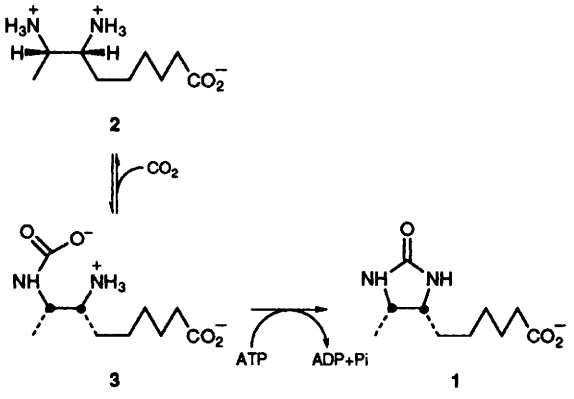
The Mechanism of *Escherichia coli* Dethiobiotin Synthetase—the Closure of the *ureido* Ring of Dethiobiotin involves Formation of a Carbamic-phosphate Mixed Anhydride

Robert L. Baxter* and Helen C. Baxter

Edinburgh Centre for Molecular Recognition, Department of Chemistry, The University of Edinburgh, West Mains Road, Edinburgh UK EH9 3JJ

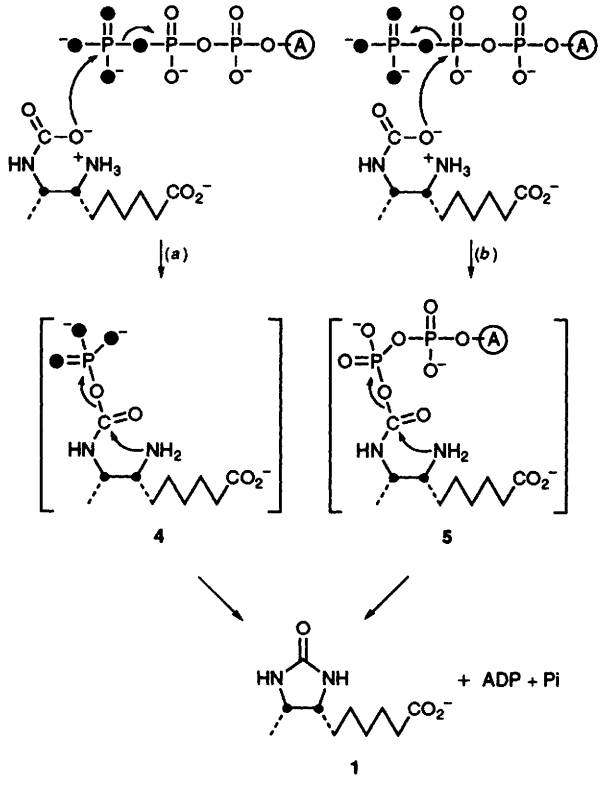
The final intermediate in the enzymatic synthesis of the *ureido* ring of D-dethiobiotin **1** from (7*R*,8*S*)-7,8-diaminononanoate **2** catalysed by *E. coli* dethiobiotin synthetase is the phosphoric acid anhydride **4** of the carbamate **3**.

Dethiobiotin synthetase (E.C. 6.3.3.3) is the penultimate enzyme of biotin synthesis, catalysing the formation of the *ureido* ring of D-dethiobiotin **1** from (7*R*,8*S*)-7,8-diaminononanoic acid **2**, carbon dioxide and ATP.[1] Recently, we have cloned and overexpressed the *E. coli* enzyme with the result that detailed studies on its mechanism of action have now become realisable. In a previous communication[2] we showed that purified *E. coli* dethiobiotin synthetase catalyses the formation of the carbamate **3** from (7*R*,8*S*)-7,8-diaminononanoate **2** and carbon dioxide *in vitro* in the absence of ATP. This indicates that formation of the carbamate **3** is the first step in the reaction sequence as shown in Scheme 1. Furthermore, we demonstrated that only one equivalent of ATP is formally hydrolysed to ADP and phosphate in the overall reaction.[2] A logical inference from these facts is that the ring closure to form the *ureido* ring of the product, D-dethiobiotin **1**, requires formation of a mixed anhydride of the carbamate **3**. In this communication we show that the phosphoric anhydride of **3** is an enzymatic intermediate in the reaction sequence catalysed by the *E. coli* enzyme.



Scheme 1 The reaction catalysed by *E. coli* dethiobiotin synthetase

Since formation of the *ureido* ring of dethiobiotin from the carbamate intermediate **3** involves formal hydrolysis of ATP to ADP and inorganic phosphate it is a reasonable premise that either a phosphoric or an ADP mixed anhydride (**4** and **5** in Scheme 2) is involved as a transient intermediate in the activation of the carbamate function for ring closure. Nucleophilic attack on the activated carbamate carbonyl, of either of these species, by the 7-amino nitrogen would afford the product **1** with displacement of phosphate or ADP, respectively. However, distinction between these mechanisms is not possible on the basis of product or kinetic analysis of the overall enzymatic reaction. To elucidate the nature of the activated intermediate we prepared γ-[$^{18}O_4$]-γβ-[^{18}O]ATP as



Scheme 2 Possible carbamic acid mixed anhydride intermediates in *ureido* ring formation; the fate of ^{18}O enriched oxygens from γ-[$^{18}O_3$]-γβ-[^{18}O]ATP is shown. (A = adenosine; ● = ^{18}O; O = ^{16}O).

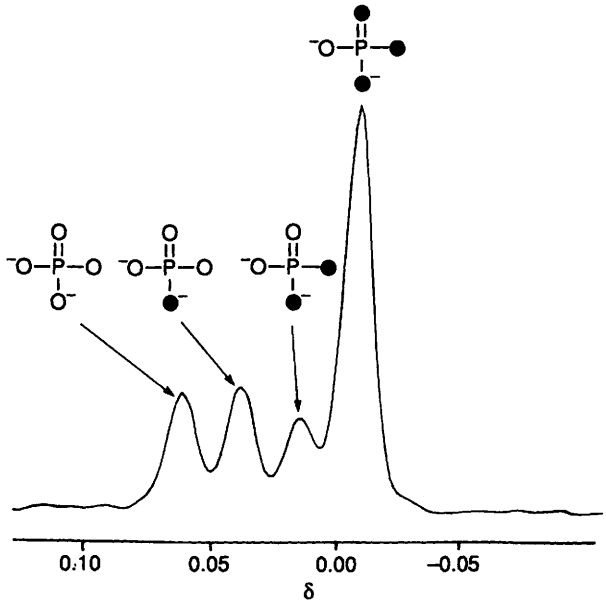


Fig. 1 146 MHz ^{31}P NMR spectrum of the inorganic phosphate produced in the *E. coli* dethiobiotin synthetase catalysed reaction; **2** + γ-[$^{18}O_3$]-γβ-[^{18}O]ATP + CO_2 → **1** + ADP + Pi. [pH 7.2, 10000 transients, aquisition time 1.02 s, spectral width 2000 Hz, line broadening factor −1.5 Hz]. Signals are referenced to external 80% H_3PO_4. The ^{18}O shift was δ −0.023 ± 0.001 and the chemical shift of unenriched phosphate was verified by spiking the sample with NaH_2PO_4.

a substrate and have examined the distribution of ^{18}O in the products formed in the dethiobiotin synthetase reaction. The logic of this experiment is apparent from Scheme 2—if an ADP anhydride is involved in the reaction, as in pathway (*b*), [$^{18}O_4$]phosphate and unenriched ADP would be produced but if a phosphoric anhydride is involved, as in pathway (*a*), [$^{18}O_3$, ^{16}O]phosphate and β-[^{18}O]ADP would be produced.

Synthesis of γ-[$^{18}O_3$]-γβ-[^{18}O]ATP (65% yield from $H_2{}^{18}O$, *ca.* 98 atom% $^{18}O_4$ on the basis of negative ion FAB MS) was carried out from $Na_2HP^{18}O_4$[3] using a modification of the methodology described by Hassett *et al.*[4] FPLC, MS, and ^{31}P NMR assays of the synthetic material showed no detectable contamination with ADP or inorganic phosphate. (7*R*,8*S*)-7,8-Diaminononanoic acid sulphate (**2**, 30 mg, 0.12 mmol) and γ-[$^{18}O_3$]-γβ-[^{18}O]ATP disodium salt (3.6 mg, 0.07 mmol) were incubated with purified *E. coli* dethiobiotin synthetase (3.4 mg, 0.75 U) in tris buffer (50 mmol dm^{-3}, pH 7.2, 3 cm^3) containing $MgSO_4$ (2 mmol dm^{-3}) and $NaHCO_3$ (10 mmol dm^{-3}). After 3 h the protein was removed by gel permeation, the deproteinated solution was concentrated by lyophilisation and the distribution of ^{18}O in the products examined by ^{31}P NMR. The phosphate resonance region of the ^{31}P NMR (Fig. 1) shows a principal peak corresponding to [$^{18}O_3$, ^{16}O]phosphate indicating that it is formed *via* pathway (*a*) in Scheme 2. This is corroborated by the observation of a single ^{18}O shift for the β-P resonance of the ADP formed.

This result, taken in conjunction with earlier work from this laboratory,[2] is consistent with an overall mechanism for the *E. coli* dethiobiotin synthetase catalysed reaction sequence which involves three bond forming steps; formation of the carbamate **3** from CO_2 and (7*R*,8*S*)-7,8-diaminononanoic acid **2**, reaction of **3** with ATP to give the phosphoric anhydride **4** and ADP and finally nucleophilic displacement of phosphate to afford dethiobiotin. Studies are underway to elucidate the identity of the amino acid residues of the enzyme structure involved in these transformations.

We are grateful to the SERC for support of this work.

Received, 30th December 1993; Com. 3/07620B

References

1 K. Krell and M. A. Eisenberg, *J. Biol. Chem.*, 1970, **245**, 6558; Y. Izumi, Y. Tani and H. Yamada, *Bull Inst. Chem. Res., Kyoto*, 1980, **58**, 434 and references cited therein.
2 R. L. Baxter, A. J. Ramsey, L. A. McIver and H. C. Baxter, *J. Chem. Soc., Chem. Commun.*, 1994, 559.
3 $Na_2HP^{18}O_4$ (*ca.* 98 atom% 18O) was prepared from $H_2{}^{18}O$ as described by J. M. Risley and R. L. Van Etten, *J. Labelled Cpds. Radiopharm.*, 1978, 15, 533.
4 A. Hassett, W. Blatter and J. R. Knowles, *Biochemistry*, 1982, **21**, 6336.