Biotin Synthase Is a Pyridoxal Phosphate-Dependent Cysteine Desulfurase

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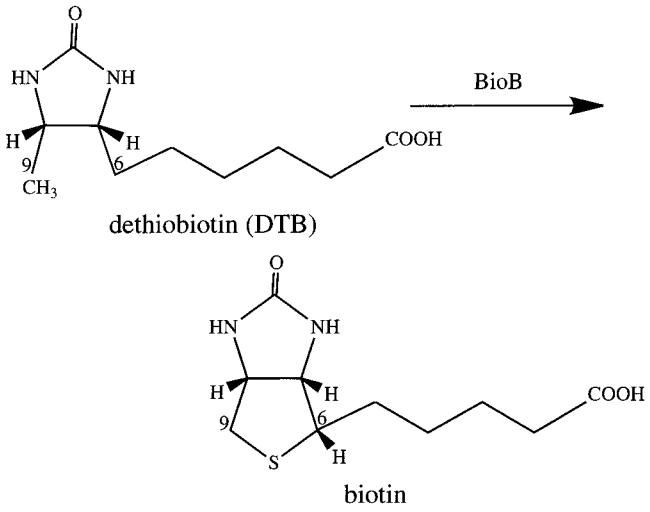
ABSTRACT: Biotin synthase (BioB) is an iron−sulfur dimeric enzyme which catalyzes the last step in biotin synthesis. The reaction consists of the introduction of a sulfur atom into dethiobiotin. It is shown here that BioB displays a significant cysteine desulfurase activity, providing it with the ability to mobilize sulfur from free cysteine. This activity is dependent on pyridoxal 5′-phosphate (PLP) and dithiothreitol and proceeds through a protein-bound persulfide. Like other cysteine desulfurases, BioB binds 1 equiv of PLP. By site-directed mutagenesis, two conserved cysteines, Cys97 and Cys128, are shown to be critical for cysteine desulfuration and are good candidates as the site for a persulfide. Since biotin synthase activity is greatly increased by PLP and cysteine, even though it does not exceed 1 nmol of biotin/nmol of monomer, it is proposed that cysteine desulfuration is intimately linked to biotin synthesis. New scenarios for sulfur insertion into dethiobiotin, in which cysteine persulfides play a key role, are discussed.

Biotin synthase (BioB)[1] catalyzes the last step of the biotin biosynthetic pathway (*1*). The reaction consists of the introduction of a sulfur atom into dethiobiotin, thus requiring activation of C−H bonds (Scheme 1). Biotin synthase from *Escherichia coli* is a homodimer of 76 kDa, with each polypeptide chain carrying an oxygen-sensitive (4Fe-4S) cluster, probably ligated by three cysteines of a CXXXCXXC box conserved among all known BioB sequences and a fourth still not identified ligand (*2*−*9*). Site-directed mutants of BioB in which these cysteines (Cys53, Cys57, and Cys60) have been changed into alanine are inactive (*8*). Recently, Ugulava and co-workers suggested that the active form of BioB contained an additional cluster, a (2Fe-2S) one, per polypeptide chain (*10*, *11*).

In recent years, *S*-adenosylmethionine (AdoMet) and an electron-donating system consisting of NADPH, flavodoxin reductase (Fpr), and flavodoxin (Fldx) were shown to be required for activity (*3*, *12*). This is strong evidence that biotin synthase belongs to a large class of enzymes, the so-called "radical-SAM superfamily", defined on the basis that (i) they use a reduced iron−sulfur center and AdoMet (also abbreviated as SAM) for catalysis and (ii) they all contain a CXXXCXXC sequence providing the cysteine ligands to the cluster (*13*−*16*). The AdoMet/Fe−S combination is supposed, in the case of prototypic enzymes such as the anaerobic ribonucleotide reductase, pyruvate formate lyase, and lysine aminomutase, to generate a 5′-deoxyadenosyl radical which initiates the reaction. In the case of biotin synthase, we have recently shown that, indeed, the reduced $(4Fe\text{-}4S)^{+}$ form of the cluster is competent for a one-electron reduction of AdoMet (*17*). This provides a clue about the mechanism of the reaction since a 5′-deoxyadenosyl radical would be suitable for activation of dethiobiotin, through hydrogen atom abstraction at C9 and C6 (*18*). The resulting carbon radicals are supposed to be reactive for incorporation of a sulfur atom.

Scheme 1: Reaction Catalyzed by Biotin Synthase (BioB)



The following intriguing questions associated with biotin synthase have been addressed over the past 10 years.

First, despite extensive studies, an efficient enzymatic system is not available yet. The reported activities with well-defined assay mixtures rarely exceed 1 nmol of biotin/nmol of monomer. This failure to obtain mutiple turnovers has sometimes been put forward to propose that biotin synthase is not an enzyme but a reactant. Alternatively, this may reflect the fact that extra important cofactors, not identified yet, are

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[1] Abbreviations: BioB, biotin synthase; PLP, pyridoxal 5′-phosphate; recBioB, reconstituted biotin synthase; DTT, dithiothreitol; DTB, dethiobiotin; AdoMet or SAM, *S*-adenosylmethionine; I-AEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; Fldx, flavodoxin; Fpr, flavodoxin reductase.

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missing, in agreement with the observation that moderately increased activities are obtained when assay mixtures are complemented with bacterial soluble extracts. Finally, one cannot exclude the possibility that an irreversible inactivation, by some unknown mechanisms, occurs in vitro.

Second, the question of the source of the sulfur atom that ends up into biotin is still a matter of controversy and investigation. Whereas whole-cell experiments suggested that in vivo cysteine is the source of sulfur for biotin biosynthesis (*3*, *19*), in vitro assays have not allowed an unambiguous identification of the final sulfur donor, even though AdoMet as well as DTT, generally present in reaction mixtures, could be excluded (*12*, *20*). The most recent scenario involves the bridging sulfur atoms of an iron−sulfur center as the species being trapped by the intermediate substrate radicals (*21*). This function has first been suggested to reside in the (4Fe-4S) cluster (*21*, *22*) and, more recently by Ugulava et al., in the (2Fe-2S) cluster present in their preparations (*10*, *11*). In *E. coli*, three additional cysteines (Cys97, Cys128, and Cys188) proved to play a key role in biotin synthesis by site-directed mutagenesis (*8*). Since they are not involved in ligation of the (4Fe-4S) cluster and not required for AdoMet reduction (*8*, *17*), they might function as a site for the (2Fe-2S) center proposed by Ugulava et al. and thus be essential during sulfur incorporation into the substrate.

In this paper, we report the discovery that BioB displays a pyridoxal phosphate-dependent cysteine desulfurase activity, which allows mobilization of the sulfur atom from free cysteine. Site-directed mutagenesis demonstrates the involvement of Cys97 and Cys128 in this activity. The implications for the mechanism of sulfur insertion into biotin are discussed.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were reagent grade and obtained from Sigma-Aldrich Chemical Co. or Fluka unless otherwise stated. 5-Deaza-7,8-demethyl-10-methylisoalloxazine (5-DAF) was prepared according to the method of Ashton et al. (*23*). AdoMet was from Boehringer Mannheim. Pyridoxal 5′-phophate was from Interchim. Flavodoxin, flavodoxin reductase, and IscS were available in our laboratory.

Preparation of Biotin Synthase. BioB was overexpressed in the BL21(DE3) *E. coli* strain carrying the pKH200 plasmid as previously described (*5*, *7*, *8*). BioB was also expressed in the iscS$^-$ *E. coli* CL100 strain lacking an active cysteine desulfurase *iscS* gene, provided by D. Dean (Blacksburg, VA) (*24*). For that purpose, the pKH200 plasmid was introduced into competent CL100 lyzogenized using a DE3 lysogenization kit (Novagen). Overexpression and purification procedures were standard (*5*, *7*, *8*).

Preparation of Reconstituted Biotin Synthase. Mutant and wild-type apoBioB proteins were prepared and reconstituted with $Fe(NH_4)_2(SO_4)_2$ and Na_2S as previously described (*5*, *7*, *8*). Reconstituted proteins were desalted over Sephadex G-25 to remove adventitiously bound iron and sulfide.

Reconstitution of Biotin Synthase with PLP. Biotin synthase proteins (apoBioB, BioB, and recBioB) were incubated anaerobically with 10 mM DTT at 20 °C in a total volume of 500 μL. After reaction for 30 min, proteins were further incubated for 4 h with 5 equiv of PLP. The excess of PLP was then removed anaerobically by dialysis against 2 × 1 L of 0.1 M Tris-HCl (pH 8) and 30 mM KCl. In some experiments, the resulting protein solution (400 μL) was treated with 1 mM $NaBH_4$ for 5 min at room temperature and analyzed for light absorption and activities, after dialysis.

Biotin Synthase Activity. The activity was assayed from the amount of biotin formed from dethiobiotin (DTB). The standard reaction mixture in a final volume of 50 μL of 0.1 M Tris-HCl (pH 8) and 50 mM KCl contained 1.7 nmol of biotin synthase (apoBioB, BioB, and recBioB), 400 μM DTB, 5 mM DTT, 150 μM AdoMet, 20 μM flavodoxin, 4 μM flavodoxin reductase, 1 mM NADPH, 2 mM cysteine, and 1 equiv of PLP. The reaction was monitored at 37 °C for 3−4 h and stopped by the addition of 10% (v/v) 1 M TCA. After centrifugation to remove precipitated proteins, the supernatant was assayed for biotin formation by a microbiological method using *Lactobacillus plantarum* (*25*). To accurately measure the amount of biotin that was synthesized, we put standards on each plate, and all the data presented in this paper represent the average of at least duplicate experiments.

Cysteine Desulfurase Activity. Biotin synthase proteins (4 nmol) (apoBioB, BioB, recBioB, or mutants) were incubated at 20 or 37 °C with 20 mM DTT in a total volume of 100 μL. After incubation for 30 min, PLP (4 nmol) and 2 mM L-cysteine were added to the reaction mixture. At time intervals, the reaction was stopped by addition of 10% (v/v) 1 M TCA and the mix left on ice for 15 min. Precipitated protein was removed by centrifugation for 15 min at 15 000 rpm and the solution dried on a speed-vac. The residue was dissolved in 150 μL of a citrate buffer (pH 2.2). A ninhydrin derivative was generated and analyzed at 570 nm by HPLC on a system 7300 Beckman apparatus working with an ion exchange column (S101036) calibrated with pure amino acid standards and developed by stepwise pH changes according to a published procedure (*26*).

Analysis. Protein concentrations (by monomer) were determined by the method of Bradford (*27*) standardized with bovine serum albumin. A correction factor of 1.1 is applied according to dry weight measurements from amino acid analysis. The amount of protein-bound iron was determined under reducing conditions with bathophenanthroline disulfonate after acid denaturation of the protein (*28*) and labile sulfide by the method of Beinert (*29*). The amount of enzyme-bound pyridoxal 5-phosphate was determined spectrophotometrically by the phenylhydrazine method (*30*).

Identification of the BioB-Bound Persulfide. The BioB-bound persulfide was identified according to the procedure of Zheng and colleagues (*31*). Inside a glovebox, BioB in 0.1 M Tris-HCl (pH 8.0) and 50 mM KCl was treated with 25 mM DTT for 1 h, isolated from the latter by NAP-10 chromatography (Pharmacia), and divided into two portions. In the first portion, 265 nmol of the protein was incubated for 1 h at 37 °C with 270 nmol of PLP and 2 mM cysteine. The second portion (500 nmol) was kept under the same conditions without PLP and cysteine. Then a 20-fold excess of the persulfide alkylating reagent *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine (I-AEDANS) (λ = 337 nm, ϵ = 5000 M^{-1} cm^{-1}) was added to both samples, and the mixtures were incubated further for 1 h at 19 °C. After removal of all low-molecular weight components by NAP-10 chromatography, the protein solutions (1 mL) were filtered on NanoSep ultrafiltration devices (2 × 1 mL). The filtrates

were checked by UV−vis spectroscopy inside the glovebox and proved to display only negligible absorption at 337 nm. Both protein solutions (400 μL) were then reacted with DTT (10 mM) for 30 min and filtered again on NanoSep cartridges. The filtrate of the PLP- and Cys-treated samples exhibited a strong absorption at 337 nm corresponding to the thio derivative *N*-(thioacetyl)-*N*′-(5-sulfo-1-naphthyl)-ethylenediamine (0.3−0.5 mol/mol of BioB monomer) derived from reduction of the mixed protein−AEDANS disulfide. In the control experiment, the absorption at 337 nm corresponded to 0.02 mol/mol of BioB monomer. The solutions were then loaded onto a LightC18 Sep-Pak (Waters) column equilibrated in water. After being washed with 4 × 1 mL of water, the 337 nm-absorbing compounds were collected in a 60:40 (v:v) CH_3CN/H_2O mixture (500 μL). After addition of 50 μL of 1 M NH_4OH and 5 μL of iodomethane, the reaction was allowed to proceed at ambient temperature for 1 h. The solutions were then freeze-dried and analyzed by mass spectrometry (ES-MS) working with negative ion detection. Only one compound ($M^- = 353.1$) corresponding to the methylthio derivative of AEDANS was detected. MS/MS of this ion gave rise to daughter peaks at 265.1 and 221.1 corresponding to the two fragments derived from cleavage at the aromatic amino position.

UV−Visible Spectroscopy. UV−visible spectra of the BioB protein, in 0.1 M Tris-HCl (pH 8) and 50 mM KCl, were recorded under anaerobic conditions directly inside the glovebox with a Uvikon XL100 instrument (Biotek).

Mass Spectrometry. The mass spectra were acquired on a LCQ-ion trap (Finnigan-Thermoquest, San Jose, CA), equipped with an electrospray source.

Electrospray full scan spectra, in the range of *m*/*z* 50−600, were obtained by infusion through fused silica tubing at a rate of 2−10 μL/min. The solutions were analyzed in the negative mode. The LCQ calibration (*m*/*z* 50−2000) was achieved according to the standard calibration procedure from the manufacturer (mixture of caffein, MRFA, and Ultramark 1621). The temperature of the heated capillary of the LCQ trap was set to 150 °C, and the ion spray voltage was in the range of 1−6 kV with an injection time of 5−200 ms.

RESULTS

Different Preparations of BioB. Three different forms of biotin synthase were used in this study. BioB designates the dimeric protein purified from an overexpressing strain of *E. coli*, as previously described (*7*). This preparation contains a small amount of iron and sulfide (1.0−1.5 per polypeptide chain) due to loss of the cluster during chromatography and accordingly displays low biotin synthase activity. ApoBioB is obtained by anaerobic reduction of BioB by photoreduced deazaflavin in the presence of 10 mM EDTA followed by chromatography on Sephadex G25 (*7*). As a consequence, it does not contain any measurable iron and sulfide. Finally, recBioB is obtained from apoBioB by treatment with a 5-fold excess of both ferrous iron and sulfide in the presence of DTT followed by desalting on Sephadex-G25, all steps carried out anaerobically within a glovebox. From one preparation to another, recBioB was found to chelate between three and four iron and sulfur atoms per polypeptide chain. Whereas the major part of the iron (60−80%) resides in a $(4Fe\text{-}4S)^{2+}$ cluster, small and variable amounts of iron are found within $(2Fe\text{-}2S)^{2+}$ clusters and mononuclear Fe^{2+} species, as shown by Mössbauer spectroscopy (*7*, *17*). The incomplete (4Fe-4S) assembly or lack of clusters altogether in some monomers probably reflects the lability of the cluster and its sensitivity to oxygen, as previously noted (*7*). However, under the reducing conditions required for activity, mobilization of iron and sulfur occurs to generate more (4Fe-4S) clusters in the +1 ($S = {}^1/_2$) oxidation state (80−90% of total iron), which is the active form of the cluster during AdoMet reductive cleavage (*7*, *17*).

BioB was also overexpressed within the *E. coli* CL100 strain, lacking an active *iscS* cysteine desulfurase gene (*24*), from which the three forms apoBioB, BioB, and recBioB were prepared.

BioB Is a Pyridoxal Phosphate-Dependent Cysteine Desulfurase. In preliminary experiments, we discovered that incubation of BioB (1.2 Fe and 1.5 S atoms per polypeptide chain) with 1 mM L-cysteine and 5 mM DTT, within an anaerobic glovebox, resulted in the slow formation of alanine (0.4−0.6 nmol/nmol of monomer BioB over 4 h at 20 °C). An approximately stoichiometric amount of sulfide was formed under these conditions. No alanine could be detected when BioB or L-cysteine was omitted from the reaction mixture, or when L-cysteine was replaced with D-cysteine. Omission of DTT resulted in greatly decreased rates of alanine formation (0.1 nmol/nmol of monomer over 4 h). Addition of dethiobiotin, AdoMet, and ATP or a reducing agent (photoactivated deazaflavin) had no effect on the reaction. Finally, since pyridoxal 5′-phosphate (PLP) is a common cofactor for cysteine desulfurase (*31*), we next included 1 equiv of PLP, with regard to BioB, in the assay mixture. This led to a large increase in activity (2 nmol/nmol of monomer over 4 h). To exclude the possibility that such a low activity would be due to the presence of IscS, a potent cysteine desulfurase, as a contaminant, recombinant BioB from the *E. coli* CL100 strain lacking an active *iscS* gene (*24*) was assayed and found to display similar properties in terms of PLP-dependent cysteine desulfurase activities.

Characterization of the Cysteine Desulfurase Activity of BioB. On the basis of the experiments described above, the cysteine desulfurase activity of BioB was studied as a function of DTT, cysteine, and PLP concentration during a 4 h incubation at 20 °C. As shown in Figure 1A, DTT (20 mM) provided a 10-fold stimulation of the cysteine desulfurase activity (1 mM cysteine, 1 equiv of PLP with regard to BioB). Figure 1B shows that the highest activity was obtained for 1 mM cysteine (in the presence of 10 mM DTT) and a slight inhibition for higher concentrations. A K_m value of 30 μM for cysteine could be derived from the data depicted in Figure 1B. Finally, Figure 1C shows that the addition of 1 equiv of PLP was sufficient to obtain the highest activity, and no inhibition was observed for higher concentrations. It should be noted that, in the presence of cysteine and DTT, a significant activity remained upon omission of PLP, suggesting that BioB contains a small amount of protein-bound PLP. On this basis, we could establish an assay with 4 nmol of BioB monomer, 20 mM DTT, 2 mM cysteine, and 4 nmol of PLP. Under these optimal conditions, the production of alanine and sulfide was linear for at least 4 h as shown by the time curve of Figure 2. Finally, as shown in Table 1, cysteine desulfurase activity proved to depend greatly on the amount of iron cluster within the protein since

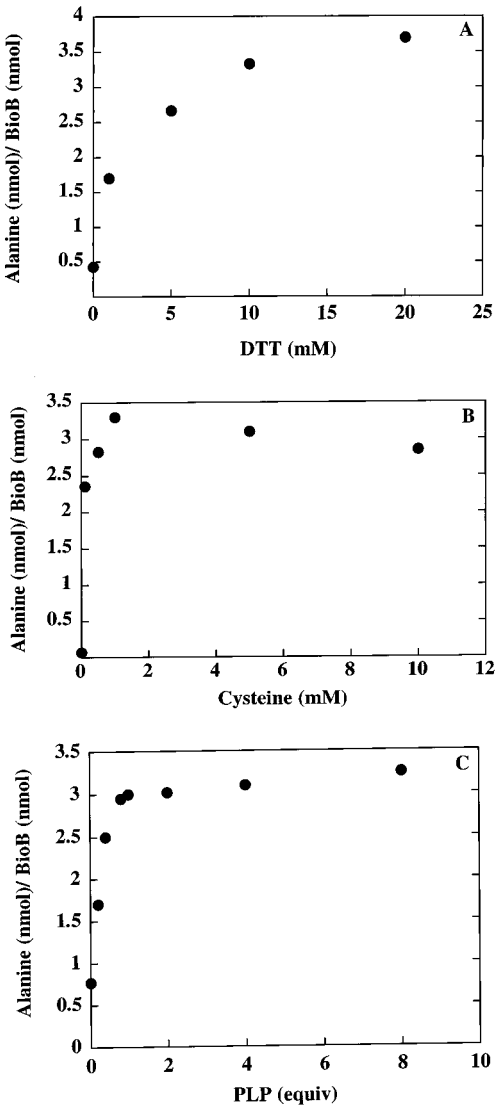

FIGURE 1: Cysteine desulfurase activity of BioB as a function of DTT (A), cysteine (B), and PLP (C). Assays were run anaerobically with 5 nmol of recBioB monomer for 4 h at 20 °C. Activities are defined as nanomoles of alanine per nanomole of monomer.

apoBioB was more than 10 times less active than BioB whereas recBioB displayed the highest activity.

Cysteine desulfurases are thought to generate protein-bound persulfides which can be detected after reaction with an alkylating probe, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)-ethylenediamine (I-AEDANS) (*31*). The product of that reaction is a mixed disulfide which, upon reduction with DTT, releases the thio derivative of the probe to be quantitated. BioB, after incubation at 37 °C for 1 h with 2 mM cysteine, in the presence of 1 equiv of PLP and in the absence of DTT was assayed for persulfide, as described in Experimental Procedures, and found to contain ~0.3−0.5 nmol of persulfide/nmol of BioB monomer. In a control experiment with the same preparation before reaction with cysteine and PLP, no persulfide could be detected.

As Cys97, Cys128, and Cys188 were previously shown to be required for biotin synthesis (*8*, *17*), the corresponding reconstituted Cys-to-Ala site-directed mutants (recBioB forms), which have no detectable biotin synthase activity, were assayed for cysteine desulfurase at 37 °C. As shown in Table 2, mutations of Cys97 and Cys128 resulted in

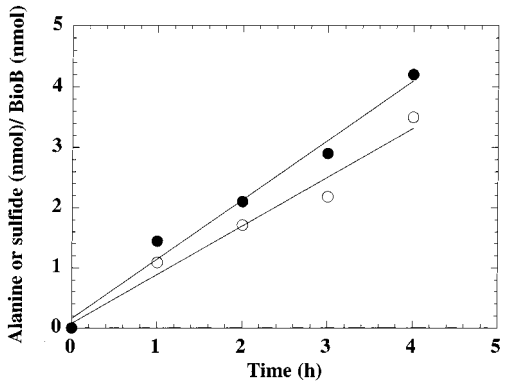

FIGURE 2: Cysteine desulfurase activity of BioB as a function of time. BioB (5 nmol of monomer) was incubated anaerobically at 20 °C with 20 mM DTT, 2 mM cysteine, and 1 equiv of PLP and the reaction stopped at different times. Activities are defined as nanomoles of alanine (●) or sulfide (○) produced per nanomole of monomer.

Table 1: Enzyme Activities[a] and Cofactor Content[b] of the Different Preparations of Biotin Synthase

preparation	Fe[b]	cysteine desulfurase activity[c]	biotin synthase activity[d]	PLP[b]
apoBioB	0	0.1	0.01	0
bioB	1.0	1.7	0.1	0.3−0.4
recBioB	3.5	6.0	1	0.7−1
recBioB with $NaBH_4$	3.5	0.07	0.006	−

[a] Enzyme assays in the presence of PLP and cysteine as described in Experimental Procedures. The amounts of enzyme were 1.7 and 5 nmol for biotin synthase and cysteine desulfurase activities, respectively, and the temperatures were 37 and 20 °C, respectively. [b] Fe in nanomoles per nanomole of monomer; PLP maximal amount in nanomoles per nanomole of monomer that protein can bind after incubation with 5 equiv of PLP and dialysis. [c] Nanomoles of alanine per nanomole of monomer after 4 h. [d] Nanomoles of biotin per nanomole of monomer after 4 h.

Table 2: Cysteine Desulfurase Activities of Site-Directed Mutants of Biotin Synthase

protein	cysteine desulfurase activity[a]
wild type	100
C97A	5
C128A	7
C188A	70

[a] Relative cysteine desulfurase activity (percentage) of wild-type and mutant recBioB. Assays were run anaerobically at 37 °C for 4 h in the presence of 20 mM DTT, 2 mM cysteine, and 1 equiv of PLP. The maximal activity observed with the wild-type protein corresponds to 9.6 nmol of alanine/nmol of recBioB monomer.

enzymes with only ~5% of the activity of the wild-type enzyme. Instead, the activity of the Cys188Ala mutant protein was only slightly affected.

PLP Binding to BioB. The discovery that PLP was a key cofactor for the cysteine desulfurase activity of BioB led us to speculate that biotin synthase is a PLP-dependent enzyme and that most of the protein-bound PLP was lost during purification. For determination of the level of binding PLP, we used a common colorimetric assay, based on titration of PLP released upon protein boiling (*30*). This assay was validated with IscS, a PLP-dependent cysteine desulfurase involved in iron−sulfur cluster assembly (*32*). PLP was found to be present in BioB (0.05 mol of PLP per monomer).

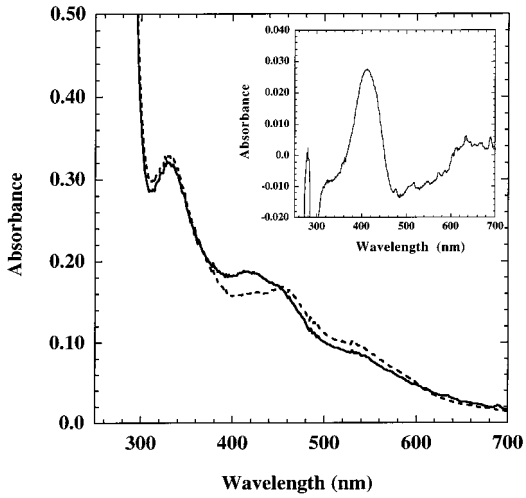

FIGURE 3: UV−visible absorbance spectra of BioB (28 μM) before (- - -) and after (—) reconstitution with PLP and dialysis at 20 °C. The difference spectrum is shown in the inset, which highlights the absorbance increase in the 410 nm region upon reconstitution. The spectral change is consistent with the presence of 0.24 nmol of PLP/monomer (ϵ_{410} = 6000 M^{-1} cm^{-1}).

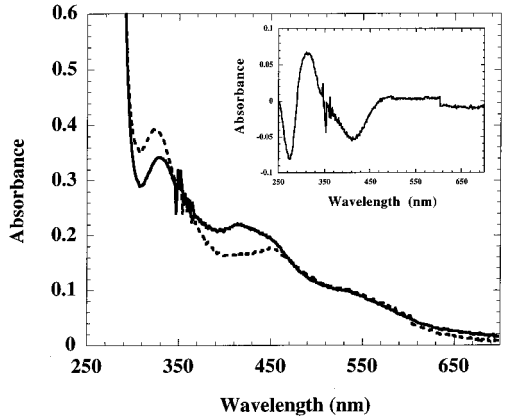

FIGURE 4: Spectral changes upon reduction of PLP-containing BioB by sodium borohydride. The UV−visible spectra of 40 μM purified biotin synthase before (—) and after (- - -) addition of 1 mM $NaBH_4$ at room temperature for 5 min. The difference spectrum (after reduction minus before reduction) is shown in the inset, which highlights the absorption decrease in the 420 nm region and the increase at 325 nm upon reduction.

However, incubation of BioB with a 5-fold excess of PLP for 4 h followed by extensive dialysis resulted in an increase in the amount of protein-bound PLP. From one preparation of BioB to another, between 0.3 and 0.4 mol of PLP was found attached to 1 mol of BioB monomer (Table 1). In Figure 3 are shown the light absorption spectra of BioB before and after treatment with PLP. They now show slight differences in the 400 nm region as a consequence of the increased level of PLP binding. The difference spectrum displayed one major band at 410 nm highly characteristic for PLP (inset of Figure 3). The absorbance at 410 nm was consistent with the amount of PLP determined colorimetrically. PLP could be irreversibly bound to BioB upon reduction with sodium borohydride, suggesting a ketinimine linkage between PLP and BioB as expected for a PLP-dependent enzyme. As shown in Figure 4, the 420 nm absorption band was bleached by addition of $NaBH_4$, concomitant with an absorption increase at 325 nm. These

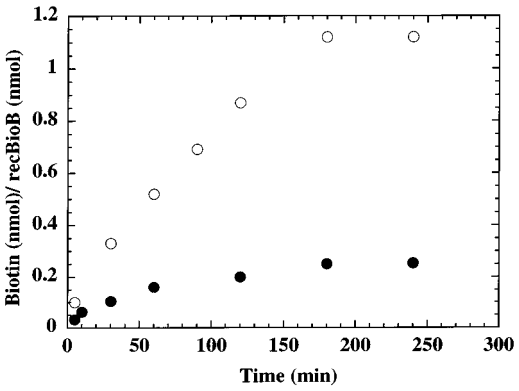

FIGURE 5: Biotin synthase activity. RecBioB (35 μM) was incubated for different periods of time at 37 °C with 400 μM DTB, 5 mM DTT, 20 μM Fldx, 4 μM Fpr, 1 mM NADPH, and 150 μM AdoMet with (○) or without (●) cysteine (1 mM) and PLP (35 μM).

changes are characteristic of reduction of an aldimine linkage between PLP and a lysine residue (*33*).

The capacity of BioB to bind PLP was found to be dependent on the total amount of protein-bound iron cluster: apoBioB was unable to bind PLP, whereas recBioB could bind as much as 0.7−1 mol of PLP per polypeptide chain, the largest amount obtained when the incubation with PLP was carried out in the presence of DTT (Table 1). When recBioB was exposed to air and desalted, a reaction resulting in the loss of ~50% of the iron and sulfide as previously shown (*7*), the maximal amount of PLP that could bind to the protein was ~0.4 mol/mol of monomer.

Stimulation of Biotin Synthase Activity by Cysteine and PLP. To check whether this cysteine desulfurase activity is involved in biotin synthesis, we studied the dependence of biotin synthase activity on cysteine and PLP. The results are shown in Figure 5, using recBioB as the enzyme form. Whereas a small activity was observed in the absence of cysteine and PLP, addition of both compounds resulted in both an important acceleration and an increased yield of biotin production. Maximal activity was obtained with the addition of 1 equiv of PLP (data not shown). However, even under these optimal conditions, the production of biotin leveled off after reaction for 3 h when ~1 nmol of biotin/nmol of BioB monomer was formed (Figure 5). As shown in Table 1, biotin synthase activity in the presence of PLP and cysteine was dependent on the amount of cluster in the protein. The BioB preparation from the $iscS^-$ *E. coli* CL100 strain displays similar PLP-dependent biotin synthase activities. Table 1 also shows that both biotin synthase and cysteine desulfurase activities are greatly decreased upon treatment of PLP-containing recBioB with $NaBH_4$.

DISCUSSION

The species that donates sulfur to dethiobiotin to form biotin has been the subject of a number of contradictory studies. Despite inconsistencies between reports, some generalizations may be drawn. First, when crude cell-free bacterial extracts were included in biotin synthase assays, the sulfur from cysteine was incorporated into biotin, as shown from experiments in which [^{35}S]cysteine was used (*3*, *34*). Second, biotin synthase activity can be assayed in the absence of crude extracts in vitro, using purified

preparations of the enzyme, dethiobiotin, NADPH, flavodoxin reductase, flavodoxin, AdoMet, dithiothreitol (DTT), cysteine, iron, and sulfide (*2*, *3*, *9*, *10*). The stimulating effects of Fe and sulfide were then supposed to reflect the necessity of maintaining an active iron−sulfur cluster during the reaction. Third, when the same assay was carried out with a partially purified biotin synthase, expressed in cells growing in the presence of [^{35}S]cysteine, efficient incorporation of the isotopic label into biotin was observed in the absence of added exogenous sulfur donors (*35*). This, together with the lack of enzyme turnover, led to the suggestion that biotin synthase was a reagent and not a catalyst and that the sulfur atom was coming either from a cysteine residue from the protein or from an iron−sulfur center. At about the same time, Marquet et al. proposed that the cluster was the sulfur donor, since transfer of ^{34}S from a biotin synthase preparation, in which the cluster was labeled with ^{34}S by chemical or enzymatic reconstitution, to biotin was demonstrated (*21*).

In all these reports, BioB was assumed to contain a single cluster. However, the type and number of iron−sulfur clusters in BioB have also been a matter of controversy. A first proposal, based on spectroscopic studies (*2*), envisioned a (2Fe-2S) center which could, under reducing conditions, convert into one (4Fe-4S) center bridging the two subunits of the dimer (*4*). This proposal was then challenged when we (*7*) and others (*9*) demonstrated that anaerobic preparations of BioB could bind as many as four Fe and four S atoms per monomer in the form of $(4Fe\text{-}4S)^{2+/1+}$ clusters which undergo degradation into (2Fe-2S) clusters upon exposure to air (*7*−*9*, *36*). Finally, a recent report from Ugulava et al. suggested the presence of two different clusters in biotin synthase, a (4Fe-4S) cluster involved in the conversion of dethiobiotin into a radical and a (2Fe-2S) one proposed to be the source of sulfur atoms for incorporation into this radical (*10*, *11*). This was supported by spectroscopic analysis of the enzyme during a single turnover, leading to the conclusion that during catalysis the (4Fe-4S) center was preserved while the (2Fe-2S) cluster was destroyed. With this hypothesis that a cluster would be the species donating sulfur to the substrate, a two-cluster model is more attractive than a one-cluster one. Indeed, in the latter, BioB would be fully inactivated after each turnover for both AdoMet reductive cleavage and sulfur transfer. On the other hand, in both scenarios, the enzyme needs to be reconstituted for multiple turnovers. Since, in general, no more than 1 mol of biotin can be formed per mole of BioB monomer in vitro, one still wonders whether BioB is a reactant or requires still unidentified specific reactivating machinery which is still unknown.

On the basis of the results reported here, we propose a novel scenario in which an unanticipated activity of biotin synthase would play an essential role during sulfur transfer. Here we show for the first time that biotin synthase displays a significant cysteine desulfurase activity in the presence of DTT: it has an intrinsic ability to mobilize the sulfur atom from cysteine by catalyzing the conversion of free cysteine into alanine and free sulfur, with L-cysteine and not D-cysteine being a substrate. Contamination by the potent cysteine desulfurase IscS is excluded since BioB preparations from an *E. coli* strain lacking an active IscS displayed similar activities. Like all cysteine desulfurases reported so far, this activity critically depends on the presence of PLP and thus certainly proceeds by similar mechanisms, namely, through the intermediate formation of a substrate cysteine−pyridoxal phosphate ketinimine adduct and subsequent nucleophilic attack by the thiolate anion of an active protein-bound cysteine (*31*). These events result in formation of a protein-bound persulfide and a PLP-bound enamine which is ultimately released as alanine. Here we show that biotin synthase binds 1 equiv of PLP and, in the presence of cysteine, is able to generate a protein-bound persulfide. Although there is in BioB no obvious sequence corresponding to PLP-binding motifs (*33*), a good candidate for a PLP binding site could be lysine 49, just upstream from the CXXXCXXC sequence, a very highly conserved lysine in BioB sequences (only BioB from *Heliobacter pylori* contains an arginine at this position). The changes observed in the visible spectrum during reaction with $NaBH_4$ are indeed characteristic of the reduction of an aldimine linkage between PLP and a lysine residue (*33*). Further experiments, including site-directed mutagenesis studies, are required to identify this site.

It is noteworthy that the presence of an iron−sulfur center is important for PLP binding and thus for cysteine desulfurase activity (Table 1). Lysine 2,3-aminomutase provides a precedent for an enzyme carrying both an iron−sulfur center and PLP (*33*). Also in this case, a correlation between PLP content and Fe and S content has been observed. The reason PLP has not been observed in earlier BioB preparations thus likely resides in the lability of the cluster. During purification, a large amount of iron and sulfide and most of the PLP are lost. Furthermore, the cluster and PLP absorb light in the same energy range, and the small amounts of PLP in the purified enzyme could not be easily detected. Finally, the importance of the cysteine desulfurase activity of BioB during biotin synthesis is suggested from the maximal acceleration of the rates of biotin formation caused by addition of 1 equiv of PLP and cysteine (Figure 5) and the rather good correlation between both activities (Table 1). Unfortunately, one must note that the PLP/cysteine combination does not by itself provide the conditions for multiple turnovers, since, again, the reaction leveled off after formation of ~1 nmol/nmol of monomer. Furthermore, the origin of the residual activity in the absence of PLP is unclear at that stage.

We recently found that in addition to the three cysteines, namely, Cys53, Cys57, and Cys60, of the CXXXCXXC sequence specific for the enzymes of the radical-SAM family (*13*), BioB from *E. coli* requires, to be active, Cys97, Cys128, and Cys188 (*8*). The first three have been shown to provide the ligands to the (4Fe-4S) center (*8*). Since mutations at the three last cysteines resulted in proteins with the ability to assemble a (4Fe-4S) center with both spectroscopic features comparable to those of wild-type BioB and good activity during AdoMet reductolysis, they were excluded as ligands to that cluster, leaving thus open the question of the nature of the fourth ligand (*8*). Now we show that Cys97 and Cys128 are critical to the cysteine desulfurase activity of BioB since replacement with an alanine resulted in a drastic impairment of that activity. It is tempting to suggest that one of these two cysteines is the site of the intermediate persulfide. It is too early to tell what the exact function of the second cysteine is, but one possibility is that it participates

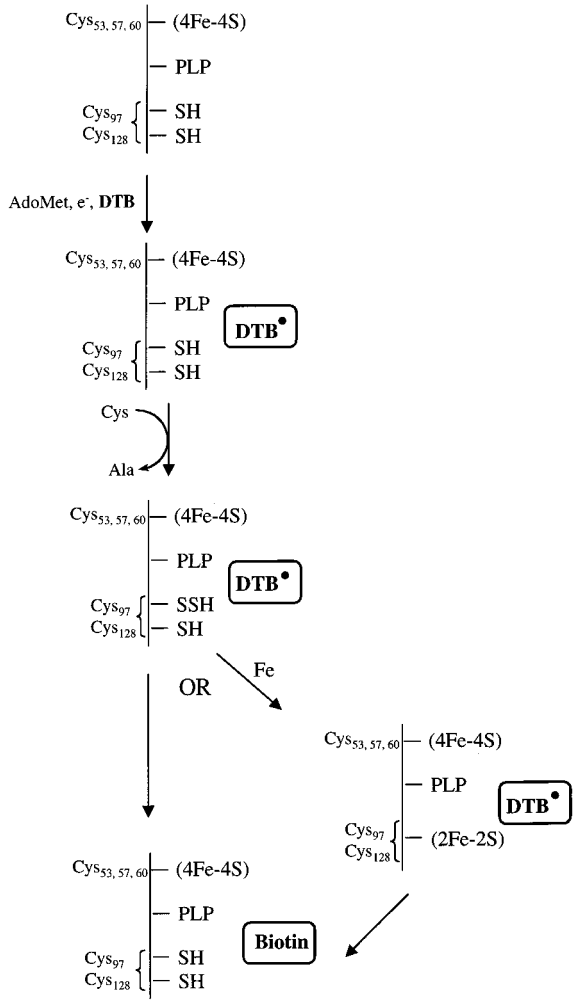

FIGURE 6: Proposed mechanisms for the persulfide-mediated formation of biotin.

in the liberation of the sulfur atom from the persulfide, resulting in the formation of a disulfide bridge between Cys97 and Cys128. A similar combination of two cysteines has been suggested during another biological sulfur transfer reaction involving transient persulfide and a disulfide bridge, namely, the last step of 4-thiouridine synthesis catalyzed by ThiI (*37*). In our case, a similar mechanism would imply an effect of the mutations more drastic than that observed here, unless both cysteines have the potential to generate a persulfide. More experiments are needed to understand the specific function of Cys97 and Cys128. The function of Cys188, which is fully conserved, remains to be identified.

The discovery of a PLP-dependent cysteine desulfurase activity for biotin synthase leads us to propose a new mechanism for biotin production shown in Figure 6. In the first step, the (4Fe-4S) cluster catalyzes the one-electron reduction of AdoMet by reduced flavodoxin. The resulting cleavage of AdoMet gives rise to methionine and the 5′-deoxyadenosyl radical which subsequently abstracts one H atom at position 6 or 9 of dethiobiotin (DTB). In a second step, sulfur transfer from cysteine to the substrate radical proceeds through the intermediate formation of a reactive protein-bound persulfide species at Cys97 or Cys128. In this mechanism, the persulfide would serve, with the collaboration of a second cysteine, as a species directly donating a sulfur atom to the radical. This is clearly inconsistent with the suggestion by Ugulava et al. that an iron−sulfur center is the sulfur donor (*10*, *11*). Nevertheless, the BioB preparation used in this study and lacking a second (2Fe-2S) cluster displays, in the presence of PLP and cysteine and with no additional iron and sulfide, reasonable enzyme activities (Figure 5). A second possibility is that persulfides would be intermediates during synthesis in the same polypeptide of a (2Fe-2S) cluster which then would give its sulfur atoms to the radical (Figure 6). There is an increasing amount of evidence that persulfides are intermediates during biosynthesis of iron−sulfur clusters (*38*−*40*). However, in general, the cysteine desulfurase protein is different from the iron−sulfur protein, and sulfur transfer between the two proteins proceeds by persulfide exchange from the cysteine of the first protein to a cysteine of the second one. A well-studied example is the process of cluster synthesis in IscU mediated by IscS, both proteins belonging to the ISC operon involved in iron−sulfur cluster assembly (*40*). This mechanism would be much more consistent with Ugulava's proposal (*10*). However, it raises the question of the origin of the iron needed to make an additional (2Fe-2S) center in our assay and would thus suggest that a significant part of the (4Fe-4S) center as well as iron impurities would be mobilized for that purpose, which we find to be rather unlikely. Further experiments are clearly needed to find out how to reconcile these seemingly divergent observations. Nevertheless, our results suggest that sulfur insertion into biotin follows the general persulfide-dependent mechanism operating in the biosynthesis of other sulfur-containing compounds (*41*).

As BioB behaves as a very versatile system with at least three different activities (AdoMet reduction, cysteine desulfuration, and biotin synthesis), it is likely that a complex and novel chemistry, involving free radicals, persulfides, and iron−sulfur clusters, is operating. More experiments are needed to address, in addition to those mentioned above, the problems of sluggishness and the apparent inactivation of the enzyme.

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