Biotin synthase mechanism: on the origin of sulphur

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Abstract Biotin synthase catalyses the last step of the biosynthesis of biotin in microorganisms and plants. The active protein isolated from Bacillus sphaericus and Escherichia coli contains an iron-sulphur (FeS) cluster. The native enzymes were depleted of their iron and inorganic sulphide and the resulting apoenzymes were chemically reconstituted with FeCl₃ and Na₂[³⁴S] to give labelled (Fe³⁴S) enzymes. These enzymes were functional and when assayed in vitro produced labelled biotin containing about 65% of ³⁴S. These data strongly support the hypothesis that the sulphur of biotin is derived from the (FeS) centre of the enzyme.

Key words: Biotin synthase; Iron-sulfur cluster; ³⁴S reconstituted enzyme; Sulfur donor

1. Introduction

Biotin synthase catalyses the final step of the biotin biosynthetic pathway which involves the insertion of a sulphur atom into dethiobiotin (DTB), an unusual and fascinating reaction.

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\text{Dethiobiotin} \quad \text{Biotin}
\]

Detailed investigation of the catalytic mechanism of the Escherichia coli and Bacillus sphaericus enzymes has been possible only since the past few years with the availability of an active in vitro system, which has an absolute requirement for S-adenosylmethionine (AdoMet) and Na₂[³⁴S] to give labelled (Fe³⁴S) enzymes. These enzymes were functional and when assayed in vitro produced labelled biotin containing about 65% of ³⁴S. These data strongly support the hypothesis that the sulphur of biotin is derived from the (FeS) centre of the enzyme.

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2. Materials and methods

2.1. Materials

Abbreviations: (FeS), iron-sulphur cluster; AdoMet, S-adenosylmethionine; DTT, dithiothreitol; DTB, dethiobiotin; Ado-CH₃; deoxyadenosyl radical; PFL, pyruvate formate lyase; ARNR, anaerobic ribonucleotide reductase; LAM, lysine 2,3-aminomutase

We propose [4] three possibilities for the sulphur source:

1. the (FeS) centre of the protein
2. a sulphur species covalently bound to the protein, for instance of a sulphane type [16]
3. DTT present in large amounts in our in vitro system.

In this paper, we present strong experimental arguments in favour of the first hypothesis.

3. Materials and methods

3.1. Materials

Details on bacterial strains and plasmids, growth conditions, preparation of cell-free extracts, purification and characterisation of the
2.2. Analytical methods

Protein concentration was measured by the method of Bradford using bovine serum albumin as a standard [19]. Iron was assayed by the method of Fish [20] and inorganic sulphide was quantified spectrophotometrically as described by Beinert [21].

2.3. Assay of biotin synthase of B. sphaericus (assay 1) [4]

The reaction mixture in a final volume of 100 μl contained 50 μM biotin synthase, 50 μM (+)DTB, 0.1 mM AdoMet and 5 mM DTT in 100 mM Tris-HCl buffer pH 8.0. The mixture was allowed to stand at room temperature under a stream of moist argon for 30 min. 5 μl of a 0.6 mM solution of 5-deazaflavin, previously degassed in the dark for 30 min, was added to the assay mixture using a gas-tight syringe. The reaction was initiated by photoreduction using a fluorescent lamp. After 2 h, the reaction was stopped by precipitation with trichloroacetic acid. Precipitated proteins were removed by centrifugation and the amount of biotin formed was determined in the supernatant by the paper disc-plate method using Lactobacillus plantarum [22].

2.4. Assay of biotin synthase of E. coli (assay 2) [8]

The reaction mixture in a final volume of 100 μl consisted of 12 μM biotin synthase, 50 μM (+)DTB, 0.2 mM AdoMet, 10 mM DTT, 2 mM NADPH, 2 μM flavodoxin, 0.2 μM flavodoxin reductase and 0.2 mM Fe(NH)₄(SO₄)₂ in 40 mM Tris-HCl buffer pH 8.0. The incubation was carried out anaerobically for 2 or 4 h (see Section 3) at 30°C. Biotin was determined as described in assay 1.

2.5. Effect of free Fe³⁺ or Fe²⁺ and S²⁻ on the activity of biotin synthases

For B. sphaericus, the reaction mixture is the same as described in assay 1. After degassing the reaction mixture for 30 min, 100 μM FeCl₃ was added to 1 mM Na₂S were added. The latter components were from stock solutions of 1 mM FeCl₃ in H₂O) and 10 mM Na₂S (in 50 mM Tris-HCl pH 8) previously degassed under a stream of moist argon for 30 min. The reaction mixture was incubated for a further 30 min before the addition of deazaflavin. For E. coli, the reaction mixture was the same as described in assay 2. The mixture was left to stand under a stream of moist argon for 30 min and 500 μM Na₂S was added from a degassed 10 mM stock solution. The reaction was allowed to proceed for 4 h.

2.6. Synthesis of Na₂S⁴⁰⁸

Na₂S⁴⁰⁸ was synthesised from 5 mg of elemental ⁴⁰⁸S (purity 99.91% from Euriso-top, France) and excess sodium in liquid ammonia according to [23]. The solid white Na₂S⁴⁰⁸ was dissolved in 1 ml of 50 mM Tris buffer containing 40 mM DTT pH 8 to give a 150 mM stock solution. The concentration of sulphide ions was verified using the method of Beinert [21]. This solution was kept under argon at 4°C.

2.7. ³⁴S incorporation from free Na₂S⁴⁰⁸ into biotin

The reaction mixture was as described in assay 1, with an assay volume of 200 μl. Prior to the addition of deazaflavin, 100 μM FeCl₃, followed by 1 mM Na₂S⁴⁰⁸ were added to the reaction mixture from degassed stock solutions of 1 mM FeCl₃ and 10 mM Na₂S⁴⁰⁸. The mixture was left to stand 30 min, deazaflavin was added and the reaction started by illumination. Experiments were multiplied such as to cumulate 70 nmol of biotin for the purification procedure.

2.8. Preparation of apoenzymes

The degradation of the cluster during reduction by dithionite [3] has been exploited for the preparation of apo-biotin synthases. All procedures were carried out at room temperature in 50 mM Tris-HCl pH 8.0, hereafter referred to as buffer. A solution of 5 mg (130 nmol) protein in 2 ml of buffer was left under a stream of wet argon for 1 h. A 50-fold excess of both sodium dithionite and EDTA (3.25 mM final concentration) which were also deoxygenated separately under a stream of argon were then added to the enzyme via a gas-tight syringe. The reaction was allowed to proceed anaerobically for 3 h for the enzyme of B. sphaericus and 20 h for the E. coli enzyme. The cluster from the latter enzyme is stabler and thus requires more time for its degradation. The mixture was desalted on a Sephadex G-25 column with buffer containing 5 mM DTT and the eluted apoprotein was concentrated on Centriprep 30 (Amicon), frozen in liquid nitrogen and stored at −80°C before use.

2.9. Reconstitution of the (Fe³⁴S) centres into apoenzymes

500 μl aliquots of apo-biotin synthases (260 μM), 20 mM Na₂S⁴⁰⁸ in argon-saturated buffer, 20 mM FeCl₃ and 100 mM DTT (both dissolved in water) were deoxygenated in separate tubes under a stream of wet argon for 1 h. After this time, DTT was added to the apoenzymes to a final concentration of 5 mM and this mixture was incubated for a further 30 min. Optimised concentrations of FeCl₃ and Na₂S⁴⁰⁸, that is, a 1.5-fold excess with respect to the B. sphaericus enzyme and a 3-fold excess for the E. coli enzyme, were then added to the solution. The mixture was allowed to proceed for 90 min after which EDTA (1 mM final concentration) in buffer was added and incubated for 30 min to chelate the free iron. The mixture was desalted on a PD 10 column (Pharmacia) with buffer containing 5 mM DTT and concentrated on Centriprep 30.

2.10. ³⁴S incorporation from (Fe³⁴S) biotin synthases into biotin

The reconstituted (Fe³⁴S) biotin synthases were assayed using the protocols described in assays 1 and 2 except that the reaction volumes were increased to 200 and 400 μl respectively. Assay 1 also contained additional 100 μM Fe²⁺ to increase biotin production.

2.11. Isolation and purification of [³⁴S]biotin

After the removal of proteins, the supernatant was loaded on a C₁₈ reversed phase silica column (1.5×20 cm). The column was succes-
sively washed with 250 ml of a solution of 1% acetic acid and 100 ml water; elution was then performed with a solution of 10% methanol in water. The solvent was removed under vacuum and the residue containing biotin was redisolved in 1 ml methanol. After esterification with diazomethane, the sample was chromatographed on an HPLC reversed phase (Lichrospher 100 RPS, 250 × 4 mm) column (Merck). Chromatography conditions were 65% A and 35% B, isocratic over 25 min at a flow rate of 1 ml/min. Solvent A consists of H$_2$O/TFA 0.1% and B of H$_2$O/TFA 0.1%; CH$_3$CN (50:50). Absorbance was monitored at 213 nm. Fractions were collected and 10 µl aliquots of those having the same migration time as a biotin methyl ester standard were saponified and tested in the L. plantarum biotin assay. The biotin-containing fractions were evaporated in a speed-vac, pooled and studied by mass spectrometry on a triple quadrupole tandem mass spectrometer R30-10 NERMAG, Quad Service (Poissy, France). The $^{34}$S/$^{32}$S ratio in methylated biotin was determined from ammonia chemical ionisation mass spectra. Purity of $^{32}$S and $^{34}$S methylated biotin molecules was confirmed by MSMS experiments from MH$^+$ ions, respectively $m/z$ 259 and $m/z$ 261.

3. Results

3.1. Biotin synthase of B. sphaericus

In the in vitro assay of biotin synthase (assay 1: DTB, AdoMet, DTT and photoreduced deazaflavin), the enzyme is not catalytic and the amount of biotin formed is low. This low yield can be increased 3–4-fold by addition of optimised concentrations of FeCl$_3$ (100 µM) and Na$_2$S (1 mM) (Fig. 1A)$.^2$ As we have observed by UV-Vis spectroscopy that the (FeS) cluster was partially degraded under the irradiation conditions (data not shown), we postulated that this improved yield could be attributed to some reconstitution of the (FeS) cluster, hence of the active enzyme during reaction. If this is the case and if according to our first hypothesis, the sulphur of biotin originates from the (FeS) centre, the added sulphide should, via this cluster, be incorporated to some extent into biotin. We thus added 1 mM Na$_2$[34S] to the reaction medium. After precipitation of the proteins, the biotin formed was purified as its methyl ester. Mass spectral analysis was performed to test its purity and to calculate the ratio $^{34}$S/$^{32}$S which was found to be 40%. As it is very unlikely that Na$_2$S itself can trap a carbon radical (Scheme 1), this experiment strongly favours our hypothesis.

For further confirmation, we prepared the apoenzyme whose (FeS) centre was then reconstituted with Na$_2$[34S]. The apoenzyme was obtained by reduction with an excess of sodium dithionite in the presence of EDTA. This enzyme was reconstituted chemically with FeCl$_3$ and Na$_2$[34S] in the presence of DTT to yield an active enzyme having an iron-sulphur content (Table 1) and UV/Vis spectrum quite similar to the native holoenzyme (Fig. 2). We then repeated the above experiment (assay 1) with this $^{34}$S reconstituted enzyme, except that no Na$_2$S was added to the incubation mixture. The percentage of labelled biotin was found to be 67%.

3.2. Biotin synthase of E. coli

An in vitro assay described by Sanyal et al. [8] consists of biotin synthase, flavodoxin, flavodoxin reductase, NADPH, DTB, AdoMet, Fe(NH$_4$)$_2$(SO$_4$)$_2$, DTT, l-cysteine, fructose 1,6-bisphosphate (FBP) and KCl. They reported the formation of 1 mol of biotin per mol of enzyme after 4 h, but under these conditions we never obtained more than 0.5 mol of biotin per mol of enzyme. In addition, we did not notice any effect of FBP and KCl on the activity which were thus withdrawn from the assay (assay 2). Similarly, it was observed by Shaw et al. [24] that their enzyme in a desalted cell-free extract plus defined low molecular mass components also had no catalytic activity.

It was important to confirm the above described incorporation of sulphur from the (Fe$^{35}$S) reconstituted enzyme with this E. coli system which is closer to physiological conditions. The apoenzyme was prepared and its (FeS) centre reconstituted as described for the B. sphaericus enzyme (see Section 2 and Table 1). This $^{34}$S reconstituted enzyme was incubated in assay 2, from which cysteine was omitted, in spite of its two-

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$^2$ This observation was first made by T. Hoshino of Nippon-Roche.
fold contribution to the activity, so as to avoid any interference with the sulphur of the cluster. After a 4 h reaction time, biotin was isolated, purified and analysed by mass spectrometry. The extent of $^{34}$S incorporation was found to be 45%. Although high, this value is far from 100%. This may be due to a partial exchange of the $^{34}$S of the cluster with an external $^{34}$S source in the medium. Just as for the B. sphaericus enzyme, an increase in biotin production is observed upon addition of Fe$^{2+}$ and S$^{2-}$ to the enzyme, suggesting a spontaneous exchange of free ions with the elements of the (FeS) cluster (Fig. 1B). If such an exchange is responsible for the non-quantitative incorporation of $^{34}$S, a shorter reaction time, implying less exchange possibility, should lead to an increased incorporation in biotin. This was indeed the case. The biotin isolated after a 2 h incubation time with (Fe$^{3+}$S) reconstituted enzymes contained 62% of $^{34}$S.

4. Discussion

We have shown that the (Fe$^{3+}$S) reconstituted biotin synthases of B. sphaericus and E. coli produced $[^{34}$S]biotin with a high $^{34}$S incorporation, respectively 67% and 62% in the most favourable conditions studied (Table 1).

We think that these results render very unlikely the last two of the three hypotheses that we formulated concerning the origin of sulphur (see Section 1). A persulphide species (second hypothesis), which could have been present in the native protein, produced by an enzymatic process would not have survived the strongly reducing conditions used to prepare the apoenzymes and it is difficult to imagine that it could be formed again spontaneously in vitro in the reconstitution conditions in presence of DTT.

DTT (third hypothesis) cannot be the physiological sulphur donor but could be a substitute in the in vitro assays that we use. As it is present in very high amounts (5 or 10 mM) as compared to the concentration of $^{34}$S-labelled biotin synthase (50 μM for B. sphaericus and 12 μM for E. coli), the important $^{34}$S labelling of biotin that we observe excludes DTT as the sulphur source.

On the other hand, our experimental results are consistent with the first hypothesis. The biotin synthase system has a strong analogy with the PFL, ARNR and LAM systems. All four have an absolute requirement for AdoMet, the monoelectronic cleavage of which results in the formation of methionine and a deoxyadenosyl radical (Ado-CH$_2$) [9,11–13]. The Ado-CH$_2$ is then involved in the homolytic cleavage of a C-H bond (Scheme 1).

The (FeS) cluster present in PFL activase [25], ARNR [26] and LAM [27] has been assumed to participate in this one-electron transfer. It very likely plays a similar role in biotin synthase but this redox function does not exclude another role for the cluster which is to give its sulphur. This is a reasonable interpretation for the 65% incorporation of $^{34}$S into biotin from the reconstituted $^{34}$S biotin synthases. The fact that this incorporation does not reach 100% is not completely clear. We have attributed it to the residual $^{34}$S sulphide left over in the apoenzymes (Table 1) and to a ready exchange of the cluster sulphide with an external $^{32}$S sulphide source, a phenomenon already observed in other (FeS) enzymes [28,29]. This interpretation is also consistent with the incorporation of $^{34}$S in biotin when Na$_2$S$^{[34]$S] is added to the medium in assay 1.

Another interpretation could be that free S$^{2-}$ is the actual sulphur donor. With the (Fe$^{3+}$S) reconstituted enzymes, $^{34}$S$^{2-}$ would have to be released from the cluster before reaction. This is, however, not consistent with our findings in assay 2 as the amount of $^{34}$S transferred to biotin from E. coli (Fe$^{3+}$S) biotin synthase decreases with the incubation time. Moreover, the mechanism that we propose postulates that carbon radicals are trapped by the sulphur donor, which should be a radical. It is very unlikely that S$^{2-}$ itself can trap a radical. Although it would not be sound at this stage to formulate a very precise mechanism for the C-S bond formation, it is
tempting to postulate the participation of the iron atoms of the cluster to provide a radical reactivity to the inorganic sulphide. We hope to be able to further elucidate this by following the reaction with spectroscopic methods suitable for the characterisation of the (FeS) centre (ESR, Mössbauer, NMR, etc.).

Although we cannot exclude at this stage another unknown final sulphur-donating species, our hypothesis which implies that the sulphide of the cluster is progressively depleted to form biotin can explain the absence of turnover of biotin synthase in the presently described in vitro systems. To restore a catalytic activity, the (FeS) clusters would have to be efficiently regenerated. Enzyme-catalysed reactions are involved in the formation of (FeS) clusters in vivo. This problem has been well studied for instance in the case of the nitrogenase of Azotobacter vinelandii. It was proposed by Dean and collaborators [30,31] that NifS and NifU gene products (NifS and NifU) were implicated in the formation of the [4Fe-4S] cluster of nitrogenase. It has now been demonstrated that NifS catalyses the desulphurisation of t-cysteine leading finally to S²⁻ [32]. Recently, several other NifS- or NifS-like-mediated reconstitutions of [4Fe-4S] or [2Fe-2S] clusters have also been reported [33–36]. The specific role of NifU is not yet known but it has been suggested that it might function either to deliver the iron or to provide an intermediate site for (FeS) assembly.

Our previous results with crude extracts of B. sphaericus [2] and those of Birch et al. [7] with impure fractions of E. coli implicated cysteine as the precursor of sulphur in biotin. In view of the above discussion, it could be that the sulphur of the cysteine used in our experiment was removed by an enzyme system of a NifS type present in the crude extracts. (FeS) clusters have been implicated in a variety of biological functions of which the most widely known has been electron transport [37]. More recently, new roles have been attributed to these metallocentres, such as their involvement in direct enzymatic catalysis as in aconitase [38] and dihydroxy-acid dehydratase [39]. DNA recognition in endonuclease III [40] and regulatory roles in the FNR [33] and SoxR proteins [34]. Based on our experiments, we suggest a new function for the (FeS) cluster found in biotin synthase which is to provide its sulphide. This should also apply to lipoic acid synthase. It has a strong homology with biotin synthase and probably works according to the same type of mechanism.

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