Biotin synthase from *Escherichia coli*: isolation of an enzyme-generated intermediate and stoichiometry of S-adenosylmethionine use

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A cell-free extract from *Escherichia coli* containing an *E. coli* biotin synthase that was expressed to approx. 1 % of soluble cell protein by cloning the *E. coli bioB* gene was used to investigate the biotin synthase reaction. The pH optimum was between 8 and 8.5, and the reaction velocity was dependent on the concentrations of dethiobiotin, cysteine, S-adenosylmethionine and asparagine. The catalytic-centre activity of the enzyme *in vitro* was estimated to be 0.95 h⁻¹, and each molecule of enzyme turned over less than one molecule of dethiobiotin, i.e. the enzyme was not acting catalytically. HPLC analysis of reaction mixtures revealed the presence of a compound with the characteristics of an intermediate: (1) it was labelled with ¹⁴C, and therefore derived from the [¹⁴C]dethiobiotin substrate; (2) it was present only in reaction mixtures containing biotin synthase; (3) it was not derived from [¹⁴C]biotin; (4) ³⁵S from [³⁵S]cysteine was

incorporated into the intermediate during the reaction; (5) its synthesis was dependent on the presence of S-adenosylmethionine, and was decreased when free cysteine was omitted from the reaction; (6) it could be isolated from the reaction mixture by chromatography and then re-introduced into an assay as the substrate, whereupon it was converted to biotin; (7) this conversion to biotin was S-adenosylmethionine-dependent. During the reaction S-adenosylmethionine was cleaved to methionine and presumably 5'-deoxyadenosine. Observation of the intermediate allowed us to perform experiments to determine the stoichiometry of S-adenosylmethionine use. We propose that two molecules of S-adenosylmethionine are used to synthesize one molecule of biotin, i.e. one from dethiobiotin to the intermediate, and a second from the intermediate to biotin.

INTRODUCTION

Biotin is a vitamin synthesized by micro-organisms and plants. The last step in the biotin biosynthetic pathway is catalysed by biotin synthase and involves the insertion of a sulphur atom between the unactivated methyl and methylene carbon atoms adjacent to the imidazole ring of dethiobiotin. The gene for biotin synthase has been cloned from a number of micro-organisms [1–10] and from the plant *Arabidopsis thaliana* [11,12]. The biotin synthase gene from *Escherichia coli* has also been cloned into *Arabidopsis*, where it was able to complement a biotin synthase-deficient mutant [13]. Only recently has measurement of biotin synthase activity *in vitro* been possible [5,14–17]. The enzymes from *E. coli* [18] and *Bacillus sphaericus* [9] have been purified and partly characterized; a number of proteins and low-molecular-mass compounds that are essential for the activity of the *E. coli* enzyme *in vitro* have been identified [16,17,19,20]. These are flavodoxin, ferredoxin (flavodoxin) NADP⁺ reductase, a third protein fraction that requires thiamine pyrophosphate to maintain its activity during purification, cysteine, S-adenosylmethionine, Fe²⁺, thiamine pyrophosphate, one of the amino acids asparagine, aspartate, glutamine or serine, and NADPH. The involvement of the thiamine pyrophosphate-dependent protein fraction, thiamine pyrophosphate itself and the amino acids is a matter of controversy, as is the nature of the sulphur donor for the reaction. A number of groups have provided evidence that cysteine is the source of the sulphur atom in biotin, but there are other reports that contradict this [5,17,20–22].

To help clarify the reaction mechanism for biotin synthase, a number of putative intermediates have been synthesized and tested as substrates, both in whole-cell systems and *in vitro* [23,24]. One possible intermediate, 9-mercaptodethiobiotin, supported the growth of an *E. coli* mutant lacking the entire biotin

pathway but carrying a plasmid encoding the *E. coli* biotin synthase gene, indicating that the compound might be able to substitute for dethiobiotin [25]. Similar results were obtained with *B. sphaericus* [24]. Further evidence supporting 9-mercaptodethiobiotin as an intermediate comes from experiments in which the label from [³⁴S]mercaptodethiobiotin was incorporated into biotin by a cell-free system from *B. sphaericus* [26], and the isolation of a reaction intermediate from plant cells that was proposed to be 9-mercaptodethiobiotin [27].

The involvement of flavodoxin, ferredoxin (flavodoxin) NADP⁺ reductase, S-adenosylmethionine and NADPH suggests similarities between biotin synthase and other enzymes such as pyruvate formate-lyase [28,29], anaerobic ribonucleotide reductase [30] and lysine 2,3-aminomutase [31], whose mechanisms involve the formation of either an enzyme-bound (pyruvate formate-lyase and anaerobic ribonucleotide reductase) or substrate-bound (lysine 2,3-aminomutase) radical. On the basis of experiments in which the stereochemical information from a chiral (isotopically labelled) methyl group in dethiobiotin was lost on enzymic biotin formation, it was proposed that the biotin synthase reaction proceeded via the formation of an intermediate with a conformationally labile methylene radical, but these experiments were not followed up [32]. So, despite the advances in our understanding of biotin synthase in the past few years, much remains to be learnt about its structure, interaction with other proteins, and reaction mechanism.

METHODS

Materials

Details of chemicals, bacterial strains and plasmids, growth of cells, preparation of cell-free extracts, TLC and HPLC are given in [17]. S-Adenosyl-L-[methyl-¹⁴C]methionine (59.3 mCi/mmol)

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and L-[methyl- ^{14}C]methionine (44.0 mCi/mmol) were from New England Nuclear. The biotin synthase assays *in vitro* were also performed as in [17], with some modifications. The standard assay was in 100 mM Tris/HCl buffer, pH 8.0, and the amounts and concentrations of reactants were [^{14}C]dethiobiotin, 0.046 μCi , 0.8 nmol (3.2 μM); cysteine, 250 nmol (1 mM); S-adenosylmethionine, 25 nmol (100 μM); asparagine, 5 μmol (20 mM); Fe^{2+} -gluconate, 50 nmol (200 μM); thiamine pyrophosphate, 25 nmol (100 μM); NADPH, 25 nmol (100 μM); dithiothreitol, 250 nmol (1 mM). To avoid the lag phase described in the Results section, the reaction components were usually mixed and preincubated for 30 min before the addition of dethiobiotin to start the reaction. The cell-free extract used in all experiments was from *E. coli* BM4062 with plasmid pBO30A-15/9, which carries the *E. coli* biotin synthase gene. Expression of biotin synthase from this plasmid was approx. 1 % of total cell protein. The extract was usually freed of low-molecular-mass compounds before use by desalting on Sephadex G-25M gel-filtration columns (Pharmacia, PD-10). The low-molecular-mass compounds described in [17] were then required for activity.

pH optimum and kinetics

The pH optimum for the reaction was determined in the following buffers, each at 100 mM: pH 6.0 and 6.5, Mes; pH 7.0, Mops; pH 7.5, Hepes; pH 8.0, 8.5, 9.0 and 9.5, Tris/HCl. The assays were incubated for 1 h. For the kinetic experiments the amount of cell-free extract was 1.55 mg of protein per assay. Reaction products were separated by TLC and HPLC. The radioactivity in each compound was determined by scintillation counting of the spots scraped from the TLC plates after detection by autoradiography, or after HPLC with an on-line radiochemical detector. Reactions were performed at least in duplicate.

Characterization of the enzyme-generated intermediate

The ^{14}C -labelled intermediate was isolated from other radioactive compounds in reaction mixtures by chromatography on a reverse-phase column (PepRPC, HR5/5; Pharmacia) (see below for details). Elution buffer was removed by chromatography on disposable C_{18} solid-phase extraction columns, under the same conditions as those used for biotin and dethiobiotin [17]. For assays in which the ^{14}C -labelled intermediate was the substrate, the amount in each assay was 3.2 nCi (6996 d.p.m.), equivalent to 74 pmol and a concentration of 0.295 μM . For assays to determine the incorporation of sulphur into the intermediate, the amount of [^{35}S]cysteine was 2.5 μCi , 25 nmol (100 μM).

The fate of S-adenosylmethionine and stoichiometry of its use

Assays were performed as described in [17] with either [^{14}C]dethiobiotin (purified by chromatography on a PepRPC column under the conditions described below; 0.116 μCi , 2028 pmol) and S-adenosyl-L-[methyl- ^{14}C]methionine (0.1 μCi , 6700 pmol), or with S-adenosyl-L-[methyl- ^{14}C]methionine alone (0.1 μCi , 6700 pmol) as the control. The reactions were stopped with 500 μl of 12.5 % (w/v) trichloroacetic acid, precipitated protein was removed by centrifugation, and 200 μl aliquots were analysed by chromatography on a reverse-phase column (PepRPC) to separate biotin, dethiobiotin and the intermediate, and by chromatography on a cation-exchange FPLC column (MonoS, HR5/5; Pharmacia) to separate S-adenosylmethionine and methionine. To determine the amount of radioactivity present in each compound, fractions from the columns were analysed by scintillation counting. The chromatography conditions for the PepRPC column were: start buffer, 50 mM NaH_2PO_4 /20 mM

triethylamine (pH 3.5); end buffer, as for the start buffer plus 20 % (v/v) acetonitrile; flow rate, 0.5 ml/min; gradient, start buffer for 4 ml, then a 40 ml gradient to 100 % end buffer. Under these conditions S-adenosylmethionine and methionine were eluted in the first 5 ml, whereas biotin, dethiobiotin and the intermediate were separated by the gradient. The chromatography conditions for the MonoS column were: start buffer, 50 mM ammonium formate, pH 3.8; end buffer, as for the start buffer plus 1 M NaCl, flow rate, 0.5 ml/min; gradient, start buffer for 4 ml, then a 24 ml gradient to 100 % end buffer. Under these conditions biotin, dethiobiotin and the intermediate were either eluted in the first 4 ml (standards dissolved in start buffer) or did not appear at all (samples from the assays), whereas methionine and S-adenosylmethionine were separated by the gradient, and another S-adenosylmethionine degradation product was eluted in the first 4 ml. Suitable controls were performed to account for the radioactivity put into the assays and that recovered from the column fractions.

RESULTS AND DISCUSSION

Assay system

The study of biotin synthase is still at a very early stage, and although the enzyme itself has been purified it requires a complex and as yet not fully defined mixture of proteins and low-molecular-mass compounds for activity *in vitro*. One group [20] has claimed to have activity in a fully defined assay mixture, but the assay mixture contained three proteins of only 90 % purity. We have evidence (N. M. Shaw, O. M. Birch, A. Tinschert and R. Dietrich, unpublished work) that in addition to flavodoxin, ferredoxin (flavodoxin) NADP $^{+}$ reductase and the thiamine pyrophosphate-dependent protein, a fourth as yet unidentified protein is required for activity *in vitro*. So, given the present difficulties of trying to work with a fully defined assay system, we chose to study biotin synthase in a desalted cell-free extract plus defined low-molecular-mass components, with the appropriate controls.

Approx. 1 % of protein in the cell-free extract used in the assays was biotin synthase as measured by two-dimensional PAGE with silver-stained gels and quantification by computer densitometry [33].

pH optimum and kinetics

The pH optimum for the conversion of dethiobiotin to biotin was between 8.0 and 8.5 (Figure 1). The concentration of dethiobiotin at which the enzyme was estimated to be saturated was approx. 15 μM . This agrees broadly with the K_m determined in [18] (2 μM) with purified biotin synthase plus crude cell-free extract from *E. coli* to provide the remaining proteins and a microbial assay for biotin, and supports our choice of experimental system. The direct involvement of S-adenosylmethionine in the reaction has been established, but those of cysteine and especially asparagine remain contentious. There are no published kinetic constants for cysteine, S-adenosylmethionine and asparagine. To ensure that saturating concentrations of these substrates were used in assays, the concentration dependence of the reaction with these compounds was investigated. The resulting curves were not simple hyperbolae, but for each compound a concentration dependence was seen. For cysteine the enzyme was estimated to be saturated at approx. 600 μM , for S-adenosylmethionine the saturating concentration was estimated to be approx. 100 μM and that for asparagine was approx. 12 mM. More definitive kinetics will be determined when the system has been fully defined.

Figure 1 pH optimum for the biotin synthase reaction

When all the components for the assay were preincubated individually at the assay temperature, the maximum rate of biotin synthesis was not reached until approx. 10 min after the components were mixed. Preincubation of a mixture of all the components except [^{14}C]dethiobiotin for 30 min at 37 °C eliminated the lag period.

We calculated from the kinetics results that at its maximum rate 410 pmol of biotin synthase synthesized 350 pmol of biotin in 40 min. The catalytic-centre activity of the enzyme was also estimated from the results shown below in Figure 3(b) to be approx. 0.5 h^{-1} if the conversion from dethiobiotin to biotin was considered, but 0.95 h^{-1} if the conversion of dethiobiotin to biotin and the intermediate described below was considered. These calculations were based on 1 % of protein in the cell-free extract being biotin synthase, and a molecular mass for the biotin synthase monomer of 38.7 kDa, with one active site per monomer. None of our results so far have shown one molecule of enzyme turning over more than one molecule of substrate *in vitro*. The catalytic-centre activity is similar to that reported in [18], although in that paper the enzyme is reported to be catalytic, with three molecules of biotin formed per molecule of enzyme. We conclude that the enzyme is not acting catalytically *in vitro*, but other interpretations are possible. For example, if the amount of enzyme were overestimated, or if some of the enzyme were inactive or less active (two forms of biotin synthase with different activities were reported in [18]), the actual catalytic-centre activity of the enzyme would be a little higher. However, even if one of these two other possibilities were true, the catalytic-centre activity of the enzyme would still be very low.

Observation and characterization of the enzyme-generated intermediate

Analysis of the assay mixtures from the preliminary kinetic experiments by HPLC with on-line radiochemical detection showed a third major peak of radioactivity in addition to the expected radioactive peaks for dethiobiotin and biotin (Figure 2).

The characteristics of this compound, as described below, are consistent with its being an enzyme-generated intermediate. The kinetics of the appearance of the intermediate were studied in assays with three concentrations of cell-free extract. At the lowest protein concentration (1 mg per assay), the amount of the intermediate increased up to 40 min incubation time, then

Figure 2 HPLC analysis of biotin synthase assay mixtures

The 20 min time points from Figure 3 [1.0 (a), 2.5 (b) and 5.0 (c) mg of cell-free extract per incubation respectively] are shown, illustrating the synthesis of the intermediate and biotin, and the concomitant decrease in the amount of dethiobiotin.

decreased slightly, and then steadily increased again for up to 3 h (Figure 3a). With 2.5 mg of protein per assay, the amount of the intermediate increased rapidly up to 20 min incubation time, and then decreased steadily for 3 h (Figure 3b). At the highest protein concentration (5 mg), similar kinetics were observed, the intermediate reaching a maximum after 10 min, then declining steadily (Figure 3c). The total amount of radioactivity in

Figure 3 Biotin synthase reaction time courses

Reactions contained 1.0 (a), 2.5 (b) and 5.0 (c) mg of cell-free extract per incubation respectively. Reaction mixtures were analysed by HPLC with on-line radiochemical detection.

dethiobiotin, biotin and the intermediate remained approximately constant at all time points in all three incubations.

The intermediate was derived from dethiobiotin, because it was labelled with ^{14}C . Control assays showed that it was generated only in assays with cell-free extract containing over-expressed biotin synthase; it was not produced when ^{14}C biotin was substituted in the assay for ^{14}C dethiobiotin, showing that it was neither produced in a reverse step of the biotin synthase reaction nor was it a breakdown product or metabolic derivative

of biotin. Synthesis of the intermediate was dependent on the presence of *S*-adenosylmethionine.

The influence of the low-molecular-mass compounds required for biotin synthase activity on the conversion of dethiobiotin to the intermediate could not be studied in isolation because the intermediate is subsequently converted to biotin, and the low-molecular-mass compounds might also affect any processes involved in binding sulphur to a protein in the enzyme system. So, although leaving cysteine out of the reaction decreased the total amount of intermediate and biotin by up to 75 %, the effect might not be directly on this step. The fact that the intermediate and biotin are formed at all in the absence of added cysteine lends further support to the theory that cysteine or sulphur is bound to a protein in the system [20] and is then transferred to dethiobiotin.

Labelling of the intermediate with ^{35}S from $[^{35}\text{S}]$ cysteine

The intermediate from assays in which $[^{35}\text{S}]$ cysteine was the radioactively labelled component was analysed to determine whether ^{35}S was incorporated. A parallel standard assay with $[^{14}\text{C}]$ dethiobiotin was performed to provide an intermediate standard for comparison, as was a control assay with $[^{35}\text{S}]$ cysteine but no unlabelled dethiobiotin, in which no intermediate or biotin should be formed. Incubation mixtures were analysed by reverse-phase chromatography (PepRPC, FPLC) with scintillation counting of the resulting fractions, and by TLC and autoradiography (Figure 4). The standard assay with $[^{14}\text{C}]$ dethiobiotin resulted in characteristic biotin, dethiobiotin and intermediate peaks and bands on reverse-phase chromatography and TLC respectively (Figures 4a and 4c, lane 2). [Note that under the TLC conditions used, the intermediate ran with a slightly higher R_f than dethiobiotin and is therefore difficult to see on the X-ray when $[^{14}\text{C}]$ dethiobiotin is also present. A zero time point control for the $[^{14}\text{C}]$ dethiobiotin assay that has no intermediate band is shown in Figure 4(c), lane 1, for comparison.] When the assay was performed with $[^{35}\text{S}]$ cysteine in the absence of dethiobiotin, no biotin signal was seen on reverse-phase chromatography or TLC, there was no peak corresponding to the intermediate on reverse-phase chromatography (results not shown) and only one band at the higher R_f position on TLC (Figure 4c, lane 3). When the assay was performed with $[^{35}\text{S}]$ cysteine in the presence of unlabelled dethiobiotin, there were two radioactive peaks, one of which corresponded to biotin and one was in the position characteristic of the intermediate (see Figure 4b). Furthermore a characteristic biotin band was seen on TLC, together with two bands running at a higher R_f (Figure 4c, lane 4). The radioactive material in the peaks corresponding to the intermediate from reverse-phase chromatography of both the standard $[^{14}\text{C}]$ dethiobiotin assay and the $[^{35}\text{S}]$ cysteine assay with unlabelled dethiobiotin was analysed by TLC. In both cases a band with an identical R_f , running in the position characteristic of the intermediate, was seen (Figure 4c, lanes 5 and 6). These results show that sulphur from cysteine was incorporated into the intermediate, thus strengthening the case for the involvement of cysteine as a precursor, if not as the immediate sulphur donor, for biotin. Whether the sulphur atom is first transferred to the iron-sulphur centre of biotin synthase, as suggested in [20], is an intriguing possibility.

Conversion of the intermediate to biotin

The ^{14}C -labelled intermediate was separated from the other radioactively labelled compounds present in reaction mixtures by

Figure 4 Evidence for the incorporation of sulphur into the intermediate

(a) Analysis by reverse-phase FPLC (PepRPC) of the standard ($[^{14}\text{C}]$ dethiobiotin) assay; fraction size 1 ml. (b) Analysis by reverse-phase FPLC (PepRPC) of the $[^{35}\text{S}]$ cystine plus dethiobiotin assay; fraction size 1 ml. (c) TLC: lane 1, zero time control for the $[^{14}\text{C}]$ dethiobiotin assay shown in lane 2; lane 2, standard ($[^{14}\text{C}]$ dethiobiotin) assay; lane 3, assay with $[^{35}\text{S}]$ cystine minus dethiobiotin; lane 4, assay with $[^{35}\text{S}]$ cystine plus dethiobiotin; lane 5, radioactive material from the intermediate peak on PepRPC from the $[^{14}\text{C}]$ dethiobiotin assay; lane 6, radioactive material from the intermediate peak on PepRPC from the $[^{35}\text{S}]$ cystine plus dethiobiotin assay.

reverse-phase chromatography. It was then put into the biotin synthase assay as the only labelled compound; it was converted to biotin (Figure 5). Conversion of the intermediate to biotin was dependent on the presence of *S*-adenosylmethionine.

The fate of *S*-adenosylmethionine and stoichiometry of its use

Having seen that *S*-adenosylmethionine was essential both for the synthesis of the intermediate from dethiobiotin and for the conversion of the intermediate to biotin, we performed further

Figure 5 Conversion of the intermediate into biotin

TLC and autoradiography of assay mixtures. ^{14}C -labelled intermediate was introduced into the standard assay, without $[^{14}\text{C}]$ dethiobiotin. Lane 1, standard assay; lane 2, minus *S*-adenosylmethionine.

experiments with *S*-adenosyl-L-[methyl- ^{14}C]methionine to determine the fate of this compound in the biotin synthase reaction, and also the stoichiometry of its use (Figure 6 and Table 1).

Figure 6 FPLC analysis of reaction mixtures from the assays for determination of stoichiometry of *S*-adenosylmethionine use

Analyses from the assay containing both $[^{14}\text{C-methyl}]$ *S*-adenosylmethionine and $[^{14}\text{C}]$ dethiobiotin, 30 min time point: (a) reverse-phase (PepRPC) column; (b) cation-exchange (MonoS) column. Fraction size 1 ml.

Table 1 Stoichiometry of *S*-adenosylmethionine use in the biotin synthase reaction

Components in the reaction mixtures were separated by FPLC on reverse-phase (PepRPC) and cation-exchange (MonoS) columns, and the radioactivity in the fractions was determined by scintillation counting. To calculate the amount of each substance formed, the percentage of radioactivity in each peak was related to the total amount of each substrate put into the assay. Typical results are shown. The experiment for the 60 min time point was repeated and the reaction components were isolated by ion-exchange chromatography and then analysed by HPLC with on-line radiochemical detection. The results were similar to those shown, but closer to a 2:1 ratio of *S*-adenosylmethionine to biotin.

Time (min)	Amount of substance formed (pmol)					Methionine found (experiment minus control)
	Biotin	Intermediate	Methionine			
			Experiment	Control	Expected*	
15	308	237	1521	637	853 (1:1) 1161 (1:2) 1090 (2:1)	884
30	464	150	1796	590	1078 (1:1) 1542 (1:2) 1228 (2:1)	1206
60	525	170	2379	710	1220 (1:1) 1745 (1:2) 1440 (2:1)	1669

* Methionine expected if one molecule of *S*-adenosylmethionine were used to form the intermediate, and a second molecule in the transformation of the intermediate into biotin (1:1), or if one molecule of *S*-adenosylmethionine were used in the first half-reaction and two in the second (1:2), or vice versa (2:1).

These experiments were made possible only by the observation of the intermediate.

The reaction resulted in the production of [¹⁴C]methionine, the concentration of which increased with incubation time. Control reactions, in which dethiobiotin was omitted, resulted in the production of a constant amount of methionine, and the amounts of the unknown *S*-adenosylmethionine impurity or degradation product that was eluted early from the MonoS column were similar in both the experimental and the control assays. These results support the hypothesis that *S*-adenosylmethionine is cleaved to give methionine and presumably 5'-deoxyadenosine. The stoichiometry of *S*-adenosylmethionine use was compared with that of intermediate and biotin production (Table 1). For the 15 min time point the results are consistent with the consumption of one molecule of *S*-adenosylmethionine from dethiobiotin to the intermediate, and an additional molecule of *S*-adenosylmethionine from the intermediate to biotin, i.e. a total of two *S*-adenosylmethionine molecules per molecule of dethiobiotin converted to biotin. For the 30 and 60 min time points, the ratio increased to nearer three molecules of *S*-adenosylmethionine used per molecule of biotin produced. It is possible that one molecule of *S*-adenosylmethionine is used for the conversion of dethiobiotin to the intermediate and two molecules for the conversion of the intermediate to biotin, or vice versa. A third possibility is that only two molecules of *S*-adenosylmethionine are required for the dethiobiotin to biotin reaction, but as the reaction proceeds it becomes less efficient. This could be due to biotin synthase itself or it is possible that one of the accessory proteins is required for the coupling of *S*-adenosylmethionine cleavage to carbon-sulphur bond formation, and this protein is slowly inactivated during the assay. Our results do not allow us to distinguish between these possibilities, but as the

stoichiometry was two molecules of *S*-adenosylmethionine to one of biotin at the start of the reaction, we propose that the third possibility is more likely.

The molar ratio of biotin produced to biotin synthase in these assays was approx. 1:1, and therefore the ratio of *S*-adenosylmethionine consumed to biotin synthase would also be approx. 2:1. This calculation, and those above, were based on the biotin synthase monomer having a molecular mass of 38.7 kDa and one active site per monomer. The enzyme has been reported to exist as a homodimer [18]; a recent paper [34] cites unpublished evidence that the dimer shares one active site with a subunit-bridging [4Fe-4S] centre. If this is so, then the ratio of *S*-adenosylmethionine to biotin synthase would be 4:1 and the catalytic-centre activities would be double those reported above.

Implications and a working model for the biotin synthase reaction

The results presented here strengthen the connection between biotin synthase and other enzymes that involve the cleavage of *S*-adenosylmethionine to form an enzyme- or substrate-bound radical. If two radical reactions are required by biotin synthase, one to activate the methyl carbon, and a second to activate the methylene carbon, this would be consistent with the use of two molecules of *S*-adenosylmethionine per molecule of biotin formed. The formation of a sulphur-containing intermediate would also support this hypothesis.

Many groups have tried to identify a reaction intermediate for biotin synthase, which would help to elucidate the reaction mechanism [24,25,27,35,36]. Our observation of an intermediate was made possible only by the use of the biotin synthase assay that we developed with [¹⁴C]dethiobiotin as substrate. All other groups working in this area are, to our knowledge, working with microbiological methods of detecting the biotin formed during the reaction, which would be unable to detect the intermediate. The intermediate is produced in the assay in only nanogram amounts, and so far we have been unable to isolate enough to identify it by methods such as NMR and liquid chromatography-MS. These techniques will also show whether only sulphur or the whole cysteine molecule is attached to dethiobiotin. We note that the compound isolated from plant cells described in [27] and proposed to be 9-mercaptodethiobiotin ran at a different position on silica-gel TLC than the compound that we isolated.

On the basis of our results and those of other groups working in this area, we propose the working model shown in Scheme 1. The sulphur from cysteine is bound to a protein in the system, perhaps in an Fe²⁺- and asparagine-dependent process. The conversion of dethiobiotin to the intermediate, and of the intermediate to biotin, follows, each step involving the generation of an enzyme- or substrate-bound radical via the reductive cleavage of *S*-adenosylmethionine to form methionine and 5'-deoxyadenosine, a process that also requires the provision of an electron by the flavodoxin/ferredoxin (flavodoxin) NADP⁺ reductase system. The model does not take the thiamine pyrophosphate-dependent protein, or the fourth protein that we propose to be involved in the system, into account. Pyruvate formate-lyase has an iron-dependent activating enzyme [34], and one of the biotin synthase accessory proteins could have a similar role.

Much more work is required to solve the mystery of biotin synthase. All the protein components need to be identified and purified to homogeneity and their functions determined, artifacts eliminated and a fully defined assay system reconstituted *in vitro*. Biotin synthase itself requires further characterization with respect to the multiple forms resulting from overexpression of the enzyme, the relative activities of these forms, and the number

Scheme 1 Working model for the biotin synthase reaction

Abbreviations: Fld, flavodoxin; Fpr, ferredoxin (flavodoxin) NADP⁺ reductase.

and nature of the active sites. The intermediate that we have described here must be identified, and the identification of the sulphur donor must be conclusive. If the sulphur donor is cysteine, the fate of the rest of the molecule should be studied. All this will then allow the complete reaction mechanism to be elucidated.

Shortly before submission of our manuscript, a paper on the stoichiometry of *S*-adenosylmethionine use in the biotin synthase reaction was published [37]. Our results confirm the data and conclusions in that paper, but provide further information about the reaction, especially with regard to characterization of the reaction intermediate, and the requirement of *S*-adenosylmethionine both for its synthesis and conversion to biotin. This intermediate was probably present but not taken into account in [37], because it would not be detected by the microbiological assay for biotin. This could account, at least partly, for the 3:1 ratio of *S*-adenosylmethionine to biotin observed in [37], because *S*-adenosylmethionine would also be used to synthesize the intermediate.

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