

Mutagenesis of the proposed iron-sulfur cluster binding ligands in *Escherichia coli* biotin synthase

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Received 11 October 1999; received in revised form 15 December 1999

Edited by Richard Cogdell

Abstract Biotin synthase (BioB) is a member of a family of enzymes that includes anaerobic ribonucleotide reductase and pyruvate formate lyase activating enzyme. These enzymes all use *S*-adenosylmethionine during turnover and contain three highly conserved cysteine residues that may act as ligands to an iron-sulfur cluster required for activity. Three mutant enzymes of BioB have been made, each with one cysteine residue (C53, 57, 60) mutated to alanine. All three mutant enzymes were inactive, but they still exhibited the characteristic UV-visible spectrum of a [2Fe-2S]²⁺ cluster similar to that of the wild-type enzyme.

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Key words: Biotin synthase; Iron-sulfur cluster; Mutagenesis of iron-sulfur cluster ligand

1. Introduction

Biotin synthase (BioB) participates in the final step of the biotin biosynthetic pathway, where a sulfur atom is inserted between the unactivated methyl and C6 methylene group of dethiobiotin (Fig. 1). Catalytic activity in vitro has not been observed for this reaction [1–3].

BioB is a homodimeric protein with a subunit molecular mass of 38.7 kDa and contains an iron-sulfur (Fe-S) cluster that has been the focus of recent investigations. Using spectroscopic techniques [4,5], the near stoichiometric conversion of two [2Fe-2S]²⁺ clusters (one per monomer of BioB) to form a single [4Fe-4S]²⁺ cluster has been observed. As no exogenous iron or sulfide had been added, it was postulated that the [4Fe-4S]²⁺ cluster formed a bridge between the two monomers of BioB and was the active form [4]. A similar essential Fe-S cluster has also been proposed for pyruvate formate lyase activating enzyme (pfl AE) [6] and anaerobic ribonucleotide reductase activating enzyme (aRR AE) [7]. However, aRR AE has subsequently been shown to assemble one [4Fe-4S] cluster per monomer (under strictly anaerobic conditions) and this is thought to be the active species under assay conditions [8]. Külzer et al. [9] have recently demonstrated that active pfl AE

can also be reconstituted under anaerobic conditions, as a monomeric (28 kDa) holoenzyme with one [4Fe-4S] cluster per monomer. There is a high degree of sequence similarity between aRR AE, pfl AE and BioB, in particular a highly conserved cysteine triad (C-X₃-C-X₂-C) that seems likely to be a ligand to the Fe-S cluster [4] (Fig. 2).

All these enzymes share a common co-factor requirement for *S*-adenosylmethionine (SAM) and for a reducing system consisting of NADPH, ferredoxin (flavodoxin) NADP⁺ reductase and flavodoxin [3,6,10]. For all three enzymes, the proposed role of the [4Fe-4S]²⁺ cluster is the one electron reductive cleavage of SAM to methionine and a 5'-deoxyadenosyl radical. This radical may react further, either to form a carbon-centred protein radical as proposed for the mechanism of pfl AE and aRR AE [5], or directly with the substrate, as suggested for BioB [11].

The key functional difference between BioB and pfl/aRR AE is that the Fe-S cluster of BioB may also serve as the immediate sulfur atom donor during biotin formation [12]. The Fe-S cluster of BioB is therefore bifunctional.

In view of the importance of the Fe-S cluster of BioB, three mutant enzymes were made. Each had one of the cysteine residues of the cysteine triad mutated to alanine and the effect on the spectroscopic properties and activity of BioB was observed.

2. Materials and methods

2.1. Materials

DNA manipulations were carried out by standard protocols [13]. All chemicals were of reagent grade and obtained from Sigma-Aldrich Chemical Co. unless otherwise stated. Restriction enzymes and molecular biology reagents were purchased from Promega. Oligonucleotides were synthesised by Mrs V. Cooper, Dyson Perrins Laboratory, Oxford University, on an Applied Biosystems DNA Synthesiser (model 380B or 394). Protein concentrations were determined by the method of Bradford [14]. [¹⁴C]Dethiobiotin (100 µCi/mmol) was synthesised enzymatically [15].

2.2. Site-directed mutagenesis

The *bioB* gene was amplified by PCR and ligated into the p-GEM T vector. We have found that the BioB gene product contains two mutations which may have been introduced during the PCR amplification. At position 12, a valine (GTC) is mutated to an alanine (GCC) and at position 335, a proline (CCG) is mutated to a leucine (CTG). The enzyme retained the same activity as literature reports. Single-stranded DNA was produced by standard techniques using helper phage R408. Site-directed mutagenesis was performed using the U.S.E. Mutagenesis kit (Amersham Pharmacia Biotech). In this technique, one primer, the target primer, introduces the desired mutation into the plasmid DNA. The other, the selection primer, mutates a unique non-essential restriction site which serves as a basis for selec-

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Abbreviations: SAM, *S*-adenosylmethionine; Fe-S, iron-sulfur cluster; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; pfl AE, pyruvate formate lyase activating enzyme; aRR AE, anaerobic ribonucleotide reductase activating enzyme

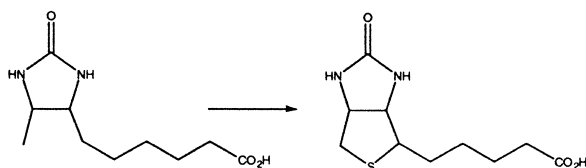


Fig. 1. The conversion of desethiobiotin to biotin.

tion of mutated plasmids. Each of the cysteines from the sequence, ACPEDCKYCP, was mutated individually to give three mutant enzymes, each with one cysteine to alanine mutation: target primer, first cysteine, C53A: 5'-GCA ATC TTC CGG AGC AGC TCC GG-3'. Target primer, second cysteine, C57A: 5'-CGG GCA GTA TTT AGC ATC TTC CGG-3'. Target primer, third cysteine, C60A: 5'-GCG CGA GCT TTG CGG AGC GTA TTT GC-3' (mutations are underlined).

Positive mutants, as shown by restriction digest, were confirmed by DNA sequencing.

2.3. Expression and purification

For expression studies, the wild-type and mutant *bioB* genes were subcloned into the pET 24d(+) vector (Novagen) after digestion of the p-GEM T vector with *Nco*I/*Bam*HI restriction enzymes.

Plasmids were transformed into BL21(DE3) and grown at 37°C in 2TY media containing 30 µg/ml kanamycin. When the OD₆₀₀ reached 1.0, the temperature was reduced to 30°C, the cells were induced with 0.5 mM isopropyl-β-D-thiogalactoside and harvested after 2.5 h by centrifugation in a JA-14 rotor at 12000 rpm for 30 min at 4°C.

BioB/BL21(DE3) cells were thawed and resuspended in 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol (DTT). Chicken egg white lysozyme was added (0.5 mg/ml), the mixture stirred for 15 min and Triton X-100 added to 1% (w/v). After a further 15 min stirring, the cell debris was removed by centrifugation in a JA-14 rotor at 12000 rpm for 30 min at 4°C. Ammonium sulfate was added to 35% saturation and the centrifugation repeated. The resulting pellet was resuspended in buffer A (50 mM Tris-HCl, pH 7.5) and an equal volume of buffer B added (50 mM Tris-HCl, pH 7.5, 0.5 M (NH₄)₂SO₄). This was loaded onto a Source Phe column (Pharmacia, 150 ml) that had been equilibrated with buffer B, and protein was eluted with a 0–100% gradient of buffer A. The purest samples, as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis, were combined, concentrated to 50 mg/ml and chromatographed on a Superdex-S200 column (Pharmacia, 700 ml) that had been equilibrated with buffer A. The purest fractions, >95% pure by SDS–PAGE analysis, were combined and concentrated to 50 mg/ml and stored at –80°C. The mutant proteins were purified in a similar way.

2.4. Enzyme assays

Assays of either wild-type or mutant BioB were performed by the method of Birch et al. [3], including co-factor requirements, incubation time and isolation of biotin. Lysate assays included 2.25 mg of BioB containing lysate supplemented with a further 2.25 mg of BL21(DE3) cell free extract. The wild-type enzyme produced 2.6 nmol of biotin (0.44 mol of biotin per mol of BioB over a 1 h assay, assuming a 10% expression level).

Purified BioB, either wild-type or mutant, was assayed using 0.225 mg of purified bioB and supplemented with BL21(DE3) cell free lysate to bring the total protein content to 4.5 mg. The wild-type enzyme produced 3.18 nmol of biotin (0.54 mol of biotin per mol of BioB over a 1 h assay).

2.5. Iron quantification

Iron content was measured using both inductively coupled plasma optical emission spectroscopy on a Jobin-Yvon 36 spectrometer at 259.4 nm and the method of Fish [16].

2.6. Spectroscopy

UV-visible absorbance spectra were measured over the scan range 200–800 nm using a Shimadzu UV-1601 spectrophotometer at protein concentrations of 3.0 mg/ml in 50 mM Tris-HCl buffer, pH 7.5. CD

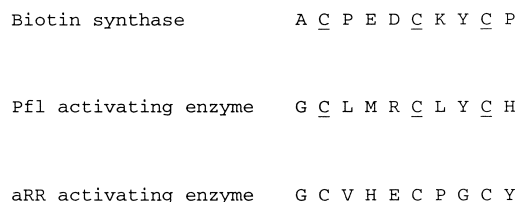


Fig. 2. The conserved cysteine triad motif from BioB, pfl AE and aRR AE.

spectra were recorded using a Jasco 720 spectropolarimeter at 20°C in a 1 mm path length cell over the range 190–250 nm at a protein concentration of 0.2 mg/ml in 10 mM Tris-HCl buffer, pH 7.5.

3. Results

3.1. Expression and purification

BioB was overexpressed in *Escherichia coli* BL21(DE3) as approximately 10% of the total soluble protein, as judged by SDS–PAGE analysis, and was the most abundant band. Growth of cells containing the mutant proteins C53A, C57A and C60A gave comparable levels of expression (Fig. 3). The mutant proteins exhibited similar chromatographic properties as the wild-type enzyme during purification, having similar elution profiles from the Source Phe and Superdex-S200 columns. Purification of the wild-type and mutant enzymes gave samples of >95% purity, as judged by SDS–PAGE analysis, that were suitable both for assays and for spectroscopy. BioB is reddish brown due to the presence of the Fe-S cluster. The mutant proteins were of a similar, but less intense colour than the wild-type, suggesting that some form of Fe-S cluster remained. This was further investigated by spectroscopic techniques.

3.2. Spectroscopy and iron quantification

The UV-visible absorption spectrum of the wild-type BioB was consistent with the presence of a [2Fe-2S]²⁺ cluster, with absorbance maxima at 330 and 420 nm, as previously observed [4]. The mutant enzymes exhibited spectra with an identical form, but with a decrease in the extinction coefficients over the wavelengths scanned (Fig. 4). Iron quantification by either method indicated that for the wild-type, and the mutant enzymes, the iron content was lower than if each monomer bound two atoms of iron, as expected. However, for the mutant enzymes C53A and C57A, the iron content

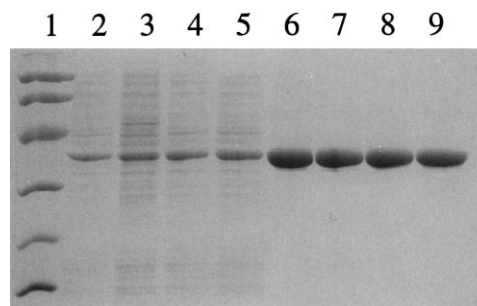


Fig. 3. Coomassie blue-stained 12.5% SDS–PAGE gel of wild-type and mutant BioB cell lysates and purified enzyme. Each lane was loaded with 15 µg of protein. (1) Molecular weight markers, 94, 67, 43, 30, 20.1 and 14.4 kDa; (2) wild-type; (3) C53A; (4) C57A; (5) C60A cell lysates; (6) wild-type; (7) C53A; (8) C57A; (9) C60A purified enzymes.

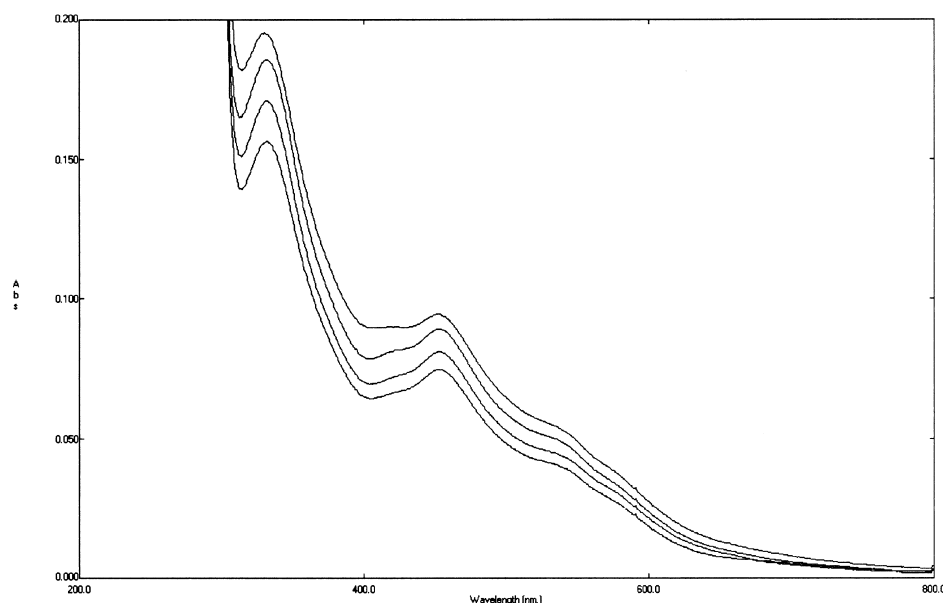


Fig. 4. Comparison of the UV-visible spectra of the wild-type BioB and the cysteine mutant enzymes at 3.0 mg/ml. From top to bottom trace, wild-type, C60A, C53A, C57A.

was approximately half that of the wild-type enzyme (Table 1), and for C60A, there was slightly more iron present than in the other mutant enzymes. These values are reflected in the UV-visible absorption spectra. Comparison of the CD spectra of the wild-type and the mutant enzymes indicated no significant secondary structure alteration (data not shown).

3.3. Radiochemical assays

All three mutant enzymes were inactive when assayed in cell lysates, in direct comparison with the wild-type enzyme (Fig. 5). The purified wild-type and mutant enzymes were all inactive when assayed in isolation without the other proteins necessary for BioB activity [3]. However, upon addition of *E. coli* BL21(DE3) cell lysate, which contains these additional proteins, activity was restored to the wild-type purified enzyme, but not for the mutant enzymes.

4. Discussion

This is the first experimental evidence that BioB cysteine residues C53, C57 and C60 are essential for activity and is consistent with their proposed role as ligands to the Fe-S cluster.

UV-visible spectroscopy and iron quantification suggested incomplete formation of the $[2\text{Fe-2S}]^{2+}$ cluster upon replacement of a single cysteine with alanine. The low iron content

for both wild-type and mutant enzymes was probably due to cluster degradation during aerobic purification. Cluster degradation has been observed for aRR AE [17], where there was a direct correlation between activity and iron content. Implementation of anaerobic purification procedures may be required to reduce such iron loss.

Although reconstitution of the BioB Fe-S cluster by Tse Sum Bum et al. [11] demonstrated that the Fe-S cluster was the immediate sulfur donor to dethiobiotin, it did not result in catalytic activity in vitro. To explain this, it was suggested that regeneration of the intact Fe-S cluster was a pre-requisite for multiple turnovers. The mechanism of Fe-S cluster formation or regeneration in vivo is still unknown. Several gene products, e.g. from the *isc* gene cluster, may be involved [18–20].

Resonance Raman [4] and Mössbauer studies [5] are consistent with incomplete cysteinyl co-ordination of the $[2\text{Fe-2S}]^{2+}$ cluster, with an oxygenic ligand, possibly serine, functioning as the fourth cluster ligand. A mechanism has been proposed for the reductive dimerisation of two such $[2\text{Fe-2S}]^{2+}$ clusters to form a single $[4\text{Fe-4S}]^{2+}$ cluster bridging

Table 1
Comparison of the iron content of the wild-type and BioB mutants, C53A, C57A and C60A

Protein	Iron quantification (mol Fe/mol bioB) by method:	
	1	2
Wild-type	0.39	0.33
C53A	0.23	0.17
C57A	0.21	0.17
C60A	0.28	0.22

Method 1: inductively coupled plasma optical emission spectroscopy, method 2: Fish [16].

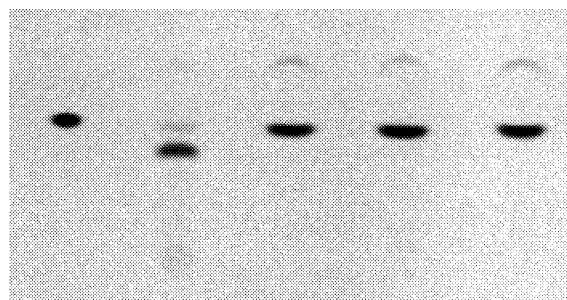


Fig. 5. Results of radiochemical assay with cell lysates. (1) $[^{14}\text{C}]$ dethiobiotin; (2) wild-type; (3) C53A; (4) C57A; (5) C60A. Radiolabelled compounds were analysed by thin layer chromatography and autoradiography. The wild-type enzyme (2) showed a ^{14}C -labelled compound with the same R_f value as biotin.

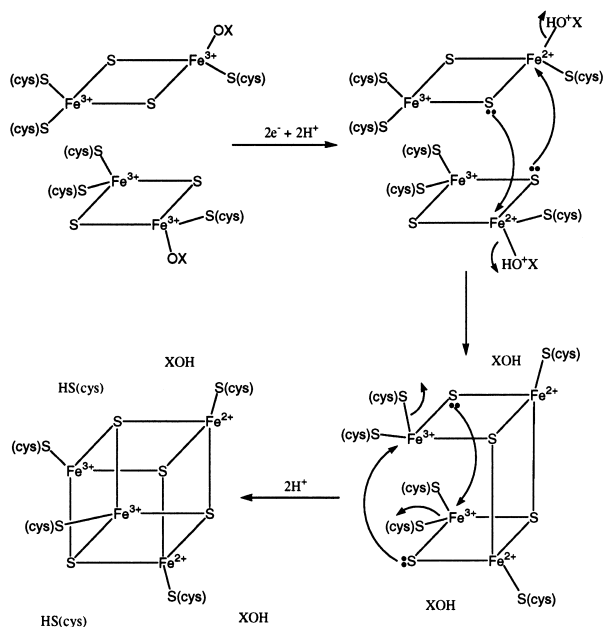


Fig. 6. Reductive dimerisation of two $[2\text{Fe-2S}]^{2+}$ clusters to a single $[4\text{Fe-4S}]^{2+}$ cluster bridging the dimer of BioB, as proposed by Johnson et al. [6].

the monomers of BioB (Fig. 6). This mechanism results in displacement of the oxygenic ligand upon $[4\text{Fe-4S}]^{2+}$ cluster formation. The $[4\text{Fe-4S}]^{2+}$ cluster is proposed to be the form of BioB involved in catalysis [4]. It seems reasonable to suggest that the mutants described here are unable to undergo this rearrangement and are therefore also unable to participate in the conversion of dethiobiotin to biotin. Work is in progress to elucidate the exact form of the Fe-S cluster in the mutant proteins and the nature of the fourth cluster binding ligand.

It is possible that a spatially close alternative residue is acting as a ligand to the Fe-S cluster, as a substitute for the mutated cysteine, and there are a total of eight cysteine residues in BioB. This phenomenon has been observed for *Azotobacter vinelandii* ferredoxin I [21], which binds one $[4\text{Fe-4S}]$ and one $[3\text{Fe-3S}]$ cluster per monomer. This ferredoxin has C20 as the distant ligand to the $[4\text{Fe-4S}]$ cluster which, upon mutation to an alanine, still binds a $[4\text{Fe-4S}]$ cluster. Structural rearrangement of the protein, as determined by X-ray crystallography, allowed a 'free cysteine', C24, to replace C20 as a cluster binding ligand.

Mutagenesis of cysteinyl Fe-S cluster binding ligands to serine residues has also been reported. Külzer et al. [17] have mutated all of the cysteine residues in pfl AE to serines. They observed that three out of the six cysteine residues were essential for activity which in a sequence alignment are equivalent to C53, C57 and C60 of BioB. In studies on other proteins, the mutagenesis resulted in an Fe-S cluster similar to that of the wild-type protein that could support biological activity ([22] and references therein). There are also naturally occurring proteins that contain non-cysteinyl ligands to Fe-S clusters. *Pyrococcus furiosus* ferredoxin, which contains a single $[4\text{Fe-4S}]$ cluster, possesses three cysteine residues with an aspartate as the fourth cluster binding ligand [23]. Rieske $[2\text{Fe-2S}]$ clusters have two cysteines and two histidines as their binding ligands [24]. One aspartate and two serine residues are

close to the cysteine triad in the *E. coli* BioB sequence, AC-PEDCKYCPQSS. It is therefore possible that one of these residues could substitute for a mutated cysteine, but it is unclear if such a rearrangement would be observed by CD spectroscopy. The X-ray crystal structure of BioB has not yet been reported and hence investigation by this method is not possible.

Fe-S cluster enzymes are widespread in biology and function primarily as electron transporters with the ferredoxins ([25] and references therein). Fe-S cluster enzymes are directly involved in enzyme catalysis, e.g. conversion of citrate to isocitrate by aconitase [26], and both SoxR [27] and fumarate nitrate reduction protein (FNR) [28] are proposed to have roles in transcriptional regulation. Exposure of *E. coli* FNR protein to oxygen converts the regulatory $[4\text{Fe-4S}]^{2+}$ cluster to a single $[2\text{Fe-2S}]^{2+}$. Reconstitution of the active FNR form requires incubation with cysteine, iron, DTT and the NifS protein. The first three of these are required as co-factors for the in vitro BioB assay.

The Fe-S cluster of BioB is required not only as the electron donor to SAM, but also as the sulfur donor to dethiobiotin. The apparent interconversion between the two different cluster forms, $[2\text{Fe-2S}]$ and $[4\text{Fe-4S}]$, may also be necessary for the function of BioB. Further mutagenesis investigations will provide additional information on the role of the Fe-S cluster in the formation of biotin.

Acknowledgements: We would like to thank the BBSRC and Lonza U.K. for financial support for K.S.H and the Royal Society for a University Research Fellowship to P.L.R.

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