Biotin Synthase: Purification, Characterization as a [2Fe-2S]Cluster Protein, and *in Vitro* Activity of the *Escherichia coli bioB* Gene Product

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Received August 13, 1993; Revised Manuscript Received December 14, 1993®

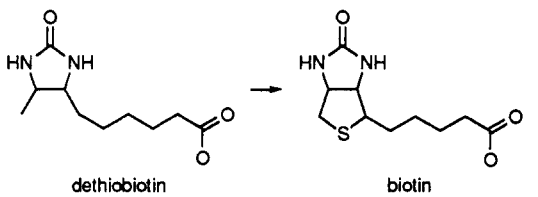
ABSTRACT: We report here the first purification of the protein encoded by the *Escherichia coli bioB* gene. One species of this protein runs on native gels with an electrophoretic mobility typical of a protein with m = 82 kDa, suggesting the protein is a dimer (gene sequence predicts m = 38.7 kDa). There are two iron- and two acid-labile sulfur atoms per protein monomer. Solutions containing the protein are red and have an absorbance spectrum characteristic of proteins with [2Fe-2S] clusters. In its oxidized native state, the protein is EPR-silent. Upon addition of dithionite, the protein's UV–visible absorbance spectrum is very slowly bleached, and an EPR active species is produced that displays a signal at g_{avg} = 1.95. All these results are consistent with this protein containing one [2Fe-2S] cluster per monomer. The EPR spin quantitation is only 5–10% of expected. Since this protein loses iron upon reduction with dithionite, the low-spin quantitation is probably due to cluster instability in the reduced state. Another species of the *bioB* gene products has also been purified which runs on native gels with an electrophoretic mobility typical of a protein with m = 104 kDa. This species appears to be a dimer with one [2Fe-2S] cluster per dimer. The 104-kDa protein can be converted to the 82-kDa protein upon incubation with Fe^{3+} and S^{2-}. The *bioB* gene product we have isolated is active in the conversion of dethiobiotin to biotin *in vitro* in the presence of NADPH, AdoMet, Fe^{3+} or Fe^{2+}, and additional unidentified factors from the crude extracts of *E. coli*. The K_m for dethiobiotin in this reaction has been found to be 2 μM.

The last step in the biotin biosynthetic pathway is shown in Scheme 1 (Eisenberg, 1987).

In the reaction depicted in Scheme 1, biotin is formed from dethiobiotin by the chemically difficult insertion of a sulfur atom between the unactivated methyl and methylene carbon atoms adjacent to the imidazole ring of dethiobiotin. The enzyme that carries out this reaction has been given the name biotin synthase although this enzyme has never been isolated. Little is known about the mechanism of this reaction. The source of the sulfur atom is not known (Demole & Shive, 1983; White, 1982; Nimura *et al.*, 1964). Parry's group has shown that except for the two protons that are removed in this reaction to accommodate the addition of sulfur, all the other protons on the two carbons to which the sulfur is added and the carbon-bound protons on the imidazole ring are preserved (Parry, 1983). Recently, this reaction has been observed for the first time in cell-free extracts. Ifuku and co-workers have reported the conversion of dethiobiotin to biotin in crude extracts of *Escherichia coli* cells which overexpress the *bioB* gene product. Fe^{2+} or Fe^{3+}, NADPH, AdoMet, KCl, and fructose 1,6-bisphosphate were reported to stimulate this reaction (Ifuku *et al.*, 1992).

There is definitive genetic evidence that in *E. coli* the *bioB* gene encodes a protein which is essential for the biotin synthase reaction, and it is common practice in the literature to refer to the *bioB* gene product as biotin synthase (Eisenberg, 1987; Ifuku *et al.*, 1992). However, because of the accumulating evidence that other proteins in addition to the *bioB* gene product may be involved in the biotin synthase reaction, we will refer in this paper to the product of the *bioB* gene as the *bioB* gene product or protein rather than biotin synthase. Perhaps it is best to reserve the name biotin synthase for the complete complex which carries out the conversion of dethiobiotin to biotin.

Scheme 1



The *bioB* gene has been cloned and sequenced from *E. coli* (Otsuka *et al.*, 1988). Highly homologous genes have been identified, cloned, and sequenced from *Bacillus sphaericus* (Ohsawa *et al.*, 1989) and *Saccharomyces cerevisiae* (Zhang *et al.*, 1994). These genes have been also been found to encode proteins that are intimately involved in the biotin synthase reaction (Fujisawa *et al.*, 1993; Zhang *et al.*, 1994).

From the sequence of the *E. coli bioB* gene, its gene product is expected to be a 38.7-kDa peptide. We have used this information along with the expected NH_2-terminal sequence to devise a protein-based assay for the *bioB* gene product. Using this assay, the *bioB* gene product has been purified to near-homogeneity for the first time. The protein is red, contains an [2Fe-2S] cluster, and is a homodimer. This purified protein is functional in the conversion of dethiobiotin to biotin in the presence of other unknown factors from cell-free extracts.

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® Abstract published in *Advance ACS Abstracts,* March 1, 1994.

0006-2960/94/0433-3625$04.50/0

MATERIALS AND METHODS

Strains and Growth Conditions. The plasmid pAOB7a containing the entire *bio* operon was a kind gift from Dr. Anthony J. Otsuka. A 2.2-kb *NcoI*, *BglII* fragment from this plasmid containing the *bioB* and *bioF* (encodes 7-keto-8-aminopelargonic acid synthase) genes was cloned into the plasmid pET-11d (Sambook *et al.*, 1989). The new plasmid, named pBioBF2, was used to transform *E. Coli* strain HMS 174. HMS174/pBioBF2 cells were grown at 37 °C in a fermentor in minimal medium with the addition of 2% glucose, 2% casamino acids, 1% glycerol, and ampicillin (100 μg/mL). When the A_{600} of the culture reached 5, the cells were induced by isopropyl β-D-thiogalactopyranoside, and harvested 3 h later. These cells were used as the source of the *bioB* gene product.

Analytical Methods. Protein concentrations were taken to be the value given by Bradford's method (Bradford, 1976) standardized against bovine serum albumin and multiplied by 1.1 [a correction factor determined by dry weight measurements (Nozaki, 1986) on purified *bioB* gene product].

Analysis for a range of metals in the *bioB* gene product was investigated by inductively coupled plasma (ICP) atomic emission spectroscopy. Wet chemical analysis for iron was routinely carried out by heating the protein in acid, reducing the released iron with hydroxylamine, and then detecting the iron as a complex with *o*-phenanthroline by measuring the absorbance of the complex at 512 nm (Lovenberg *et al.*, 1963). Labile sulfide was determined by the method of Beinert (1983).

Routine polyacrylamide gel electrophoresis was performed on a Pharmacia PhastSystem. In preparation for protein sequencing, samples were run on Daiichi gels in an Enprotech electrophoresis apparatus and blotted on an ABI Problot membrane using a BioRad Miniprotean II apparatus.

NH_2-terminal sequencing was performed on blotted samples in a Beckman LF 3000 gas-phase protein sequencer with a Model 126 pump, Model 166 detector, and Ultrasphere 2 × 250 mm PTH amino acid reverse-phase column.

BioB Gene Product Detection and Purification. The *bioB* gene product from column fractions was detected as follows. First, a preliminary sodium dodecyl sulfate (hereafter SDS) electrophoresis run on a PhastSystem was used to identify fractions containing a prominent 39-kDa peptide. Fractions containing such a peptide were electrophoresed again on Daiichi gels and blotted, and the NH_2-terminal sequence of the 39-kDa peptide was determined. Those fractions containing a prominent 39-kDa protein whose NH_2-terminal sequence matched that predicted from the gene sequence of the *bioB* gene were judged to contain the *bioB* gene product.

The chromatographic steps used in the purification were performed at room temperature on a Pharmacia BioPilot chromatographic system. The supernatant of the protamine sulfate precipitation of crude extract was loaded onto the bottom of a 3-L DEAE-Sepharose column equilibrated with 50 mM Tris-HCl, pH 8.1 (hereafter, T8 buffer). The column was eluted in an upward direction with an increasing gradient of KCl in T8 buffer.

Fractions containing the *bioB* gene product were combined, brought to 10% (w/v) with $(NH_4)_2SO_4$, and loaded on the top of a phenyl-Sepharose column. This column was eluted with a decreasing gradient of $(NH_4)_2SO_4$ in T8 buffer. The fractions containing the *bioB* gene product were combined and concentrated to about 10 mg of protein/mL. This solution was loaded onto a Superdex 35/600 column 10 mL at a time and eluted with T8 buffer. The fractions containing the *bioB* gene product were combined, concentrated to about 40 mg of protein/mL, and frozen as beads in liquid N_2.

Table 1: Purification of the *E. coli BioB* Gene Product

purification step	amount of protein (mg)	estimated purity (%)
crude extract	12800	4
protamine sulfate precipitation	9200	6
DEAE-Sepharose	2200	23
phenyl-Sepharose	810	56
Superdex	520	90

Spectroscopic Methods. UV–visible absorbance spectra were obtained on a Cary 2290 spectrophotometer attached to an IBM computer using SpectraCalc version 2.12 software (Galactic Industries Corp.). EPR spectra were measured on a Bruker ER 200D spectrometer connected to an IBM Instruments 9000 computer. Temperatures in the range of 6–80 K were obtained using an Air Products Heli-Tran LTR-3 liquid helium cryostat and Series 5500 temperature controller. Reduction with dithionite was carried out in an anerobic glovebox (Coy Laboratories).

Conversion of the 104-kDa Protein to the 82-kDa Protein. In an anaerobic glovebox at room temperature, dithiothreitol was added to T8 buffer to a concentration of 1 mM followed by the addition of 104-kDa protein to a concentration 0.8 mg/mL. This mixture was incubated for 10 min, and then $FeCl_3$ was added to a concentration of 0.1 mM and Na_2S was added to a concentration of 0.05 mM. After incubation for 4 h, this mixture was passed through a gel filtration column (PD-10) to separate the protein from the excess iron and sulfide.

Assay for Biotin Synthase Activity. Unless otherwise noted, the assays were carried out in 100 mM Tris-HCl buffer (pH 7.5) with 2 mM dithiothreitol in the presence of 50 μM dethiobiotin, 5 mM $Fe(NH_4)_2(SO_4)_2$, 1 mM NADPH, 0.1 mM AdoMet, 5 mM fructose 1,6-bisphosphate, and 10 mM KCl. The crude extract required for the biotin synthase assay was prepared by sonicating KS302Δ*bio* cells resuspended in 3× volume of 100 mM Tris-HCl buffer (pH 7.5) containing 2 mM dithiothreitol. A supernatant fraction (containing ~3 mg of protein/mL) was prepared by centrifugation; 160 μL of supernatant was added to the assays. The final volume of the assay mixture was 400 μL. At the end of the incubation period, the reaction was stopped by heating to 95 °C for 5 min. The conversion of dethiobiotin to biotin was determined by a microbiological assay using *E. coli* KS302Δ*bio* as the assay microorganism. This organism lacks the biotin operon and consequently has an absolute requirement for biotin.

RESULTS

Purification of the BioB Gene Product. We were able to detect the *bioB* gene product by a combination of SDS electrophoresis and NH_2-terminal sequencing in fractions from columns through which extracts of HMS174/pBioBF2 were chromatographed. With this assay, the purification scheme for the *bioB* gene product as shown in Table 1 was developed. The *bioB* gene product in the column fractions and in the purified state has the following NH_2-terminal sequence: M-A-H-R-P-R-W-T-L-S-Q-V-T-E-L-F-E. This NH_2-terminal sequence is identical to that expected from the sequence of the *bioB* gene (Otsuka *et al.*, 1988).

We found two chromatographically distinct species of the *bioB* gene product in the extracts of HMS174/pBioBF2. These two species are not well resolved on the DEAE-Sepharose column, so fractions containing both species are combined in

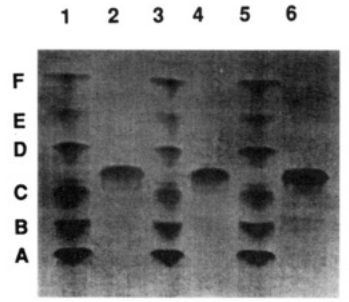

FIGURE 1: SDS–polyacrylamide gel electrophoresis of purified *bioB* gene product. Lanes 2, 4, and 6 each contain 1.5 μg of purified *bioB* gene product. Lanes 1, 3, and 5 each contain the following mixture of molecular weight markers: *A*, phosphorylase *b* (M_r 94 000); *B*, bovine serum albumin (M_r 67 000); *C*, ovalbumin (M_r 43 000); *D*, carbonic anhydrase (M_r 30 000); *E*, soybean trypsin inhibitor (M_r 21 100); *F*, α-lactalbumin (M_r 14 400). The gel origin is at the bottom of the picture.

the purification procedure. There is almost base-line resolution of the species on the phenyl-Sepharose column, so the fractions containing the two species were combined separately. The two species are partially resolved on the Superdex column, suggesting they differ in molecular mass or shape. Native gel electrophoresis gives a value of m = 82 kDa for the minor species which elutes first from the phenyl-Sepharose column and second from the Superdex column, and a value of m = 104 kDa for the major *bioB* gene product species which elutes second from the phenyl-Sepharose column and first from the Superdex column. In this paper, these two species will be referred to as the 82- and 104-kDa proteins although their precise m is not known with certainty. Both species migrate the same on SDS gels with m = 39 kDa. Figure 1 shows an SDS gel run on the purified 82-kDa protein along with molecular mass standards.

Elemental Analysis of the 82- and 104-kDa Proteins. ICP analysis for iron, cobalt, copper, nickel, molybdenum, and zinc was run on three samples of the 82-kDa protein. Iron was found in the amount of 1.7 mol of iron/mol of protein monomer; 2.1 mol of iron/mol of monomer was found in the same samples by the *o*-phenanthroline method. The acid-labile sulfide content of the samples was 2.0 mol of sulfide/mol of monomer. The 104-kDa protein was found to contain half the amount of iron and sulfide for monomer as the 82-kDa protein.

Spectroscopic Characterization of the BioB Gene Product. Solutions containing the 82- and 104-kDa proteins have a distinct red color. The UV–visible absorbance spectrum of the native 82-kDa protein is shown in Figure 2. In addition to the protein absorbance at 275 nm ($\epsilon = 3.3 \times 10^4$ M^{-1} cm^{-1}, based on monomer molecular mass), there are peaks at 330 nm ($\epsilon = 1.4 \times 10^4$ M^{-1} cm^{-1}), 420 nm (sh, $\epsilon = 6.0 \times 10^3$ M^{-1} cm^{-1}), 453 nm (ϵ 7.1×10^3 M^{-1} cm^{-1}), and 540 nm (sh, $\epsilon = 3.5 \times 10^3$ M^{-1} cm^{-1}). Most proteins with [2Fe-2S] clusters have extinction coefficients in the 400–450-nm region of (4–5) $\times 10^3$ M^{-1} cm^{-1}/iron in the +2 oxidation state (Orme-Johnson & Orme-Johnson, 1982). The extinction coefficient of the 82-kDa protein in this wavelength region is somewhat lower, but consistent with the presence of one [2Fe-2S] cluster per monomer. The spectrum of the 104-kDa protein is almost identical to the 82-kDa protein (shown in Figure 2) except the extinction coefficients for the nonprotein peaks (>300 nm) are approximately half that of the 82-kDa protein. The spectra of the 82- and 104-kDa proteins are similar to other proteins which contain [2Fe-2S] clusters, *e.g.*, ferredoxins (Palmer, 1973), Rieske proteins (Fee *et al.*, 1984), and spinach dihydroxy-acid dehydratase (Flint & Emptage, 1988).

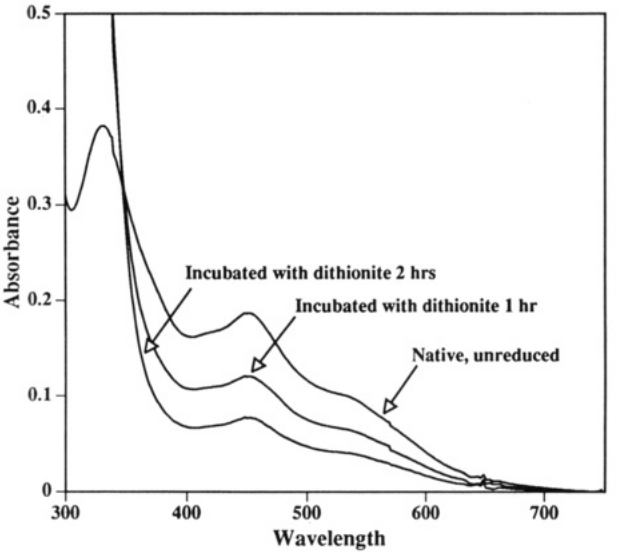

FIGURE 2: UV–visible absorbance spectra of native and dithionite-reduced 82-kDa protein. The absorption spectrum of the 82-kDa protein (1 mg/mL) in T8 buffer is shown it its native state and after incubation with 10-fold excess sodium dithionite for 1 and 2 h.

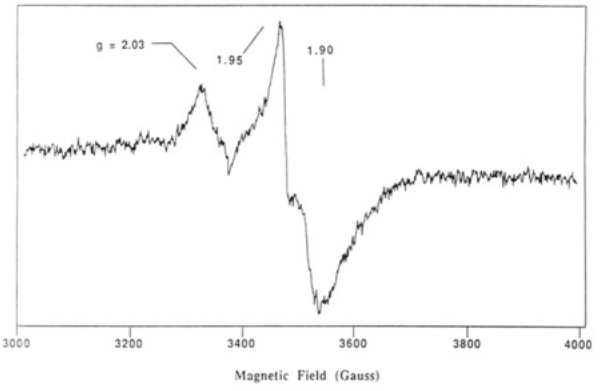

FIGURE 3: EPR spectrum of dithionite-treated 82-kDa protein. EPR spectrum of the 82-kDa protein (100 μM) reduced with a 10-fold excess of sodium dithionite in T8 buffer. The *g* values of the prominent features are indicated. Experimental conditions for obtaining the EPR spectrum were 10 K, 1-mW microwave power, 6.435-G modulation amplitude, and 9.43-GHz microwave frequency.

When either species is treated with excess dithionite anaerobically with or without the mediator methyl viologen, there are no detectable changes in the absorbance spectrum for several minutes. However, over a period of hours, the absorbance above 300 nm slowly decreases until it completely disappears. The nature of these changes is shown in Figure 2.

EPR spectra of the 82-kDa protein were measured. No signal was detected in the native state in T8 buffer. When the sample was reduced with excess dithionite with or without methyl viologen, no signal was observed for several minutes. However, after incubation for more extended periods of time, it exhibited a signal with g_{avg} = 1.95 at liquid helium temperatures as shown in Figure 3. The EPR spectrum was typical of [2Fe-2S] cluster containing proteins, which generally show an EPR signal in the g = 2 region upon reduction, and is characteristic of an $S = {}^1/_2$ state (Fee *et al.*, 1984; Flint & Emptage, 1988). However, in the case of the 82-kDa protein, the spin quantitation of samples after extended reduction was only 0.05–0.1 spin/mol of monomer in spite of the fact that the UV–visible spectrum of each sample had been significantly bleached by the dithionite. No EPR signal was observed at liquid nitrogen temperatures. This is untypical

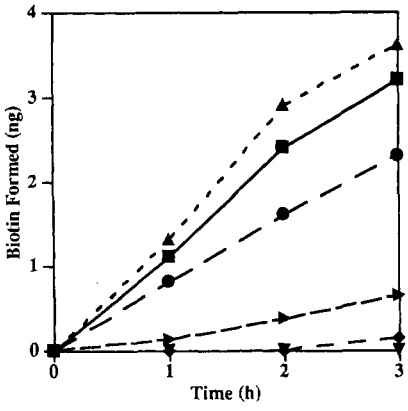

FIGURE 4: Effect of certain compounds on the biotin synthase reaction with time. Complete reaction mixture consisted of 6×10^{-10} mol of 82-kDa protein, and crude extract, KCl, NADPH, FBP, AdoMet, and $Fe(NH_3)_2(SO_4)_2$ at the concentrations indicated under Materials and Methods. (■) Complete mixture; (▲) mixture without FBP; (●) mixture without KCl; (▶) mixture without NADPH; (♦) mixture without AdoMet; (▼) mixture without $Fe(NH_3)_2(SO_4)_2$.

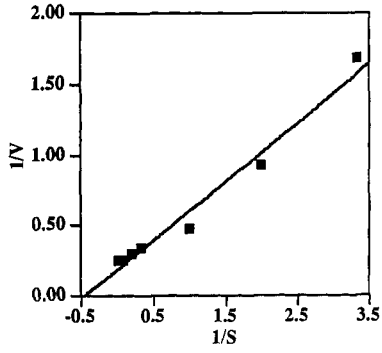

FIGURE 5: Double-reciprocal plot of the biotin synthase reaction. 1/velocity of the biotin synthase reaction in picomoles per minute plotted against 1/[dethiobiotin] in micromolar. The K_m determined from the plot is 2 μM.

Table 2: Effect of Amount of 82-kDa Protein on the Synthesis of Biotin

mol of 82-kDa protein in assay	mol of biotin formed in assay	mol of biotin formed/mol of 82-kDa monomer	turnovers h^{-1} mol of 82-kDa monomer
6×10^{-11}	2×10^{-10}	3	1
6×10^{-10}	5×10^{-10}	0.8	0.3
6×10^{-9}	10×10^{-10}	0.2	0.06

of a protein with a [2Fe-2S] cluster, but not unexpected given the low spin quantitation at liquid helium temperatures.

This result coupled with the observation that the UV–visible absorbance above 300 nm gradually decreases to zero upon reduction with dithionite, and the observation that the original spectrum of the *bioB* gene product cannot be regenerated after removal of excess dithionite and introduction of oxygen, suggested the chromophore of the *bioB* gene product was not stable upon reduction by dithionite. To investigate this further, the *bioB* gene product was reduced with excess dithionite for 2 h at room temperature. By this time, the UV–visible absorbance spectrum above 300 nm had been reduced to about 40% of the original intensity. This sample was then passed through a gel filtration column (anaerobic), and the iron content of the protein and low molecular weight fraction was measured. Almost 60% of the iron originally present in the *bioB* gene product was in the low molecular weight fraction, while only 40% remained in the protein fraction. Iron is lost from the *bioB* gene product following reduction by dithionite.

Activity of the BioB Gene Product in the Conversion of Dethiobiotin to Biotin. We found that under the conditions described by Ifuku *et al.* (1992), crude extracts from the *E. coli* strain HMS174/pBioBF2 were as active in the conversion of dethiobiotin to biotin as the crude extracts from their strain in which the *bioB* gene product was overexpressed. These results confirm the importance of the *bioB* gene product in the conversion of dethiobiotin to biotin. However, the 82-kDa protein we purified did not catalyze the conversion of dethiobiotin to biotin in the presence of the cofactors in the standard assay. Furthermore, the addition of the purified 82-kDa protein to crude extracts of HMS174/pBioBF2 did not increase the rate of biotin production in these extracts.

To further investigate the enzymatic activity of the purified 82-kDa protein, it was added to crude extracts of KS302Δ*bio*. This strain lacks the *bioB* gene product, and crude extracts from this strain were completely inactive in the conversion of dethiobiotin to biotin. When 3×10^{-10} mol of 82-kDa protein was added to crude extracts of KS302Δ*bio* cells in the presence of the other cofactors in the standard assay, the mixture was very active in the conversion of dethiobiotin to biotin. The results from a typical experiment are shown in Figure 4. This figure also shows the effect of not supplying each of the compounds added by Ifuku *et al.* in their experiments. When the 82-kDa protein was added to extracts of KS302Δ*bio* cells, fructose 1,6-bisphosphate is not required for the synthesis of biotin from dethiobiotin. The synthesis appears to be stimulated to a small extent by KCl, but in many experiments it was less than that shown in Figure 4. The synthesis is always greatly stimulated by NADPH, although a small amount of biotin is usually made when it is not added as seen in Figure 4. The synthesis of biotin is always dependent on Fe^{3+} or Fe^{2+}, and AdoMet. The oxidation state of the active form of the iron in the reaction mixture is not known. If Fe^{2+} or Fe^{3+} is mixed with the intact 82-kDa protein and the mixture is passed through a PD-10 column (Pharmacia), the only iron associated with the protein is in the Fe-S cluster (results not shown).

The K_m for dethiobiotin in the biotin synthase reaction catalyzed by the mixture of the crude extract of KS302Δ*bio* and the 82-kDa protein was found to be 2 μM. The data used to obtain this value are shown in Figure 5 in the form of a double-reciprocal plot. The effect of the amount of 82-kDa protein added to the assay mixture was investigated, and the results are shown in Table 2. There were 3 turnovers/82-kDa monomer in assays in which 3×10^{-11} mol of 82-kDa protein was incubated for 3 h with crude extract from KS302Δ*bio* for a turnover number of 1 h^{-1}. It is significant that there are more moles of biotin produced than 82-kDa protein added; i.e., the 82 kDa protein acts catalytically, not stoichiometrically. Since it can be calculated from data in the report of Ifuku and co-workers, on the *in vitro* conversion of dethiobiotin to biotin, that only 0.3 turnover per mole of *bioB* gene product occurred during their incubations (Ifuku *et al.*, 1992), this is the first report where the *bioB* gene product has been shown to act catalytically.

Conversion of the 104-kDa Protein to the 82-kDa Protein. Since the 104-kDa species appeared to be composed of a dimer with only one cluster present, an attempt was made to convert the 104-kDa species to the 82-kDa species under conditions known to be conducive to cluster formation (see Material and Methods). In the presence of Fe^{3+} and S^{2-}, the 104-kDa species was converted to a species that was indistinguishable from the 82-kDa protein isolated directly from cells. The

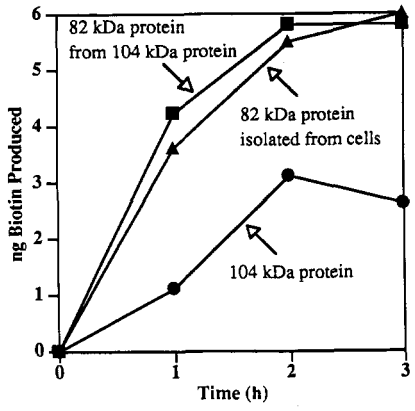

FIGURE 6: Plot of the amount of biotin made with time. Conversion of dethiobiotin to biotin in the presence of (●) 104-kDa protein, (■) 82-kDa protein reconstituted from 104-kDa protein, or (▲) 82-kDa protein isolated from cells. In each case, the proteins were incubated with crude extract from KS302Δ*bio* cells under standard assay conditions in a volume of 400 μL. The amount of biotin formed in 10 μL.

iron and sulfur content of the 104-kDa species changed from one iron and one sulfur per monomer to two irons and two sulfurs per monomer; the extinction coefficients of the peaks and shoulders of the UV–visible absorbance spectrum above 300 nm doubled (e.g., ϵ at 453 nm increased from 3.5×10^3 to 7.1×10^3 M^{-1} cm^{-1}), and the molecular mass of the protein on native gels decreased from 104 to 82 kDa.

Samples of the 82-kDa protein derived from 104-kDa protein, the 82-kDa protein isolated from cells, and the 104-kDa protein isolated from cells were added to crude extracts of KS302Δ*bio*, and their activity in the conversion of dethiobiotin to biotin was compared. In this experiment, 3×10^{-10} mol of each protein monomer was added. The amount of biotin made over 3 h with the addition of these proteins is shown in Figure 6. This figure shows all three proteins are active in the conversion of dethiobiotin to biotin. The 82-kDa protein derived from the 104-kDa protein is as active as the 82-kDa protein isolated from cells. The 104-kDa protein is only about half as active as the 82-kDa protein in the conversion of dethiobiotin to biotin.

DISCUSSION

The *bioB* gene product has been purified for the first time. A protein-based assay was used for the purification. The enzyme was identified by the congruity of its NH_2-terminal sequence to that predicted from the *bioB* gene sequence. The 82-kDa form of the *bioB* gene product appears to be a homodimer since its molecular mass is close to twice the value of 38.7 kDa predicted from the gene sequence (Otsuka *et al.*, 1988). The protein contains two $[2Fe\text{-}2S]^{2+}$ clusters per dimer. The evidence for the cluster is 3-fold: (1) there are two iron-labile and two acid-labile sulfur atoms per monomer; (2) the UV–visible absorbance spectrum is typical of proteins with [2Fe-2S] clusters; and (3) the EPR spectrum of the reduced protein is typical of proteins with [2Fe-2S] clusters (Palmer, 1973; Fee *et al.*, 1984)).

The EPR spectrum of the dithionite-reduced protein does not give a quantitative number of spins. Our explanation for this is illustrated by the following proposed reaction sequence where BBGP refers to the *bioB* gene product:

$$\text{BBGP}[2Fe\text{–}2S]^{2+} \xrightarrow[\text{(slow)}]{\text{dithionite}} \text{BBGP}[2Fe\text{–}2S]^{1+} \xrightarrow{\text{(faster)}} \text{BBGP}[<2Fe\text{–}?S]$$

If the rate of cluster reduction is slow compared to the rate of cluster destruction, the level of reduced enzyme with intact reduced cluster would inevitably remain low. This reaction sequence accommodates the following four observations: (1) The spectrum of the *bioB* gene product is slowly bleached on the addition of dithionite, indicating reduction occurs; (2) the reduced cluster loses Fe to solvent; (3) the original spectrum is not regenerated when the reduced protein is reoxidized; (4) the EPR spin quantitation is low. We are not aware of any other case where a reduced [2Fe-2S] cluster is unstable.

The 104-kDa protein appears to be a species of the *bioB* gene product that lacks a complete complement of cluster. It seems likely it is a homodimer with only one cluster per dimer. The lack of a full cluster complement may result in a more extended peptide conformation which could account for the apparent higher molecular weight of this species as determined by its electrophoretic mobility on native gels and its elution position from the Superdex column. The existence of this species could be due to the rate of cluster formation lagging behind the rapid rate of *bioB* gene product formation following induction in our high-expression strain. If this is the case, it is also possible that some of the *bioB* gene product formed in the HMS174/pBioBF2 cells was completely devoid of cluster.

The 82-kDa protein we have isolated functions in the conversion of dethiobiotin to biotin in crude extracts devoid of the *bioB* gene product. This demonstrates that the isolated protein is competent in the biotin synthase reaction, and provides the first definitive biochemical evidence that the *bioB* gene product participates in the biotin synthase reaction. However, the results reported in this paper also demonstrate that the 82-kDa protein alone is not sufficient to catalyze the biotin synthase reaction since additional unidentified factors from the crude extract are required.

In addition to these unidentified factors, both iron and AdoMet appear to be essential for the reaction, and NADPH greatly stimulates it. The required iron is in addition to that in the cluster of the 82-kDa protein. It seems likely that this additional iron functions in association with either the 82-kDa protein or a protein from the crude extract. The iron appears to be loosely bound if either is the case since (1) iron mixed in solution with the 82-kDa protein is readily removed by passage through a PD-10 column (except for the iron in the Fe-S cluster); and (2) if iron is required by a protein from the crude extract that functions in the biotin synthase reaction, the iron is not retained by the protein through the process of preparing the crude extract. The role of the additional iron is unknown, although it is worth noting that isopenicillin *N*-synthase, which catalyzes a reaction similar to that catalyzed by biotin synthase (sulfur added to an unactivated carbon atom), also requires a loosely bound iron (Chen *et al.*, 1989). This raises the possibility that there are some similarities between the mechanism of these two reactions. However, we have found unlike the isopenicillin *N*-synthase reaction which requires O_2, the biotin synthase reaction proceeds at the same rate in both anaerobic and aerobic conditions.

A turnover number of 1 h^{-1} based on the amount of 82-kDa protein is unusually low for an enzyme. There are several possible explanations. As suggested by the data in Table 2, in our assays, the 82-kDa protein is likely to be in excess compared to other required proteins from the crude extract. Although the 82-kDa protein is obviously an essential component for the biotin synthase reaction, another required protein from the crude extract could be limiting in our reaction conditions. It is even possible the 82-kDa protein is not the catalytic subunit of biotin synthase, in which case the protein

would not have a turnover number in the strict sense. We are obviously in the initial stages of understanding the biotin synthase reaction, and it is likely that a higher turnover number for this reaction will likely be found once all the proteins necessary for it are purified, the catalytic subunit is identified, and the conditions for the reaction are optimized. However, using the numbers reported in this paper, the k_{cat}/K_m based on the 82-kDa protein is 130 M^{-1} s^{-1}, which is within an order of magnitude of the k_{cat}/K_m values of 400–1500 M^{-1} s^{-1} that can be calculated from data on early reports of isopenicillin *N*-synthase (Pang *et al.*, 1984; Baldwin *et al.*, 1987). As the assays for isopenicillin *N*-synthase have been optimized, the reported k_{cat}/K_m values have increased to around 1×10^4 M^{-1} s^{-1} (Ming *et al.*, 1991; Castro *et al.*, 1988), and the value for the biotin synthase reaction may similarly increase. Even so, low turnover numbers may be characteristic of all the enzymes in the biotin biosynthetic pathway. For example, the turnover number for dethiobiotin synthase is 100 h^{-1} (Krell & Eisenberg, 1970), and that for 7,8-diaminopelargonic acid synthase is 300 h^{-1} (Stoner & Eisenberg, 1975).

Six cysteine residues are conserved in the predicted amino acid sequences of the *bioB* gene product from *E. coli* (Otsuka *et al.*, 1988) and *B. sphaericus* (Ohsawa *et al.*, 1989), and the *BIO2* gene of *S. cerevisiae* (Zhang *et al.*, 1994). Since resonance Raman spectroscopy of the *E. coli bioB* gene product (in collaboration with Michael Johnson and Lisa Brinkman, University of Georgia) indicates there are four cysteinyl ligands to the [2Fe-2S] clusters of this protein (unpublished result), four of the six conserved cysteines must be ligands to the cluster. Because of their strong sequence homology to the *E. coli bioB* gene product, it seems likely that the *B. sphearicus* and yeast *BIO2* gene products also have similar iron–sulfur clusters. Three of the conserved cysteines in these three proteins are in a unusual Cys-X-X-X-Cys-X-X-Cys motif. This motif is also found in the lipoic acid synthase protein (*lipA gene*) which catalyzes the addition of sulfur atoms to unactivated carbon atoms in octanoic acid to give lipoic acid, a reaction similar to biotin formation (Hayden *et al.*, 1992; Reed & Cronan 1993). Perhaps lipoic acid synthase also has a similar iron–sulfur cluster.

A significant issue is the unstable nature of the [2Fe-2S] cluster of the *bioB* gene product upon reduction. There is a remote possibility that the sulfur added to dethiobiotin comes from the Fe-S cluster, and its instability is associated with the loss of sulfur from the cluster during catalysis. If the sulfur does come from the cluster, then the cluster would have to be "remade" following each turnover of the enzyme. If this is the case, one of the functions of the other factor(s) required from crude extracts could be cluster resynthesis.

Recent reports on lysine 2,3-aminomutase (Frey & Reed, 1993) and anaerobic ribonucleotide reductase (Mulliez *et al.*, 1993) show that there are similarities between the reactions catalyzed by these two enzymes and the reaction catalyzed by biotin synthase; namely: (1) the enzymic catalysts contain an Fe-S cluster; (2) AdoMet is required; and (3) a hydrogen is removed from an unactivated carbon atom in the course of the reaction. The function of AdoMet and the Fe-S cluster of lysine 2,3-aminomutase and anaerobic ribonucleotide reductase appears to be in the formation of a radical involved in the mechanism of the enzymes. This brings up the possibility that the function of the [2Fe-2S] cluster in the *bioB* gene product and the requirement for AdoMet in the biotin synthase reaction are to participate in the generation of a radical that is required for the reaction.

There is also a possibility that AdoMet is the sulfur donor in the biotin synthase reaction. However, prior evidence indicates the sulfur in methionine (the obligatory precursor of AdoMet) is not the source of sulfur in the biotin synthase reaction in *E. coli* (Demoll & Shive, 1983; White, 1982), so on this evidence it seems unlikely that AdoMet is the sulfur donor. If AdoMet is not the sulfur donor, it could be involved in the activation of the enzyme.

The requirement for an apparently loosely bound iron, the requirement for AdoMet, the presence of a [2Fe-2S] cluster in the *bioB* gene product, and the requirement for additional protein factors from the crude extract of *E. coli* all indicate the mechanism of the biotin synthase reaction may be complex. It will be interesting to see if the biotin synthase reaction shares any mechanistic similarities with isopenicillin *N*-synthase, lysine 2,3-aminomutase, or anaerobic ribonucleotide reductase.

ACKNOWLEDGMENT

We thank Winona Wagner for the 200-L fermentation of pBioBF2 cells. We also thank Thomas Miller for N-terminal sequencing and James Berry for ICP analysis. Technical help by Edith Duplessis, Joseph Tuminello, and Gregory Beatty is deeply appreciated. We are also indebted to Drs. Mark Emptage (DuPont), Michael Johnson (University of Georgia), and John Mangum (Brigham Young University) for helpful discussions.

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