J Biochem 130, 627–635 (2001)

Structure–Function Studies of *Escherichia coli* Biotin Synthase *via* a Chemical Modification and Site-Directed Mutagenesis Approach[1]

Lynn Farh,[*] Shao-Ying Hwang,[†] Larry Steinrauf,[†] Huey-Jenn Chiang,[‡] and David Shiuan[†,‡,2]

[*]*Department of Natural Science Education, National Pingtung Teachers College, Pingtung, Taiwan,* [†]*Department of Biological Science and Center for Biotechnology, National Sun Yat-Sen University, Kaohsiung, Taiwan, and* [‡]*Department of Life Science and Institute of Biotechnology, National Dong Hwa University, Hualien, Taiwan*

Received July 23, 2001, accepted August 22, 2001

In *Escherichia coli*, biotin synthase (*bioB* gene product) catalyzes the key step in the biotin biosynthetic pathway, converting dethiobiotin (DTB) to biotin. Previous studies have demonstrated that BioB is a homodimer and that each monomer contains an iron-sulfur cluster. The purified BioB protein, however, does not catalyze the formation of biotin in a conventional fashion. The sulfur atom in the iron-sulfur cluster or from the cysteine residues in BioB have been suggested to act as the sulfur donor to form the biotin molecule, and yet unidentified factors were also proposed to be required to regenerate the active enzyme. In order to understand the catalytic mechanism of BioB, we employed an approach involving chemical modification and site-directed mutagenesis. The properties of the modified and mutated BioB species were examined, including DTB binding capability, biotin converting activity, and Fe^{2+} content. From our studies, four cysteine residues (Cys 53, 57, 60, and 97) were assigned as the ligands of the iron-sulfur cluster, and Cys to Ala mutations completely abolished biotin formation activity. Two other cysteine residues (Cys 128 and 188) were found to be involved mainly in DTB binding. The tryptophan and histidine residues were suggested to be involved in DTB binding and dimer formation, respectively. The present study also reveals that the iron-sulfur cluster with its ligands are the key components in the formation of the DTB binding site. Based on the current results, a refined model for the reaction mechanism of biotin synthase is proposed.

Key words: biotin synthase, chemical modification, iron-sulfur cluster, site-directed mutagenesis.

The vitamin biotin is an essential factor for carboxylase-catalyzed reactions in all living cells (*1*). The biotin biosynthetic pathway in bacteria, especially that in *Escherichia coli* and *Bacillus sphericus*, has been thoroughly investigated. The biosynthetic pathway from pimeloyl-CoA to biotin is catalyzed by the product of the *bioABFCD* operon (*2–6*). However, the steps leading to the formation of pimeloyl-CoA in *E. coli* are still unclear. Transcription of the biotin operon is divergent from a single regulatory region between the *bioA* and *bioB* gene. The biotin repressor (also functions as biotin-protein ligase, encoded by the *birA* gene, located separately from the biotin operon), working together with its co-repressor biotinyl 5′-adenylate, binds to the operator and represses transcription in both directions (*7–10*)

Biotin synthase (BioB), the *bioB* product, has been proposed to catalyze the last step in the biosynthetic pathway, converting dethiobiotin (DTB) to biotin. This step catalyzes the chemically difficult reaction involving the insertion of a sulfur atom between the unactivated methyl and methylene carbon atoms adjacent to the imidazole ring of DTB to form the thiolane ring of the vitamin biotin (Scheme 1). In order to understand the mechanism of this step, several groups have purified BioB and examined its properties. Native gel electrophoresis and gel filtration data have suggested that the protein is a dimer (*11*). Spectroscopic analysis has indicated that there is one iron-sulfur cluster per protein monomer. Several forms of the iron-sulfur cluster have been detected, including [2Fe-2S] and [4Fe-4S], with different statuses of spin and oxidation potential of the iron (*12, 13*). The current hypothesis favors the presence of a reduced $[4Fe\text{-}4S]^{2+}$ cluster in BioB as a bio-reactive form (*14*). A CxxxCxxC motif present at amino acid positions 53 to 60 of BioB has been suggested to be involved in the coordination of the $[4Fe\text{-}4S]^{2+}$ cluster (*15*).

The BioB protein alone cannot carry out biotin formation activity. A number of factors have been shown to be necessary to facilitate the reaction, including flavodoxin, ferredoxin $NADP^{+}$ reductase, MioC, and yet unidentified proteins (*11, 16, 17, 32*). Several small molecular weight

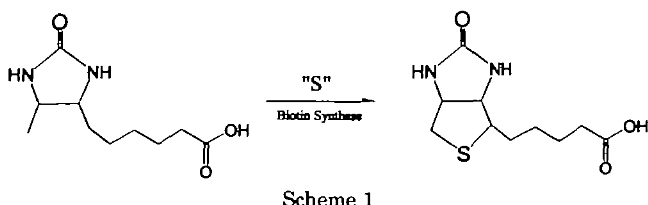
[1] The authors thank National Science Council of the Republic of China for partial support of the work through grants NSC87-2311-B 110-008 and NSC87-2622-B 110-001.

[2] To whom correspondence should be addressed at: Department of Life Science and Institute of Biotechnology, National Dong Hwa University, Hualien, Taiwan 974 Tel: +886-3-8662500 (Ext 21313), Fax. +886-3-8662620, E-mail shiuan@mail ndhu.edu tw

Abbreviations DTB, dethiobiotin; NBS, *N*-bromosuccinimide; DEPC, diethylpyrocarbonate; PLP, pyridoxal 5′-phosphate; BNPS-skatole, 2-(2-nitrophenylsulfonyl)-3-methyl-3-bromoindolenine-skatole

© 2001 by The Japanese Biochemical Society

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 30, 2012



Scheme 1

factors, such as $NADP^+$, NADPH, FAD, fructose 1,6-bisphosphate, other than DTB have been suggested to stimulate biotin synthesis in cell free extracts (*16, 18*). Critical roles of Fe^{2+}, *S*-adenosylmethionine (AdoMet) and an unidentified enzyme in addition to the *bioB* product are also thought to be obligatory for the conversion of DTB to biotin (*16, 18, 19*). Furthermore, a labile low molecular weight product of the 7,8-diaminopelagonic acid transferase (*bioA* product) has been reported to stimulate the rate of biotin formation in the defined biotin synthase reaction mixture and increase the final amount of biotin formed by threefold (*16*).

The sulfur donor for dethiobiotin is another unsettled problem. AdoMet probably initiates the reaction by abstracting an electron from the C-9 of dethiobiotin, thus forming a radical. This radical would then capture a sulfur atom, probably from a nearby cysteine (*17, 20*). However, the $Fe^{34}S$-reconstituted BioB is also able to incorporate ^{34}S into biotin (*21*). Accessory proteins such as NifS from *A. vinelandii* have been demonstrated to mediate the reconstitution of the iron-sulfur center in the BioB apo-protein using cysteine as the sulfur donor (*22*). A general scheme has therefore emerged that the sulfur for biotin is abstracted directly from BioB, which then needs to be regenerated by enzymes such as NifS using an outside sulfur-provider such as cysteine. Some unidentified accessory enzymes must be present since NifS is unable to regenerate BioB more than once (*22*).

Understanding the structure–function relationship of biotin synthase is essential to unravel the complex molecular mechanism leading to the conversion of dethiobiotin to biotin. In the present study, the *E. coli bioB* gene was overexpressed in *E. coli* and purified using the QIAexpress expression and purification system. Chemical modifications, site-directed mutagenesis, and then enzyme structure and activity assays were performed to examine the protein structure–function relationship. Here we present results that suggest the identification of the amino acid residues in BioB that serve as the ligands for the iron-sulfur cluster and DTB binding.

MATERIALS AND METHODS

All chemicals were of reagent grade and used without further purification The restriction enzymes and the *pfu* DNA polymerase for the polymerase chain reaction were obtained from Boehringer Mannheim (Germany). [α-^{35}S]dATP (>1,000 Ci/mmol) was purchased from Amersham (USA).

Bacterial Strains and Plasmids—The strains used in the study include R901 (Sm^R, *Δbio*) (*4*) for bioassay, CJ236 (*ung⁻ dut⁻ thi-1 relA1* pCJ105[Cam^R]) for site-directed mutagenesis (*23*), JM101 (*SupE thi Δ[lac-proAB] F'[traD36 proAB lacI^q lacZΔM15]*) for mutagenesis and cloning. Plasmid pEC30 carrying the *bioABFCD* genes of *E. coli* K-12 was constructed as described previously (*4*). The pQE30 expression vector and Ni-NTA columns were purchased from QIAGEN Inc (USA).

Overexpression and Purification of Biotin Synthase—The *BioB* gene coding for *E. coli* biotin synthase was amplified from pEC30 (*4*) using a polymerase chain reaction and subcloned into the expression vector pQE30 (Qiagen). To facilitate purification, an oligonucleotide sequence containing six histidine residues was engineered at the 5′ end of the Bio B gene. The plasmid, named pQE30-BioB, was transformed into the biotin operon-deficient strain R901 and incubated on a minimal plate supplemented with the biotin precursor DTB (final concentration 50 ng/ml) and a tetrazolium chloride indicator (0.004%). Colonies that could express sufficient amounts of biotin turned red. The plasmids from the red colonies were recovered and transformed into JM101 and cultured in LB (per liter contains : 10 g tryptone, 10 g NaCl and 5 g yeast extract) with 25 μg/ml kanamycin and 100 μg/ml ampicillin, and agitated at 160 rpm. Five milliliters of the overnight culture was innoculated into 500 ml of fresh LB medium with antibiotics and agitated at 160 rpm, overnight at 37°C. The overnight culture (500 ml) was then innoculated into 2.5 liters of prewarmed LB medium with both antibiotics and incubated at 37°C with agitation at 100 rpm for 2 h until the OD_{600} reached approx. 0.7 Induction was then initiated and continued for 4 h after the addition of 2 mM IPTG. The cells were harvested, washed with buffer A [25 mM Tris-HC1, pH 7.5, 2 mM Na_2EDTA, 1 mM dethiothreitol, 15% (w/w) glycerol], and lysed using a French press.

To purify the BioB protein, 10 ml of Ni-NTA agarose resin (Qiagen) was packed in a BioRad column (1.5 × 6 cm) and equilibrated with buffer A. The lysate was applied to the column at a rate of 0.5 ml/min. Bound BioB appeared as a red-brown protein band retained in the column. The column was washed with 100 ml of buffer B (same as buffer A, except the pH was adjusted to 6.0) at approx. 1.5 ml/min. BioB was eluted with a gradient of 0 to 0.5 M imidazole in 30 ml buffer A at 0 8 ml/min. The purified protein was concentrated by passing it through a centricon column (30 kDa size, AMICOM) and then flash frozen and stored at −70°C in 20% v/v glycerol. The protein concentration was determined by the Lowry reaction (*24*).

SDS-PAGE and Non-Denaturing Gel Electrophoresis Analysis—SDS-PAGE analysis was performed (*25*) and the protein bands visualized with Coomassie Brilliant Blue. Discontinuous alkaline non-denaturing polyacrylamide gel electrophoresis (*26*) was used to analyze the dimer form of biotin synthase. A 10% separating gel (acrylamide:bisacrylamide = 30.1; 236.75 mM Tris, pH 8.5) and 4.8% stacking gel (4.8% acrylamide, 39.5 mM Tris, 0.064 N Na_2HPO_4, pH 6.9) was cast and the samples were dissolved in 1/10 10× sucrose buffer (50% sucrose, 0.1% bromophenol blue). The buffer pH in the upper and lower tanks for the electrophoresis was 8.9 (37.6 mM Tris, 40 mM glycine) and 7.5 (63 mM Tris, 50 mM HC1), respectively. After electrophoresis, the gel was processed as in the case of SDS-PAGE.

Bioassay for Biotin Synthase Activity—Purified biotin synthase can convert DTB to biotin only in the presence of unidentified cellular cofactors. Therefore, biotin synthase activity was assayed with the addition of cellular component(s) from a *bio*-operon deficient strain R901. The quan-

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 30, 2012

tity of biotin synthesized is proportional to the ability to support R901 growth (*27*). To prepare the biotin-free cellular fraction, R901 were cultured in minimal medium containing 20 pg/ml biotin until the OD_{600} reached 0.8 (about 36 h). Cells were washed three times with buffer A at 4°C and resuspended in buffer A (5 ml/g cells), then lysed with a French press. Debris was removed by centrifugation at 150,000 ×*g*, at 4°C for 1 h. The cytosolic fraction was dialyzed four times against 1,000 volumes of buffer A using MWCO 4000 tubing. The dialyzed lysate was then passed through a 30 kDa centricon filter (Amicon) and the molecular mass fraction smaller than 30 kDa was used as a supplement in the biotin synthase assay.

To perform the assay, 7.5 μg of purified biotin synthase was added to 1 ml (final volume) of reaction mixture containing the previously described cellular fraction, 10 μM NADPH, 50 μM DTB, and 25 mM Tris-Cl, pH 7.5. For a standard reaction, the mixture was incubated at 37°C for 30 min. Then the reaction mixture was mixed with R901 (fresh 24 h culture, washed three times with minimal medium and adjusted to 5×10^5 cells/ml) for 30 min at 37°C. This mixture was plated on the minimal plate and the number of colonies was counted after 2–3 days incubation at 37°C. The quantity of biotin synthesized was estimated from the standard curve obtained with known biotin concentrations under the same conditions.

DTB Binding Assay—The ability of BioB to bind the substrate DTB was assayed by a molecular sieve and the amount of bound DTB was measured by the competitive ELISA method (*28*). In brief, the BioB protein (0.2 μM, 7.5 μg per reaction) was mixed with DTB (50 μM) in a total volume of 1 ml and incubated for 30 min at 37°C. It is to be noticed that no cytosolic fraction is needed for this assay The bound DTB was separated from the free DTB by gel filtration (3 ml Sephadex G-25 coarse, Pharmacia). The void volume was collected and incubated at 50°C for 10 min to dissociate DTB from BioB The DTB concentration was then measured as described (*28*).

Measurement of the Fe^{2+} Content in BioB by 1,10-Phenanthroline—The compound 1,10-phenanthroline is highly reactive with Fe^{2+}, forming a red-brown colored complex with a characteristic absorbance at 512 nm (*29*). To measure the Fe^{2+} content in BioB, purified BioB (500 μg) was incubated with 1 mM 1,10-phenanthroline (1 M stock in ethanol) at room temperature for 10 min in a final volume of 1 ml and the mixture was subjected to spectrophotometric analysis at 512 nm. Linear relationships between the concentration of the Fe^{2+}-phenanthroline complex and Fe^{2+} concentration from 1 to 80 μM were observed. To examine whether 1,10-phenanthroline removes Fe^{2+} from BioB, BioB (1 mg in 1 ml 25 mM Tris-HC1, pH 7.5) was reacted with 1 mM 1,10-phenanthroline at room temperature for 10 min. The mixtures were fractionated (or a 3 ml Sephadex G-50 column, Pharmacia) and collected (each 0.5 ml, 20 fractions), and the absorbance of each fraction was analyzed at 512 and 275 nm (for protein concentration determination).

Chemical Modification of BioB—To modify the cysteine residues, purified BioB protein (0.2 nmol) was incubated with 0.5 to 20 mM DTNB (5,5′-dithiobis[2-nitrobenzoic acid], 1 M stock solution) in a volume of 20 μl at 37°C for 0 to 30 min. The reaction was stopped by rapidly diluting the mixture to 1 ml with the bioassay mixture, and the bioassay was conducted to determine the modified enzyme activities. Modifications by NBS (*N*-bromosuccinimide), DEPC (diethylpyrocarbonate), PLP (pyridoxal 5′-phosphate), BNPS-skatole [2-(2-nitrophenylsulfonyl)-3-methyl-3-bromoindolenine-skatole], and phenylglyoxal were performed in a similar fashion (*30*) under the conditions listed in Table I. The control reactions for each chemical modification were performed using the chemical agents at the concentration after rapid dilution. The experimental data (as shown in Table II) represent the results normalized to control conditions.

Site-Directed Mutagenesis of BioB Protein—Oligonucleotide-directed mutagenesis was used to generate the Cys to Ala mutation (*23*). In brief, the *bioB* gene on plasmid

TABLE I **Chemical agents for modification.**

Chemical agent	Target	Reaction conditions	Stock
DTNB	Cys	10 mM, 37°C, 30 min	1 M
NBS	Trp, Tyr, His	10 mM, 37°C, 30 min, in the dark	1 M
BNPS–skatole	Trp	10 mM, 37°C, 30 min	1 M in 25% acetic acid
DEPC	His	10 mM, 4°C, 45 min, using enterokinase treated BioB	1 M in absolute ethanol
PLP	Lys, Arg	37°C, 30 min, in the dark	250 mM
Phenylglyoxal	Arg, Gly	37°C, 30 min, in the dark	250 mM

TABLE II. **Effects of chemical modifications on the catalytic activity, DTB binding, and dimer formation of BioB.**

Chemical agent		BioB	Modified BioB	DTB pre-incubation		NADPH pre-incubation		Dimer formation
				5 μM	50 μM	5 μM	10 μM	
DTNB	Activity %[a]	100	2.40	16.67 ± 1.1	77.96 ± 0.98	11.14 ± 0.78	10.37 ± 0.47	Not affected
	DTB binding[b]	9.4 ± 1.10	1.05 ± 0.11	–	10.15 ± 0.56	–	–	
NBS	Activity %	100	0	0.28 ± 0.15	0.38 ± 0.06	28.70 ± 0.15	48.81 ± 2.31[c]	Degraded[d]
	DTB binding	9.40 ± 1.10	0	–	–	–	8.41 ± 0.26	
DEPC	Activity %	98.15 ± 1.70	9.40	7.3 ± 1.5	7.7 ± 2.3	42.1 ± 2.35	54.5 ± 3.26	No dimer formation[e]
	DTB binding	9.40 ± 1.10	11.15 ± 0.64	–	–	–	–	
BNPS-skatole	Activity %	95.25 ± 3.16	24.91	25.63 ± 2.14	26.18 ± 2.64	27.56 ± 3.14	26.51 ± 2.16	Not affected
	DTB binding	9.40 ± 1.10	2.4 ± 0.16	–	–	–	2.5 ± 0.28	

[a]0.2 μM BioB was used for a typical bioassay, and the activities of BioB in the solvent before and after modification were compared with that of untreated BioB [b]5.26 nmol of nickle-NTA column twice-purified BioB was used for the assay. The amount of bound DTB is expressed in nmol. [c]20 μM NADPH. [d]Preincubation of BioB with NADPH protects BioB from being degraded and the BioB retains the dimer form [e]Preincubation of BioB with NADPH retains the dimer form. [f]The bar "–" represents no measurement.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 30, 2012

pQE30-BioB was subcloned into M13mp18, and used to infect *E. coli* CJ236. Single-stranded DNA containing uridines afterward were purified from CJ236. Eight oligonucleotide primers, each complementary to the ssDNA template but containing 1 or 2 nucleotide substitutions in order to change the codon of each Cys to Ala, were annealed individually to the template, and the second strand was synthesized by T4 DNA polymerase The double stranded DNA was then transformed into JM101 and the mutated DNA was recovered from the phage plaques. The sites of mutation were confirmed by single-strand DNA sequencing (*31*), and the correct clones were subcloned into pQE30 to obtain mutant proteins.

RESULTS

Biological Activities of BioB—In order to study the mechanism of biotin synthase, *E. coli bioB* was first overexpressed using the pQE gene expression system. A 6xHis tag was engineered at the N-terminal of the protein to facilitate purification via a nickel-NTA column. One-step purification achieved approx. 95% protein purity as judged by Coomassie Billiant Blue staining of SDS-PAGE (data not shown). The purified protein displayed an obvious red-brownish color. The properties of the His-tagged protein were very close to the tag-removed protein, including the finding that the His-tagged BioB clone could complement the growth defect of strain R901 as well as wild-type BioB (data not shown). Therefore, we used the purified, tagged protein for all of the following experiments unless stated otherwise. The purified BioB was analyzed by non-denaturing PAGE and two forms, monomer and dimer, in an approx. 5 to 1 ratio, appeared. This result agrees with the previous findings of several groups (*11, 13*). As shown in Fig. 1A, the gel-purified dimer form was readily (within 10 min) transformed to the monomer form and remained in the same 1:5 ratio as in the original purified status during a 90-min incubation. However, the maximal conversion ratio of gel-purified monomer to dimer was only enhanced to approx. 20% by the addition of 10 μM NADPH or the cytosolic fraction (Fig. 1B).

A sensitive bioassay was developed to determine the biotin formation activity of BioB (*27*). The components in our cell-free, semi-defined assay extracts were closest to the assay conditions of Flint (*11*) In this system, the purified BioB (monomer:dimer = 5.1) converted a fixed amount of DTB to biotin, and the reaction approached a maximum rate after 1 min, despite wide variations in the concentration of the DTB (Fig 2). These results strongly suggest that BioB is the limiting factor in the assay and did not turn over properly. The conversion ratio of DTB to biotin per BioB monomer was calculated to be 0.2, and the turnover number of BioB reached 13.89 h^{-1} in our assay system using the first minute reaction in the calculation, consistent with previous findings (*20, 21*).

Stoichiometric Binding of DTB with BioB—The DTB binding assay was performed to determine the relationship of BioB with DTB. The BioB protein was incubated with DTB and then the bound and free DTB were separated *via* a molecular sieve The amount of BioB-bound DTB was assayed using a competitive ELISA method (*28*). Approx. two DTB molecules per BioB monomer were detected (Table II). To explore the BioB turnover problem, the DTB

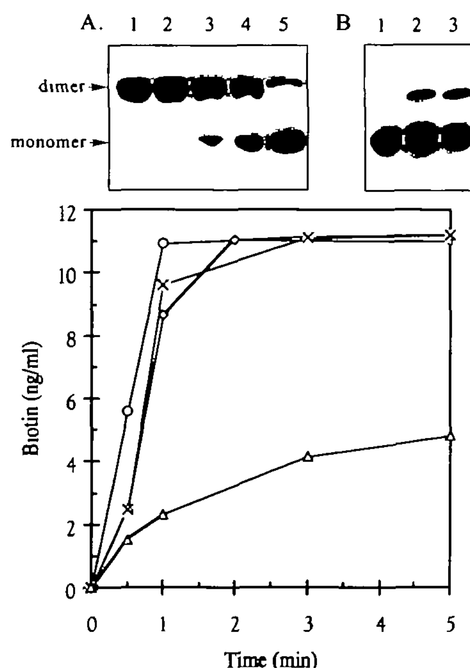


Fig 1 **Non-denaturing PAGE analysis of the BioB protein.** (A) Time dependence of the conversion of the BioB dimer to monomer. In each reaction, 50 μg of gel-purified BioB dimer was incubated in buffer A at 37°C for 0 min (lane 1), 5 min (lane 2), 15 min (lane 3), 30 min (lane 4), and 90 min (lane 5) (B) The dimerization of the BioB monomer in the presence of various cofactors. Fifty micrograms of gel-purified BioB monomer was incubated in buffer A (lane 1), with *E coli* R901 cytosolic fraction (lane 2), and with 10 μM NADPH (lane 3) for 20 min at 37°C The samples were then analyzed *via* non-denaturing PAGE and processed as described in "MATERIALS AND METHODS." Arrows indicate the respective monomer and dimer positions

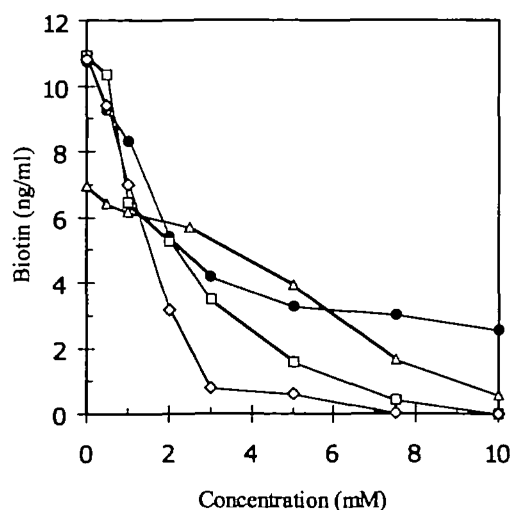


Fig 2. **Dependence of substrate (DTB) concentration on the formation of biotin.** Bioassays were performed as described in "MATERIALS AND METHODS" using 0.2 μM BioB protein, and the reaction was stopped at the indicated times. In each reaction, 0.2 μM BioB and 0 4 μM (Δ), 2 μM (×), 10 μM (□), or 50 μM (◇) of DTB were used.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 30, 2012

binding ability of BioB was also examined after the bioassay The BioB was re-purified after the bioassay via the Ni-NTA column and examined for DTB binding ability. No DTB was found to bind to BioB at the end of the bioassay (data not shown). In a control group, BioB purified twice via the nickel column showed only a slight decline in its DTB binding capacity. Therefore, the poor turnover rate of BioB could be due to the loss of its DTB binding capacity after reaction. It is conceivable that the substrate-binding site of BioB might be altered significantly after biotin formation. A possible structural change in the DTB binding pocket and its relationship to the iron-sulfur cluster of BioB will be proposed in the "DISCUSSION."

Amino Acid Residues Involved in the Biological Activities of BioB—The amino acid residues that play critical roles in BioB function can be identified through chemical modifications. As listed in Table I, a panel of chemical agents was chosen to modify different groups of amino acids. Each chemically modified BioB was then examined for its ability to catalyze biotin synthesis. Four chemical agents, including DTNB, DEPC, NBS, and BNPS-skatole, had significant impacts on the biotin formation activity of BioB BioB treated with this group of agents displayed a time-dependent and concentration-dependent loss of activity (Fig. 3). However, PLP and phenylglyoxal had no apparent effect (data not shown). This implies that cysteine, histidine, tryptophan, and possibly tyrosine are involved in the enzymatic activity of BioB, and that arginine, lysine and glycine are apparently not important for BioB function.

The DTB binding assay and non-denaturing PAGE were also performed to investigate the functional and structural changes in BioB after chemical modification. Upon treating BioB with DTNB, an agent that reacts specifically with cysteine, the BioB retained its dimer form but lost its DTB binding capacity (Table II). Interestingly, incubating BioB with DTB prior to DTNB treatment protected BioB from the loss of biotin formation activity and DTB binding capacity (Table II). Factors such as NADPH and the *E. coli* cytosolic fraction did not alter the effect of DTNB (Table II and data not shown). These results suggest that certain cysteine residue(s) are very close to the DTB binding pocket of BioB and possibly participate in DTB binding; however, cysteine residues are probably not involved in dimer formation.

Similar experimental strategies were employed to analyze the effects of NBS, DEPC and BNPS-skatole. From the results summarized in Table II, the following conclusions can be drawn. First, amino acid residues (Trp, Tyr, and/or His) modified by NBS are involved in both dimer formation and DTB binding. Second, some histidine residues are involved in BioB dimer formation, and NADPH might be able to stabilize the dimer conformation The data also reveal that the loss of the dimer form is not the only factor responsible for the loss of biotin formation activity of BioB, since only 50% activity was detected in the NADPH protection experiment in which the dimer form was identical to that of the untreated protein. Finally, tryptophan residue(s) might be not involved in dimer formation but are associated with DTB binding. Trp could be accessed by BNPS-skatole even when the DTB binding site was occupied. Modifying this tryptophan residue before conducting the

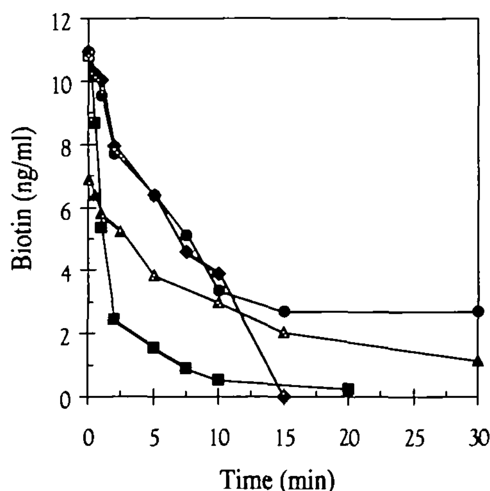


Fig 3. **Effects of chemical modification on BioB activity.** (A) Concentration dependence of chemical modification. For each reaction, 7.5 μg of BioB was incubated with DEPC (△), BNPS-skatole (○), DTNB (□), or NBS (◇) for 30 min at the indicated concentration. (B) Time dependence of chemical modification The above chemical agents (10 mM) were used for the indicated times for the modification reaction. The bioassays were then performed as described in "MATERIALS AND METHODS."

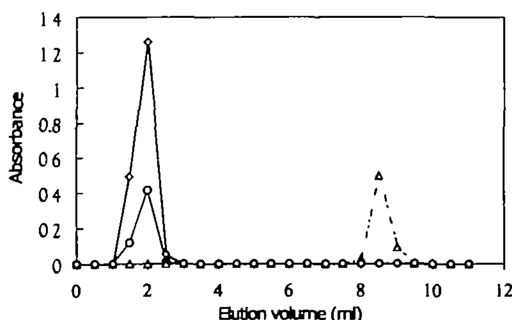


Fig 4 **Association of the Fe^{3+} chelator (1,10-phenanthroline) with BioB.** One milligram of BioB was incubated with 1 mM 1,10-phenanthroline in Tris buffer (25 mM, pH 7 5) at room temperature for 10 min, and then the mixture was fractionated by gel filtration (G-25 coarse column) Fractions were measured at 512 nm (○) and 275 nm (◇) For the control, $FeCl_2$ complexed with 1,10-phenanthroline (△, 512 nm)

TABLE III. **The effect of the iron chelator 1,10-phenanthroline on BioB.**

	BioB	Modified BioB	DTB preincubation		NADPH preincubation		Fe^{3+} compensation	
			50 μM	100 μM	10 μM	40 μM	50 μM	500 μM
Activity %	98 6	0.12	55 03	70 04	1 01	1 21	20.7	75.4
DTB binding[a] (nmol)	9.40 ± 1.10	0	8 55 ± 0 56	–	–	–	7 65±1.85	–
OD_{512} %[b]	0	100	47.5	30 7	96 9	100 3	–	–

[a]5.26 nmol of BioB purified twice on a Ni-NTA column was used for the assay. [b]The absorbance measured at 512 nm was normalized to the value obtained for modified BioB (treated with 10 μM 1,10-phenanthroline). [c]The bar "-" represents no measurement.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 30, 2012

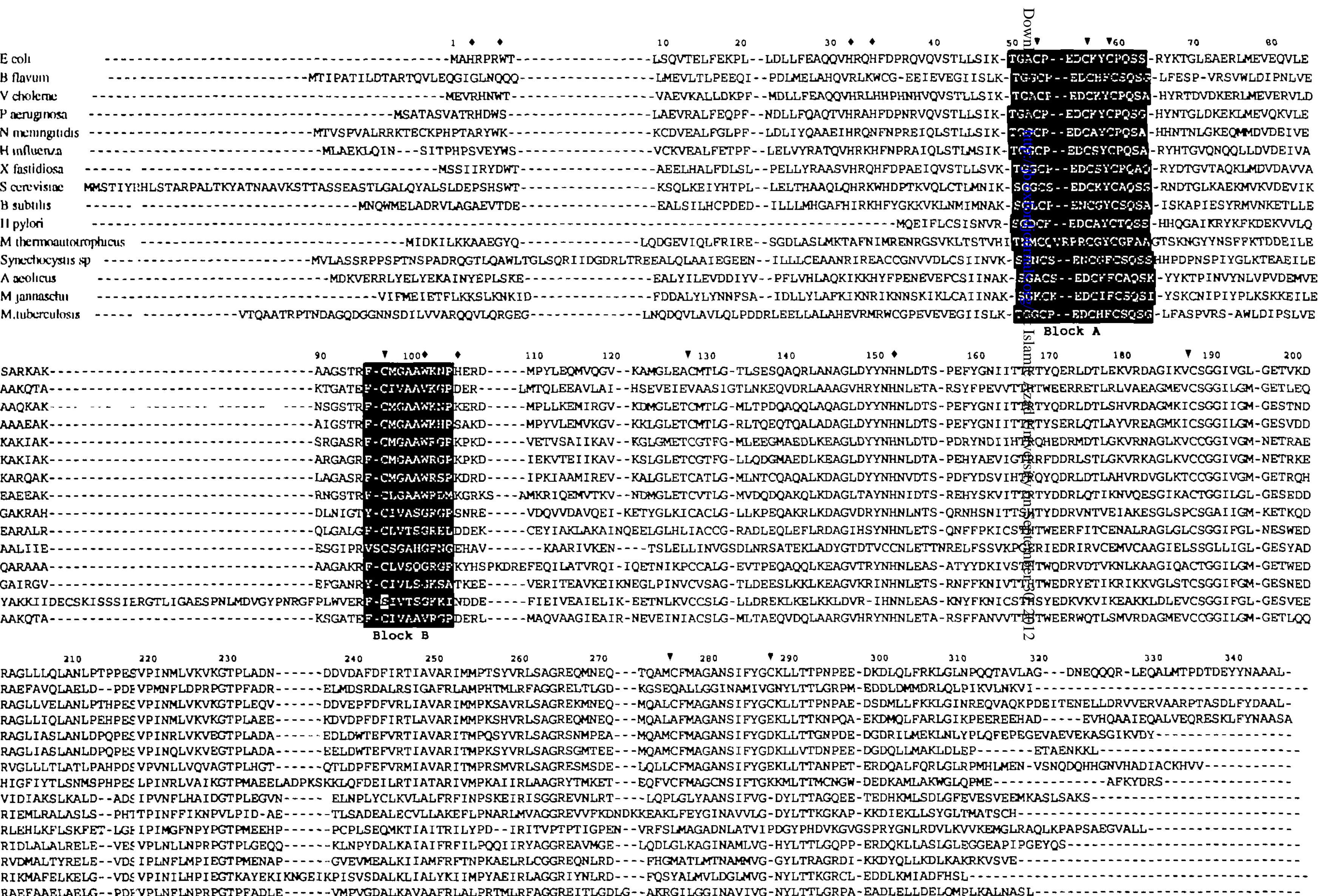


Fig. 5. **Alignment of the amino acid sequences of currently known biotin synthases.** The alignment was performed using the Clustal W program (from the proteomic tools, ExPASy Molecular Biology Server, <http://www.expasy.org>) Block A and Block B represent the two sequence regions containing the consensus cysteine residues. The black arrows indicate the cysteine residues that were mutated in this study. The possibly modified Trp and His are labeled (◆) The amino acid residues are numbered according to the *E. coli* BioB sequence.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 30, 2012

TABLE IV. **Properties of BioB cysteine mutants.**

	Wild-type	C53A	C57A	C60A	C97A	C128A	C188A	C276A	C288A
Activity %	100	0	0	0	0	20 0	44 0	98.6	96 8
DTB binding (nmol)[a]	0 56	0 39	0 41	0 37	0.42	0.168	0 214	0.54	0 57
Fe^{2+} *content* %[b]	*100*	*41 12*	*54 51*	*45 26*	*55 39*	*98 23*	*96 15*	*96 78*	*101 64*

[a]0 27 nmol of BioB (calculated as monomer) was used for DTB binding assay. [b]500 μg BioB and 10 mM 1,10-phenanthroline were used for each measurement

TABLE V **Protection of the iron-sulfur cluster of BioB by DTB.**

	BioB	C53A	C57A	C60A	C97A	C128A	C188A	C276A	C288A
OD_{512}*[a]	0 171	0 253	0 321	0 282	0 330	0 439	0 493	0.167	0 169
OD_{512}	0.684	0 273	0 342	0 307	0.376	0 676	0 658	0.668	0 669
Protection[b]	75	7 32	6 14	8 14	12 23	35 06	25.08	75	74.74

[a]OD_{512} of BioB (500 μg) preincubated with 100 μM DTB then with 10 μM 1,10-phenanthroline. [b]Protection percentage was calculated as $(1-OD_{512}^{*}/OD_{512}) \times 100\%$

DTB binding assay would block the entrance of DTB.

Iron-Sulfur Cluster Is Involved in DTB Binding—From sequence comparison, UV-visible spectroscopy and EPR studies, BioB has been demonstrated to be an iron-sulfur cluster protein (*11–13*). We used a strong Fe^{2+} chelator, 1,10-phenanthroline, to investigate the relationship of the iron-sulfur cluster with BioB function. Coordination of Fe^{2+} with 1,10-phenanthroline produces a characteristic red-brown color, and the concentration of the complex is proportional to the absorbance measured at 512 nm. The addition of 1,10-phenanthnroline at a very low concentration (10 μM) almost abolishes the biotin formation activity of BioB within 30 seconds (Table III). The BioB activity can be reconstituted to approx. 75% by supplementation with 500 μM of $FeCl_2$ after chelation. BioB loses its DTB binding capability after treatment with 1,10-phenanthroline; however, this effect can be compensated for by pre-incubation of BioB with DTB before treatment (Table III). This may indicate that the iron is removed by 1,10-phenanthroline, and, therefore, the loss of the iron-sulfur cluster is responsible for the loss of activity; aternatively, 1,10-phenanthroline may actually block DTB from occupying the substrate-binding site.

The above two hypotheses can be clarified using a molecular sieve method. After reacting BioB with 1,10-phenanthroline, the reaction mixture was passed through a G-25 Sephadex column. If 1,10-phenanthroline chelates the iron out of the protein and forms a small iron-phenanthroline complex, it should be trapped in the column matrix and eluted later. However, as shown in Fig. 4, the results clearly indicate that 1,10-phenanthroline stays in the protein complex, demonstrating that the iron-sulfur cluster of BioB participates in DTB binding. The affinity of the iron(s) for the sulfur atoms in the protein must be higher than its affinity for 1,10-phenanthroline. Probably the K_d is less than 10^{-15}, as suggested (*33*), and a 1,10-phenanthroline-iron-sulfur complex may be formed.

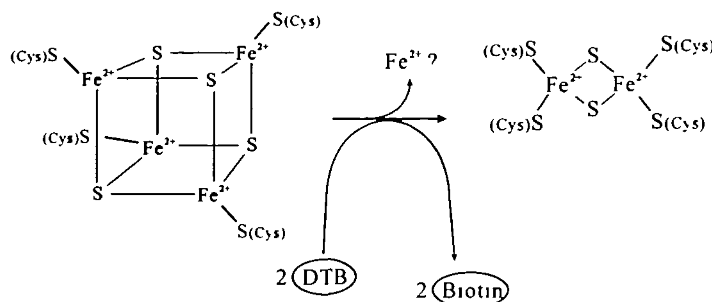
The Role of Cysteine Residues in BioB—There are eight cysteine residues (at positions 53, 57, 60, 97, 128, 188, 276, and 288) in the BioB sequence of *E. coli* (Fig. 5). In an attempt to assign the function of each cysteine, we employed site-directed mutagenesis to replace each cysteine with alanine. Eight mutant proteins were generated and all could be expressed to a similar extent. For each mutant protein, the biotin formation activity, DTB binding ability, and Fe^{2+} content were examined. Among these mutants, C53A, C57A, C60A plus C97A completely lost their biotin formation activity (Table IV). Cysteine residues at the positions 53, 57, and 60 have been assigned as the ligands for the iron-sulfur cluster (*15, 34*), here we propose that the cysteine at position 97 is the fourth ligand The data obtained with the C97A mutant displayed all the same characteristics as C53A, C57A, and C60A in retaining approx. 70% of DTB binding capacity, and 50% of Fe^{2+} content as estimated by 1,10-phenanthroline chelation (Table IV) and the loss of the brown-colored appearance These findings suggest that when one iron ligand is lost, the microstructure of the DTB binding site is somehow maintained. The decrease in the iron content of these mutants might reflect the easier exchange of iron(s) with the environment upon the loss of one ligand. This supports our finding that the affinity of iron for the ligands is so high, that when one ligand suffers a mutation, the remaining ligands are sufficient to hold the iron-sulfur cluster and, therefore, maintain the local structure. Although the four mutants retain 70% DTB binding capacity, the DTB in these mutants fails to compete with 1,10-phenanthroline (Table V). Again, this result supports evidence that the iron-sulfur cluster and the ligands in the cluster are the key components for DTB binding.

The other two cysteine mutants, C128A and C188A, show decreased biotin converting activity and DTB binding ability (Table IV), and the percentages of the activity loss are roughly proportional to the decrease in DTB binding. The Fe^{2+} contents in this mutants are close to that of the wild type protein, and incubation with DTB prior 1,10-phenanthroline treatment provides only moderate protection against Fe^{2+} loss (Table V). Therefore, we conclude that Cys128 and Cys188 are associated with DTB binding, but probably do not participate in biotin conversion. This could also explain the results of the C188S mutant described previously (*15*). The last two cysteine mutants, C276A and C288A, were indistinguishable in every way from the wild-type (Tables IV and V), indicating that these cysteine residues play no significant role in the enzymatic mechanism of biotin synthase.

DISCUSSION

In this work, we employed chemical modifications and site-

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 30, 2012



Scheme 2.

directed mutagenesis to decipher the structure-and-function role of amino acid residues in BioB. Although the chemical agents may not be very specific for the target amino acid residues, the site-directed mutation data reconfirmed the experimental results unequivocally Based on the results, cysteine residues at positions 53, 57, 60, and 97 were assigned as the ligands for the iron-sulfur cluster. Cysteine residues at positions 53, 57, and 60 had been assigned in the context of CxxxCxxC (Fig. 5, block A), a conserved motif serving as three ligands for an iron-sulfur cluster (*11*). Now we have identified Cys 97 as the fourth ligand to form the stable coordination with the iron atoms in a typical iron-sulfur cluster, both [2Fe-2S] or [4Fe-4S]. Although Cys97 is not present in the BioB of *M. jannaschii* (*35*), it is conserved among all other known BioB sequences, and the amino acid sequence surrounding Cys97 also displays a consensus nature (Fig. 5, block B). It is possible that in *M. jannaschii*, the serine residue at the position corresponding to Cys97 acts as one of the iron-sulfur cluster ligands, as in the case of anaerobic ribonucleotide reductase, which is an iron-sulfur cluster containing enzyme and uses a hydroxyl group as one of the iron ligands in iron-sulfur cluster (*36*).

The observation that BioB loses its DTB binding capacity after catalyzing the formation of biotin provides some insight into the current understanding of the BioB mechanism. Two forms of iron-sulfur clusters, a reduced $[4Fe\text{-}4S]^{2+}$ form and an oxidized $[2Fe\text{-}2S]^{2+}$ form have been suggested for BioB (*12, 13*). Evidence also revealed that BioB accommodates the conversion of $[2Fe\text{-}2S]^{2+}$ to $[4Fe\text{-}4S]^{2+}$ when reduced with dithionite (*ibid*). Our finding that two DTB molecules bind to each native BioB monomer suggests that the native BioB monomer is in its reduced $[4Fe\text{-}4S]^{2+}$ form, and may create an active site with more space (model shown in Scheme 2). Upon converting DTB to biotin, the two sulfur atoms in the $[4Fe\text{-}4S]^{2+}$ cluster probably are incorporated into each of the newly formed biotin molecules, and the active site is oxidized simultaneously to $[2Fe\text{-}2S]^{2+}$. In this conformation, the space in the active site is significantly less and can no longer accommodate DTB binding. This model fits the current hypothesis that postulates that the sulfur atom of biotin originates from the iron-sulfur cluster in BioB (*21*). A more accurate measurement of the change in iron content of BioB would provide further support for this hypothesis. Other forms of the iron-sulfur cluster, such as $[3Fe\text{-}4S]^0$ and $[4Fe\text{-}4S]^+$, may also have existed in our BioB preparations, and these would be able to bind DTB, but be unable to convert DTB to biotin. This could also explain the fewer than two biotin molecules formed per BioB monomer in the bioassay. The ultimate answer relies on detailed chemical analysis, including crystallographic structures, to examine the BioB conformation change before and after biotin formation.

Protein factors such as flavodoxin, ferredoxin $NADP^+$ reductase, MioC, and NifS have been reported to be required to regenerate the reduced $[4Fe\text{-}4S]^{2+}$ reaction center of BioB *in vitro*, but the biotin synthase still does not behave as an enzyme and turn over efficiently. The number of biotin molecules formed per BioB monomer, as calculated from different *in vitro* assays, ranges from 0.2 to 1 5 (*11, 16, 19*). An unidentified factor could exist that allows BioB to proceed with biotin formation *in vitro*. A fast turnover bioassay will be necessary to understand the components involved in the biotin synthase holoenzyme and how biotin synthase turns over *in vivo*.

In the present study, similar chemical modification and site-directed mutagenesis experiments were also performed to examine the functional role of histidine and tryptophan residues. The results indicate that some His residue(s) are important in holding the dimer form of BioB, and that Cys128 and Cys188 along with certain Trp residues are possibly engaged in DTB binding Individual His residues (at positions 3, 31, 34, 106, and 152) and Trp residues (at positions 7 and 102) were mutated and the preliminary results reveal that the dimer conformation of BioB was retained and the enzyme was able to complement the growth defect in R901, a biotin operon deletion strain. Further biochemical properties of this set of mutants are currently under investigation

We thank Miss Yu-Ron Hsu for technical assistance

REFERENCES

1 Knowles, J R. (1989) The mechanism of biotin-dependent enzymes. *Annu. Rev Biochem* **58**, 195–221
2 Cronan, J.E (1989) The *Escherichia coli* biotin operon transcriptional repression by an essential protein modification enzyme *Cell* **58**, 427–429
3. Gloekler, R., Ledoux, C., Bernard, S , Zinsius, M , Villeval, D., Kisou, T., Kamogawa, K., and Lemoine, Y (1990) Cloning and characterization of *Bacillus sphericus* genes controlling the bioconversion of pimelate into dethiobiotin *Gene* **87**, 63–70
4 Shiuan, D and Campbell, A. (1988) Transcriptional regulation and gene arrangement of *Escherichia coli*, *Citrobacter freundii* and *Salmonella typhimurium* biotin operons. *Gene* **67**, 203–211
5 5 Wu, C H , Chen, H.Y., and Shiuan, D (1996) Isolation and characterization of *Erwinia herbicola bio* operon and the sequence of *bioA* and *bioB* genes *Gene* **174**, 251–258
6. Ohsawa, I., Speck, D., Kisou, T., Hayakawa, K., Zinsius, M , Gloeckler, R., Lemoine, Y , and Kamogawa, K. (1989) Cloning of the biotin synthetase gene from *Bacillus sphericus* and expres-

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 30, 2012

sion in *Escherichia coli* *Gene* **80**, 39–48
7 Ketner, G and Campbell, A. (1975) Operator and promoter mutations affecting divergent transcription in the biotin gene cluster of *Escherichia coli* *J Mol Biol* **96**, 13–27
8 Guha, A., Saturen, Y., and Szybalski, W (1971) Divergent orientation of transcription from biotin locus of *Escherichia coli* *J. Mol Biol* **56**, 53–62
9. Otsuka, A.J and Abelson, J (1978) The regulatory region of the biotin operon in *Escherichia coli*. *Nature* **276**, 689–694
10. Lin, K.C and Shiuan, D (1993) DNaseI footprinting studies of *Escherichia coli* biotin repressor-operator interactions. *J Biochem* **114**, 670–676
11. Sanyal, I, Cohen, G, and Flint, D H. (1994) Biotin synthase purification, characterization as a [2Fe-2S] cluster protein, and *in vitro* activity of the *Escherichia coli bioB* gene product *Biochemistry* **33**, 3625–3631
12 Duin, E C, Lafferty, M E, Crouse, B R, Allen, R M, Sanyal, I., Flint, D H, and Johnson, M K. (1997) [2Fe-2S] to [4Fe-4S] cluster conversion in *Escherichia coli* biotin synthase. *Biochemistry* **36**, 11811–11820
13 Ugulava, N.B., Gibney, B.R., and Jarrett, J T. (2000) Iron-sulfur cluster interconversions in biotin synthase dissociation and reassociation of iron during conversion of [2Fe-2S] to [4Fe-4S] clusters *Biochemistry* **39**, 5206–5214
14 Ollagnier-de Choudens, S, Sanakis, Y, Hewitson, K.S, Roach, P, Baldwin, J E, Munck, E, and Fontecave, M. (2000) Iron-sulfur center of biotin synthase and lipoate synthase. *Biochemistry* **39**, 4165–4173
15 McIver, L, Boxter, R L, and Campopiano, D J. (2000) Identification of the [Fe-S] cluster-binding residues of *Escherichia coli* biotin synthase *J Biol Chem.* **275**, 13888–13894
16 Sanyal, I, Gibson, K.J, and Flint, D H (1996) *Escherichia coli* biotin synthase: an investigation into the factors required for its activity and its sulfur donor *Arch Biochem Biophys.* **326**, 48–56
17 Birch, O M, Fuhrmann, M, and Shaw, N M (1995) Biotin synthase from *Escherichia coli*, an investigation of the low molecular weight and protein components required for activity in vitro *J. Biol Chem* **270**, 19158–19165
18 Ifuku, O, Kishimoto, J., Haze, S I, Yanagi, M, and Fukushima, S (1992) Conversion of dethiobiotin to biotin in cell-free extracts of *Escherichia coli* *Biosci. Biotechnol Biochem.* **56**, 1780–1785
19 Shaw, N M, Birch, O M, Tinschery, A., Venetz, V, Dierich, R, and Savoy, L.A (1998) Biotin synthase from *Escherichia coli* isolation of an enzyme-generated intermediate and stoichiometry of *S*-adenosylmethionine use. *Biochem. J* **330**, 1079–1085
20. Gibson, K.J., Pelletier, D.A, and Turner, Sr I M (1999) Transfer of sulfur to biotin from biotin synthase (BioB protein) Biochem *Biophys. Res. Commun.* **254**, 632–635
21. Tse Sum Bui, B, Florentin, D, Fournier, F, Ploux, O, Mejean, A., and Marquet, A. (1998) Biotin synthase mechanism on the origin of sulphur. *FEBS Lett* **440**, 226–230
22. Tse Sum Bui, B, Escalettes, F, Chottard, G, Florentin, D, and Marquet, A. (2000) Enzyme-mediated sulfide production from the reconstitution of [2Fe-2S] clusters into apo-biotin synthase of *Escherichia coli* Sulfide transfer from cysteine to biotin *Eur J Biochem* **267**, 2688–2694
23 Kunkel, T.A. (1987) Rapid and efficient mutagenesis without phenotypic selection *Methods Enzymol.* **154**, 367–382
24 Lowry, O H, Rosebrough, N J., Lewis Farr, A., and Randall, R.J (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265–275
25. Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4 *Nature* **227**, 680–685
26 Paech, C., Christianson, T, and Maurer, K.H (1993) Zymogram of proteases made with developed film from nondenaturing polyacrylamide gels after electrophoresis. *Anal Biochem* **208**, 249–254
27 Hwang, S Y, Su, V, Farh, L, and Shiuan, D (1999) Bioassay of biotin concentration with *Escherichia coli bio* deletion mutant *J Biochem Biophys. Methods* **39**, 111–114
28 Shiuan, D, Wu, C H, Chang, Y S., and Chang, R J (1997) Competitive enzyme-linked immunosorbent assay for biotin *Methods Enzymol* **279**, 321–326
29. Lovenberg, W, Buchanano, B.B, and Rabinowrz, J C (1963) Studies on the chemical nature of clostridial ferredoxin *J Biol Chem* **238**, 3899–3913
30 Means, G E and Feeney, R E. (1990) Chemical modification of proteins history and applications. *Bioconjug Chem* **1**, 2–12
31 Sanger, F, Nicklen, S, and Coulson, A.R (1977) DNA sequencing with the chain-termination inhibitors. *Proc. Natl Acad Sci USA* **74**, 5463–5467
32 Birch, O M, Hewitson, K.S, Fuhrmann, M, Burgdorf, K., Baldwin, J E, Roach, P L, and Shaw, N (2000) MioC is an FMN-binding protein that is essential for *Escherichia coli* biotin activity in vitro *J Biol Chem* **275**, 32277–32280
33 Auld, D S (1988) Use of chelating agents to inhibit enzymes. *Methods Enzymol* **158**, 110–114
34 Hewitson, K.S., Baldwin, J E, Shaw, N M, and Roach, P L. (2000) Mutagenesis of the proposed iron-sulfur cluster binding ligands in *Escherichia coli* biotin synthase. *FEBS Lett* **466**, 372–376
35 Bult, C J, White, O, Olsen, G J, Zhou, L, Fleischmann, R D, Sutton, G G, Blake, J.A., FitzGerald, L M, Clayton, R.A., Gocayne, J D, Kerlavage, A R, Dougherty, B.A., Tomb, J, Adams, M D., Reich, C I, Overbeek, R, Kirkness, E F, Weinstock, K.G., Merrick, J M, Glodek, A., Scott, J D, Geoghagen, N S, Weidman, F J, Fuhrmann, J L, Nguyen, D.T, Utterback, T, Kelley, J M, Peterson, J D., Sadow, P.W, Hanna, M C, Cotton, M D., Hurst, M.A., Roberts, K.M., Kaine, B B, Borodovsky, M, Klenk, H P, Fraser, C M, Smith, H.O, Woese, C R, and Venter, J.C. (1996) Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii* *Science* **273**, 1058–1073
36 Tamarit, J, Gerez, C, Meier, C, Mulliez, E., Trautwein, A., and Fontecave, M (2000) The activating component of the anerobic ribonucleotide reductase from *Escherichia coli* An iron-sulfur center with only three cysteines. *J Biol. Chem* **275**, 15669–15675

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 30, 2012