radioactive dose over five orders of magnitude.

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