Mechanism of Dethiobiotin Synthetase—Characterisation of the 8-Aminocarbamate of (7*R*,8*S*)-7,8 Diaminononanoate as an Enzyme-bound Intermediate

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Enzymatically catalysed formation of the *ureido* ring of dethiobiotin **2** from (7*R*, 8*S*)-7,8-diaminononanoate **3** by purified *E. coli* dethiobiotin synthetase involves the formal hydrolysis of one equivalent of ATP, a combination of substrate loading and trapping experiments show that the carbamate **5** is an intermediate in the synthesis of dethiobiotin from **3**.

Dethiobiotin synthetase (EC 6.3.3.3) is the penultimate enzyme of biotin **1** synthesis in *E. coli*, *Bacillus sphaericus* and in *Pseudomonas graveolens*.[1] There is also evidence that this enzyme is produced in higher plants.[2] Earlier work on the *E. coli* enzyme by Eisenberg and coworkers[3] showed that the enzyme catalyses the formation of the *ureido* ring of dethiobiotin **2** from 7,8-diaminononanoate **3**, carbon dioxide and ATP [step i, Scheme 1]. This reaction is particularly unusual in that dethiobiotin synthetase is one of the few carboxylation enzymes that utilise carbon dioxide rather than hydrogencarbonate anion as the C_1 donor. However, in common with most enzymes of cofactor biosynthesis, dethiobiotin synthetase is expressed at extremely low levels in normal cells and a detailed examination of its mechanism has awaited the development of overexpression systems capable of producing sufficient protein for mechanistic studies.

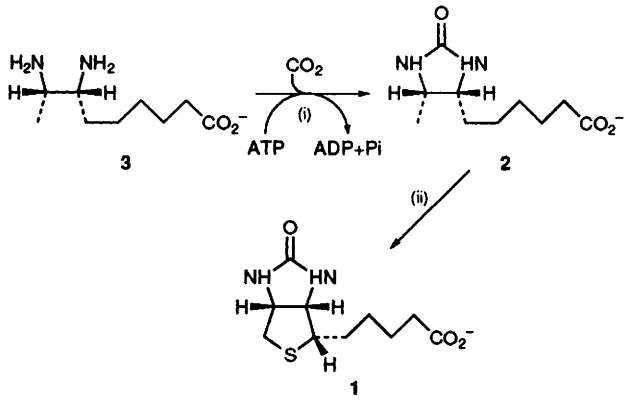
We have recently cloned the *E. coli* dethiobiotin synthetase gene (*bio*D) from λ*bio*256 into a modified pBR322 derived plasmid, overexpressed the gene and purified the protein.[4] Here we describe experiments, conducted with highly purified dethiobiotin synthetase, which provide evidence that the first covalent bond formation step of the mechanistic sequence in the synthesis of dethiobiotin **2** from (7*R*, 8*S*)-7,8-diaminononanoate **3** is the formation of the 8-amino carbamate **5**.

The stoichiometry of the overall reaction catalysed by the enzyme (as shown in Scheme 1) was determined using a linked assay by which the ATP → ADP conversion was monitored by assay of ADP produced (*via* phosphoenolpyruvate → pyruvate → lactate synthesis using a pyruvate synthetase/lactate dehydrogenase coupled system). The average of multiple experiments showed that 1.2 ± 0.1 mol of ATP were utilised for each mol of **2** produced, indicating that a single ATP molecule is formally hydrolysed to ADP and phosphate per molecule of dethiobiotin synthesised. This result indicates that a single ATP activation step is involved in formation of two carbon–nitrogen bonds.

An indication of the order in which the three substrates, 7,8-diaminononanoate **3**, carbon dioxide and ATP are utilised by dethiobiotin synthetase was obtained from a series of competition experiments (Table 1). In these experiments the enzyme was preincubated with either [^{14}C]hydrogencarbonate† alone or with [^{14}C]hydrogencarbonate in combination with either **3** (as its sulfate) or ATP. After five minutes chase solutions containing an excess of the other substrates and unlabelled hydrogencarbonate were added and the reactions was quenched after a further 30 s by addition of trichloroacetic acid. Examination of the radioincorporation from [^{14}C]hydrogencarbonate into the dethiobiotin **2** formed showed that significantly higher incorporations were obtained where 7,8-diaminononanoate **3** was preincubated with the enzyme and [^{14}C]hydrogencarbonate indicating a mechanistic sequence in which reaction between carbon dioxide and 7,8-diaminononanoate **3** precedes involvement of ATP.

These results can best be rationalised by a reaction sequence which implicates enzymatically catalysed formation of an intermediate carbamate derivative such as the species **4** or **5** from 7,8-diaminononanoate **3** and carbon dioxide [routes (a) and (b) in Scheme 2].

Distinction between the two possible intermediate species, **4** and **5**, was provided by a trapping experiment. The methylcarbamates **6** and **7** were synthesised from (7*R*, 8*S*) 7,8-diaminononanoic acid **3** by reaction with methylchloroformate.‡ Purified *E. coli* dethiobiotin synthetase (3.6 mg, 1.1 U) was incubated with [^{14}C]hydrogencarbonate (20 μCi, 5.4Ci/mol^{-1}) and (7*R*,8*S*) 7,8-diaminononanoate sulfate **3** (1.1 mmol dm^{-3}) in 50 mmol dm^{-3} tris-HCl (pH 7.2, 3 cm^3). After incubation for 10 min the solution was frozen at −78 °C and freeze dried. The lyophilised powder was suspended in ethanol, a mixture of compounds **6** and **7** (1 : 1, 15 mg) added to the suspension and the mixture was methylated with an excess of ethanolic diazomethane. The reaction mixture was filtered to remove the denatured protein, evaporated to dryness and the residue subjected to preparative TLC on silica to afford the esters **6a** and **7a**. Scintillation counting of purified samples of the esters showed an ^{14}C incorporation ratio of 95 : 5 for **7a**:**6a**.



Scheme 1. The reactions catalysed by dethiobiotin synthetase (i) and biotin synthase (ii)

Table 1 Sequence of substrate utilisation by dethiobiotin synthetase

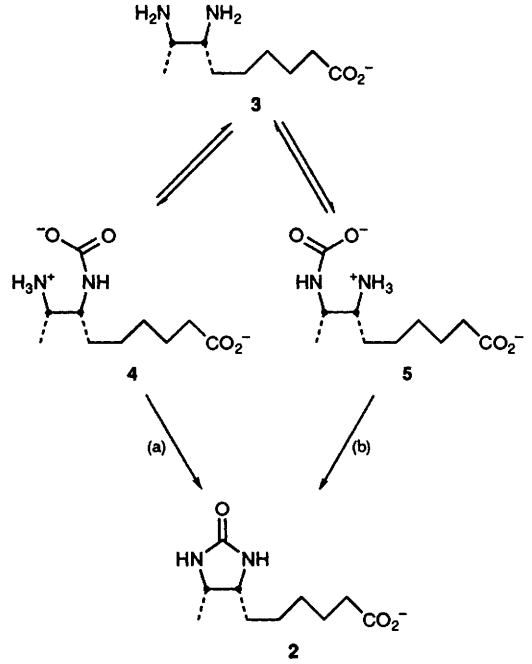
Expt.	Preincubation[a]	Chase solution[b]	^{14}C Incorporation into dethiobiotin **2** (dpm)[c]
1	[^{14}C]HCO_3^-	**3** + ATP	1550 ± 200
2	[^{14}C]HCO_3^- + **3**	ATP	13000 ± 1000
3	[^{14}C]HCO_3^- + ATP	**3**	2200 ± 220

All solutions were prepared in tris-HCl buffer (50 mmol dm^{-3}, pH 7.2) containing $MgSO_4$ (2 mmol dm^{-3}) and dithiothreitol (2 mmol dm^{-3}).

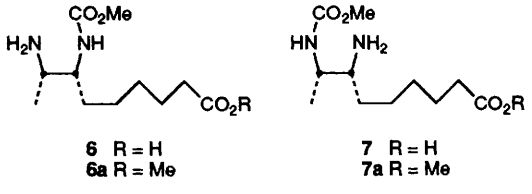
[a] Preincubation solutions (3 cm^3) contained; dethiobiotin synthetase (0.5 mg, 0.16 U) and [^{14}C]sodium hydrogencarbonate (10-μCi, 54 μCi mol^{-1}). In experiments 2 and 3 respectively; **3** sulfate (10 mmol dm^{-3}) and disodium ATP (10 mmol dm^{-3}) were added to the preincubation solutions. [b] Chase solutions (1 cm^3) contained; sodium hydrogencarbonate (10 mmol dm^{-3}) and **3** sulfate (10 mmol dm^{-3}) and/or disodium ATP (10 mmol dm^{-3}). [c] Incorporations are the average of three experiments.

The result implies that the carbamate **5** is the favoured enzyme bound intermediate formed in the first step of the dethiobiotin synthetase reaction and that formation of this intermediate does not require participation of ATP as a cofactorial activating agent. Since there are numerous examples of the formation of carbamic acid salts of aliphatic amines by reaction with carbon dioxide[5] it could be considered reasonable that formation of the carbamate **5** might be a purely chemical reaction preceding enzyme binding of the substrates. However, in control experiments in the absence of dethiobiotin synthetase we were unable to detect formation of **5** (or **4**) from **3**, implying that the reaction requires enzymatic catalysis. Thus, the first covalent bond forming step of the reaction sequence catalysed by the enzyme appears to involve formation of the carbamate **5** from **3** as indicated in Scheme 2.

Two basic mechanistic questions remain unanswered. The first is whether reaction of carbon dioxide with dethiobiotin synthetase precedes formation of the carbamate **5**. Formation of a Mg^{2+} stabilised enzyme carbamate intermediate, with for example an enzyme lysine residue, would have analogy to the first step of the reaction sequence catalysed by ribulose-1,5-diphosphate carboxylase.[6] Alternatively, the formation of the carbamate **5** could occur directly by reaction of enzyme bound **3** with carbon dioxide. The second question concerns the mechanism of the final C–N bond forming step (*i.e.* **5** → **2**). In this reaction, which appears to require ATP participation, activation of the intermediate carbamate for ureido ring closure could be achieved *via* either an ADP or a phosphoric acid mixed anhydride.



Scheme 2



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Footnotes

† At the enzyme concentrations and pH used in these experiments the equilibrium, $HCO_3^- + H^+ = CO_2 + H_2O$, maintains a sufficiently high CO_2 concentration (*ca.* 2 mmol dm^{-3}) for the reaction to proceed at close to maximum rate. (See W. A. Laing, W. L. Ogren and R. H. Hageman, *Biochemistry*, 1975, **14**, 2269 and D. Cooper, D. Filmer, M. Wishnick and M. D. Lane, *J. Biol. Chem.*, 1969, **244**, 1083) At these enzyme concentrations addition of carbonic anhydrase to the incubation does not significantly alter the rate of reaction.

‡ Concordant spectral and analytical date were obtained for all compounds synthesised.

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