The Purification and Properties of Dethiobiotin Synthetase*

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SUMMARY

The levels of dethiobiotin synthetase in Escherichia coli have been increased 6-fold over the wild type when prepared from a λ-lysogen carrying the bioD gene under phage control. The purification procedure employed gave a nearly homogeneous preparation, over 90% pure, with a 190-fold increase in specific activity. The enzyme has a molecular weight of about 42,000 and exists as a dimer. The stoichiometry of the reaction indicates that 1 mole each of ATP and CO₂ are required and that ADP is the end product of the reaction. The Kₘ values for each of the substrates were determined and ADP was found to be a competitive inhibitor. Only CTP can partially replace ATP while dianinobiotin is only 37% as effective as 7,8-diaminopelargonic acid. CO₂ is the substrate in this reaction rather than bicarbonate (HCO₃⁻). Carbamyl phosphate which gives some stimulation in cell-free extracts is inactive with the purified enzyme.

In the scheme for biotin biosynthesis postulated by Rolfe and Eisenberg (1), 7,8-diaminopelargonic acid was placed between 7-keto-8-aminopelargonic acid and dethiobiotin. This product-precursor relationship between DAPA¹ and dethiobiotin was based on indirect evidence obtained from cross-feeding experiments and excretion patterns of these intermediates in four groups of biotin auxotrophs. The group III mutant grew on either dethiobiotin or biotin and excreted DAPA and KAP into the growth medium. This evidence indicated that these mutants lacked the enzyme, dethiobiotin synthetase, required for the formation of the ureido ring of dethiobiotin. In subsequent studies with resting cells and cell-free extracts (2, 3), the direct conversion of DAPA to dethiobiotin was demonstrated. The substrates for this reaction included HCO₃⁻, ATP, and Mg²⁺. The enzyme was subject to repression by biotin. Similar results with cell-free extracts of Escherichia coli were also obtained by Pai (4).

As the result of fine structure genetic mapping (5), seven complementation groups were obtained for the biotin A operon and ordered as follows: αβλ A, B, E, F, G, C, D. The group III mutants were found to consist of one complementation group, D. The enzyme dethiobiotin synthetase was present in all mutants except members of group D and a deletion mutant T50-1. Thus, it was concluded that bioD must be the structural gene coding for dethiobiotin synthetase.

The present study describes a procedure for increasing the level of dethiobiotin synthetase in E. coli to permit its isolation. The enzyme was purified to near homogeneity and some of its physical and kinetic properties delineated.

EXPERIMENTAL PROCEDURE

Materials

d-Dethiobiotin was synthesized from d-biotin by the method of Melville et al. (6). It contained less than 0.02% biotin as determined by bioassay with strain bioB105 (see “Bioassay Organisms”). d-7,8-Diaminopelargonic acid sulfate was synthesized from d-dethiobiotin as described by du Vigneaud et al. (7). It contained less than 0.001% dethiobiotin as determined by bioassay with strain bioD302. Diaminobiotin sulfate was prepared according to the method of Hofmann, Melville, and du Vigneaud (8), and was purified by column chromatography on Bio-Rad AG 1-X8. After two crystallizations from a mixture of methanol and water, the material was found to contain less than 0.02% biotin as determined by bioassay with strain bioD302.

The following commercial products were utilized: L-leucine, diltitiun carbamyl phosphate, bovine serum albumin, chymotrypsin A (bovine pancreas), and cytochrome c (horse) from Mann; carbonic anhydrase and avidin from Worthington; crystalline serum albumin from Penfex; AMP from Sigma; ADP from Boehringer Mannheim; ATP and ITP from P-L Biochemicals; S-34C-tetralithium ATP and NaH¹⁴CO₃ from New England Nuclear; GTP, CTP, and UTP from Schwarz Bioresearch; Whatman DE 52 previously swollen microgranular DEAE-cellulose from Reeve-Angel Company, New York, New York; Bio-Gel HT hydroxyalkylate, Bio-Gel P-150 (100 to 200 mesh) polyacrylamide gel beds, and AG 1-X8 (200 to 400 mesh) ion exchange resin from Bio-Rad, Richmond, California. All other chemicals were reagent grade.

Culture Media

The basal liquid medium has been described earlier (2). Solid medium was prepared by adding 2% Bacto-agar to the above.

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² The abbreviations used are: DAPA, 7,8-diaminopelargonic acid; KAP, 7-keto-8-aminopelargonic acid; SDS, sodium dodecyl-sulfate.
Tryptone maltose broth (TM broth) contained per liter of glass-distilled water: 10 g of Bacto-tryptone, 5 g of NaCl, 2.47 g of MgSO$_4$·7H$_2$O, 1.0 mg of thiamine hydrochloride, and 2 g of maltose. The solution was adjusted to pH 7.0 with NaOH before autoclaving.

Bioassay Organisms

The organisms utilized for bioassay and bioautography have been described elsewhere (2). Strain bioD302 was routinely used for dethiobiotin determination and strain bioB105 for dianisomobiotin and biotin.

Methods

Dethiobiotin Synthetase Activity—Except where noted dethiobiotin synthetase activity was determined in 1-ml volume reaction mixtures which contained a final concentration of 0.15 mM Tris chloride buffer, pH 7.7; 10 mM ATP; 10 mM MgCl$_2$; 0.125 mM DAPA; and 40 mM NaHCO$_3$. After the mixtures had been equilibrated at 37°C in a water bath, the reaction was started by addition of the enzyme preparation. At the end of 10 min the reaction was stopped by the addition of 0.5 ml of 12% trichloroacetic acid. Samples of the reaction mixture were taken for dethiobiotin assay. Enzyme units are defined as nanomoles of dethiobiotin synthesized per 10 min.

Dethiobiotin Activity in Disc Gel Electrophoretograms—The electrophoretograms were cut into slices of approximately 1.5 to 2.0 mm thickness. The slices were sandwiched between 7-mm filter paper penicillin assay discs and placed onto the surface of a minimal medium plate inoculated with strain bioD302. The uppermost disc was moistened with 20 µl of the standard dethiobiotin assay solution described above. The plate was inverted and incubated at 37°C for 24 hours. The mean diameter of the growth area around each disc at the end of the incubation period was taken as a measure of dethiobiotin concentration.

Electrophoresis—Disc gel electrophoresis was performed according to the method of Ornstein (9) and Davis (10). Under the conditions used, the protein was concentrated on the stacking gel at pH 8.9 and separated at pH 9.5. For visualization of the protein bands, the gels were stained with Amido black. When required, the stained gels were scanned with a Gilford recording spectrophotometer at 550 nm.

SDS gel electrophoresis was carried out according to the method of Weber and Osborn (11).

Chromatography—Paper chromatography was carried out alone or combined with electrophoresis essentially as previously described (1). The solvent system used was 1-butanol-glacial acetic acid-water, 60:15:25 (v/v/v). The separation of 14C-labeled ATP, ADP, and AMP was carried out by column chromatography on AG 1-X8 resin according to the procedure of Cohn and Carter (12). The eluant was monitored continuously with an Isco model UAT ultraviolet analyzer (Instrumentations Specialties Company) at 254 nm and the fractions containing each compound were pooled and reduced to dryness. The residues were taken up in 3 ml of water and 0.2 ml samples were counted in a mixture of 2.5 ml of absolute alcohol and 10 ml of toluene liquid scintillation counting fluid.

Determination of Radioactivity—Radioactivity was determined with a Packard Tri-Carb liquid scintillation counter. The toluene liquid scintillation counting solution contained per liter of toluene: 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene and 6 g of 2,5-diphenyloxazole (Packard Instrument Company). The observed radioactivity was converted to absolute radioactivity by the use of a 14C-toluene internal standard.

Protein Analysis—Protein concentration was determined by either the biuret method (13) or the method of Lowry et al. (14). Crystalline bovine serum albumin was used as a standard.

Purification of Dethiobiotin Synthetase

Enriched Source of Enzyme. In preliminary experiments it became apparent that even though the wild-type enzyme levels could be increased 3-fold by derepression of biotin auxotrophs, large quantities of cells would still be required to obtain homogeneous enzyme preparation for subsequent studies. We therefore turned our attention to means of further increasing the levels of dethiobiotin synthetase.

A convenient source of the enzyme was found to be an E. coli K-12 biotin auxotroph, lysogenic for bacteriophage λ. The genotype of the organism designated strain N821 (1) is λ-prophage carrying a portion of the E. coli biotin operon including the D gene but not the bio operator region, thus placing the D gene under λ control. The BL57 phage has two pertinent mutations: (a) cl$_{58}$, a heat-labile repressor, which when inactivated at temperatures above 39°C allows phage induction to occur (16), and (b) S$_{5}$, a suppressor-sensitive mutation which results in defective ability to lyse the host cell. Since host cell lysis is impaired, induction leads to replication, transcription, and protein synthesis for extended periods of time. One result of these events

The authors wish to thank Dr. Max Gottesman for his helpful suggestions and for providing this lysogen.

FIG. 1. Kinetics of dethiobiotin synthetase activity after induction of strain N821 lysogen. Five 300-ml Erlenmeyer flasks containing 100 ml of TM broth were inoculated with 1 ml of an overnight culture of strain N821 cells. The flasks were then incubated at 34°C with vigorous shaking. When the cultures had reached the mid log phase of growth, they were induced by heating to 41°C for 20 min. After induction they were cooled to 34°C and the incubation was continued. At the times indicated the cultures were rapidly cooled in an ice bath and the cells were harvested by centrifugation. Dialyzed cell-free extracts were prepared and samples containing 1 mg of protein were assayed as described under "Methods," except that the DAPA concentration was 0.05 mM.
in strain N821 is a large intracellular accumulation of dethiobiotin synthetase enzyme.

**Optimal Conditions for Enzyme Formation**—The effect of prophage induction on dethiobiotin synthetase activity in the strain N821 lysogen is shown in Fig. 1. Upon induction there was an immediate rise in specific activity which, by the end of 5 hours, was equal to a 6-fold increase over the preinduced enzyme level. The induced cell-free extracts from strain N821 were 3-fold richer in enzyme than comparable derepressed A enzyme level. The induced cell-free extracts from strain N821 were 3-fold richer in enzyme than comparable derepressed A enzyme level.

**First Ammonium Sulfate Fractionation**—All steps in the purification procedure were carried out at 0°C in the presence of 0.003 M 2-mercaptoethanol except where indicated.

Cell-free extracts were prepared according to the procedure previously described (2), from cells harvested 5 hours after induction. After dialysis in 0.05 M Tris buffer, pH 7.0, at 25°C, sufficient powdered ammonium sulfate was slowly added to 30 ml of the extract with continuous stirring to give 30% saturation. The stirring was continued for another 30 min, and the suspension was centrifuged at 21,000 × g for 1 hour. Ammonium sulfate was added to the supernatant fraction (517 ml) to give 45% saturation and after 30 min the suspension was centrifuged. The supernatant was discarded and the precipitate was dissolved in 200 ml of 0.15 M Tris chloride buffer (pH 7.0) and dialyzed for 3 hours against two changes of the same buffer.

**Protamine Sulfate Treatment**—A volume of 50 ml of protamine sulfate (16 mg per ml) in 0.05 M Tris chloride buffer (pH 7.0) was slowly added with stirring to 200 ml of the 30 to 45% ammonium sulfate fraction which contained 22 mg of protein per ml. The mixture was kept cool in an ice bath and stirred continuously for 20 min. The resulting suspension was centrifuged at 21,500 × g for 60 min and the supernatant fluid was decanted.

**Second Ammonium Fractionation**—The supernatant fluid from the protamine sulfate step (256 ml containing 14.5 mg of protein per ml) was submitted to a second ammonium sulfate fractionation exactly as described for the first ammonium sulfate step. The resulting precipitate was dissolved in 35 ml of 0.05 M Tris chloride buffer (pH 7.0) and contained 89 mg of protein per ml.

**First DEAE-cellulose Column Chromatography**—The second ammonium sulfate fraction was dialyzed for 4 hours against 0.01 M Tris chloride buffer (pH 7.0) containing 0.1 M NaCl. This material was then chromatographed on a column (2.5 × 54.5 cm) of DEAE-cellulose which had been equilibrated with the same buffer. The column was eluted with a linear gradient of 0.1 M to 0.3 M NaCl in a total volume of 3 liters of Tris chloride buffer, pH 7.0. Fractions of 10 ml volume were collected at a flow rate of 1 ml per min and assayed for biological activity.

**Second DEAE-cellulose Column Chromatography**—The first DEAE fraction was rechromatographed as described above except that the column was eluted with a linear gradient of 0.02 to 0.2 M NaCl in a total volume of 2 liters of Tris chloride buffer. Fractions of 15 ml were collected at a flow rate of 1 ml per min. Again only one biologically active component was observed (Fractions 28 to 42). The enzyme was eluted over a NaCl concentration range of 0.11 to 0.13 M. The fractions containing enzyme activity were combined and the protein was precipitated by adding sufficient powdered ammonium sulfate to give 45% saturation in the manner previously described. The precipitate was dissolved in the Tris chloride buffer to give a volume of 25 ml and this solution contained 34.2 mg of protein per ml.

**Hydroxylapatite Column Chromatography**—The second DEAE fraction was dialyzed against 0.01 M phosphate buffer, pH 7.45. A volume of 5.6 ml of this solution was chromatographed on a column (1.5 × 10 cm) of Bio-Gel HTP hydroxylapatite which had been equilibrated with the same buffer. The column was eluted with a linear gradient of 0.01 to 0.04 M phosphate buffer in a total volume of 100 ml. Fractions of 1.7-ml volume were collected at a flow rate of 7.1 ml per hour. The bulk of the biological activity was obtained between Fractions 14 to 20. Disc gel electrophoretograms of the combined fractions are shown in Fig. 2. At the higher protein concentration a major band is observed with two minor bands. When duplicate gels were prepared and one assayed for enzyme activity by the slice technique described under "Methods," and the other stained and scanned, the results shown in Fig. 3 were obtained. The coincidence between the dethiobiotin synthetase activity pattern and the densitometric scan of the electrophoretogram is evident.

Over 90% of the enzymatic activity of this preparation was lost when the preparation was stored overnight at −20°C. This was accompanied by protein precipitation and suggested that the enzyme was cold-labile. Storage at 0°C decreased the rate of inactivation while at 4°C the enzyme was sufficiently stable to permit further studies. Since this behavior was in marked contrast to preparations which had been purified through the DEAE step, one could not exclude the possibility that enzyme inactivation was occurring during the hydroxylapatite chro-
FIG. 3. Dethiobiotin synthetase activity and protein distribution of disc gel electrophoretograms. Duplicate samples (25 µg of protein) of the combined peak fractions from hydroxylapatite column chromatography at 0° were submitted to disc gel electrophoresis. Dethiobiotin synthetase activity and protein concentration were determined as described under "Methods." The arbitrary enzyme units on the ordinate are defined as: analogue (mcau growth area diameter in centimeters).

Matography at 0°. Therefore a preparation was chromatographed at room temperature giving the profile shown in Fig. 4. This column was eluted in one step with 0.005 M phosphate buffer (pH 7.5) containing 0.006 M 2-mercaptoethanol. There was no apparent loss in enzyme activity. Disc gel electrophoresis of the contents of the tube with maximal activity and three tubes on either side of it showed again one or two minor bands comprising less than 5% of the total protein. The major band had a mobility identical with the major band observed in Fig. 2.

Table I summarizes the results of this purification procedure from a cell-free extract of induced strain N821 cells.

RESULTS

As the result of the purification procedure described above, it was possible to obtain about a 2% yield of the original activity with a 190-fold enrichment. The low yield and relative instability of the enzyme precluded further efforts to remove the small percentage of contaminating protein at this time.

The experiments described below utilized enzyme preparations which had been purified through the hydroxyapatite step and had specific activities of 800 to 1000 enzyme units per mg of protein.

Molecular Weight and Subunit Structure—The molecular weight of the enzyme was estimated by gel filtration chromatography using a column of porous polyacrylamide beads (17). Dethiobiotin synthetase was co-chromatographed with three enzymatically inactive proteins: bovine serum albumin, ovalbumin, and cytochrome c with mol wt of 67,000, 45,000, and 25,000, respectively. The results of this experiment are shown in Fig. 5, where elution volume is plotted as a function of molecular weight on a logarithmic scale. Only one enzymatically active component was observed and this corresponded to a molecular weight of 41,500. In a second determination, using bovine serum albumin, ovalbumin, and carbonic anhydrase (mol wt 29,000) as standards, the molecular weight of dethiobiotin synthetase was estimated to be 42,000.

The enzyme preparation was also subjected to SDS gel electrophoresis to determine if the enzyme is composed of subunits and if so, whether or not they are identical in molecular weight. The results (Fig. 6) compare the electrophoretic mobility of the protein preparation with that of suitable marker proteins. On the left is a gel containing from the top: bovine serum albumin, ovalbumin, and cytochrome c (mol wt 13,000). The center gel shows the dethiobiotin synthetase preparation and on the right, the gel contains a mixture of the marker proteins and the enzyme preparation. It can be seen that the enzyme preparation shows one major band with a barely visible minor component. A plot of the mobility of each protein against the molecular weight on a logarithmic scale indicated that the major band corresponds to a molecular weight of 25,500 and the minor band

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Total protein</th>
<th>Dethiobiotin synthetase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg</td>
<td>units</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>units/mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>Dialyzed cell-free extract of induced strain N821 cells</td>
<td>400</td>
<td>16,370</td>
<td>63,500</td>
</tr>
<tr>
<td>1st (NH₄)₂SO₄</td>
<td>250</td>
<td>5,900</td>
<td>70,820</td>
</tr>
<tr>
<td>Protamine sulfate</td>
<td>250</td>
<td>3,710</td>
<td>87,340</td>
</tr>
<tr>
<td>2nd (NH₄)₂SO₄</td>
<td>75</td>
<td>3,120</td>
<td>88,075</td>
</tr>
<tr>
<td>1st DEAE-cellulose column</td>
<td>25</td>
<td>650</td>
<td>50,000</td>
</tr>
<tr>
<td>2nd DEAE-cellulose column</td>
<td>7.5</td>
<td>50</td>
<td>12,012</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>6.3</td>
<td>15</td>
<td>1,229</td>
</tr>
</tbody>
</table>

FIG. 4. Hydroxylapatite column chromatography of dethiobiotin synthetase at ambient temperature. A volume equal to 5.6 ml of dethiobiotin synthetase solution which had been purified through the second DEAE-cellulose step was dialyzed against 0.005 M phosphate buffer, pH 7.45, containing 0.006 M 2-mercaptoethanol. After dialysis, the solution was applied to a column (1.5 X 10 cm) of Bio-Rad HT hydroxyapatite which had been equilibrated with the same buffer. The column was eluted with the same buffer mixture as above at a flow rate of 7 ml per hour.

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FIG. 5. Molecular weight determination of dethiobiotin synthetase. Gel filtration chromatography was carried out at ambient temperature on a column (1.3 × 150 cm) of Bio-Gel P-150 (100 to 200 mesh) which had been equilibrated with 0.05 M phosphate buffer, pH 7.5, containing 0.003 M 2-mercaptoethanol. A solution containing 200 µg of dethiobiotin synthetase and 4 mg each of bovine serum albumin, ovalbumin, and chymotrypsinogen A in the same buffer was applied to the column which was then eluted at a rate of 13.5 ml per hour. The protein molecular weight standards were monitored by measuring the optical density at 280 nm and dethiobiotin synthetase was monitored by disc assay.

to a molecular weight of 42,000, which is the same as that determined for the native protein. The subunits appear to be identical in molecular weight since only one major band is present.

A second SDS gel electrophoresis experiment was performed using bovine serum albumin, ovalbumin, and chymotrypsinogen A as standards. The results indicated a single component with a molecular weight of 23,500. Thus, the dethiobiotin synthetase subunit has an apparent mean molecular weight of 24,500.

**Kinetic Studies**—The results of experiments to determine the affinity constants (Kₘ) for the substrates of dethiobiotin synthetase with the purified enzyme preparation are shown in Fig. 7 as Lineweaver-Burk plots. In these studies, all substrates but the one of interest were present in saturating concentrations.

Under these conditions, all of the substrates gave straight line reciprocal plots, obeying the Michaelis-Menten equation. The Kₘ values and their standard errors as calculated by the method of least squares are: NaHCO₃, 3.4 ± 0.7 mM; ATP, 5.0 ± 2.0 µM; DAPA, 1.3 ± 0.5 µM; and Mg²⁺, 0.6 ± 0.1 mM. Mn²⁺ was about 35% as effective as Mg²⁺ in this system. None of the substrates showed inhibition of dethiobiotin synthetase at concentrations up to 10 times their respective Kₘ values. The Kₘ for DAPA is approximately the same for the purified enzyme as that estimated for cell-free extracts. However, the Kₘ values for HCO₃⁻ and ATP with the purified enzyme are 1/10 and 1/100 of that estimated for cell-free extracts, respectively (2).

**Stoichiometry of Reaction**—Experiments to determine the stoichiometry of the conversion of DAPA to dethiobiotin were performed in order to gain information about the reaction mechanism involved.

As a measure of the amount of incorporation of HCO₃⁻ or CO₂ into the ureido ring of dethiobiotin, NaH¹⁴CO₃ was used as the substrate. Both the biological activity of the dethiobiotin formed and the acid-stable radioactivity were determined in the presence of DAPA, ATP, and dethiobiotin synthetase. The results shown in Table II indicate that the net amount of acid-stable radioactivity found corresponds, within experimental error, to the quantity of dethiobiotin formed. No correction was made for the presence of unlabeled endogenous CO₂ in the reaction mixture.

By using 8-¹⁴C-ATP as substrate, it was possible both to determine the stoichiometric relationship between ATP and dethiobiotin, and whether the product of the reaction is ADP or AMP. After the reaction had been terminated, the reaction mixture was chromatographed to separate the labeled ATP and any radioactive ADP or AMP which had been formed. Since the labeled ATP used was contaminated with AMP and ADP, the necessary controls were included. No increase in AMP was found after termination of the reaction, or in control mixtures in which DAPA or DAPA and enzyme was omitted. The results of this experiment are shown in Table III. It can be seen that ADP is the end product of this reaction and equimolar quantities of ADP and dethiobiotin are formed. This is dependent upon the presence of both DAPA and dethiobiotin synthetase.

**Substrate Specificity**—Investigation of the nucleoside tri-
highly purified biotin synthetase prepared from NaI114CO3 incorporation into acid-stable lactic acid and T5O-1, an E. coli strain K-12 biotic operon deletion mutant; found that diaminobiotin was only 37 % as active as DIPA.

Table IV, re-calculated that ATP is the preferred substrate of these enzymes. The reaction mixtures were applied to column of 1% Bio-10AG enzyme were used. The velocity, V, is defined as nanomoles of mM MgCl2; 0.05 nmo 14C-ATP (1.06 x 10^5 dpm per nmole), and the experiment in which Mg2+ concentration was varied, 25 pg of 0.15 M Tris chloride, pH 7.7; 0.05 ml 1%)APA; 40 mu NaH114CO3; 10

phosphate requirement of dethiobiotin synthetase, as shown in Table IV, revealed that ATP is the preferred substrate of those tested. CTP was only one-half as active as ATP whereas TTP, UTP, and GTP had less than 10% of the activity. In the absence of added ATP, ADP showed some activity whereas AMP was inactive.

Although diaminobiotin possesses an intact tetrahydrobiotin ring and differs in this respect from DAPA, it can still be considered as a structural analogue. In addition, diaminobiotin is biologically active for bio mutants of groups I, II, and IV (1). It was found to have about 8% as much activity as DAPA when used as a substrate with strain bioB105 resting cells (2). The product of the reaction in resting cells as well as in cell-free extracts was identified as biotin by combined chromatography and electrophoresis using strain bioB105 as the bioautographic indicator organism.

The question of whether biotin synthesis from diaminobiotin and dethiobiotin synthesis from DAPA are mediated by the same enzyme, or perhaps a second enzyme involved in a salvage pathway for diaminobiotin, had to be considered. In order to resolve this question, the following preparations were tested for their ability to catalyze the formation of biotin from diaminobiotin: (a) a dialyzed cell-free extract of strain bioD302, with a point mutation in the bioD gene; (b) a cell-free extract of strain T50-1, an E. coli strain K-12 biotin operon deletion mutant; (c) a dialyzed crude cell-free extract of strain N821; and (d) a highly purified sample of dethiobiotin synthetase prepared from strain N821. Samples containing 10 µl from each reaction tube were submitted to paper chromatography. Bioautography of the chromatogram using strain bioB105 as the indicator organism revealed the presence of biotin only with the strain N821 dialyzed crude cell-free extract and the purified dethiobiotin synthetase preparation. Since the extracts from strains bioD302 and T50-1 lack an active D gene product, a logical conclusion is that biotin synthesis from diaminobiotin is the result of dethiobiotin synthetase rather than some other enzyme. Under conditions which are optimal for dethiobiotin synthesis from DAPA, it was found that diaminobiotin was only 37 % as active as DAPA. The enzymatically formed product was measured both by NaI114CO3 incorporation into acid-stable radioactivity and bioassay with bioB105. The lower activity ratio previously

![Fig. 7. Substrate kinetics of dethiobiotin synthetase.](image)

**Table II**

Stoichiometry of HCO3- incorporation into dethiobiotin

The reaction mixture (1 ml) contained a final concentration of 0.15 M Tris chloride, pH 7.7; 10 mM ATP; 10 mM MgCl2; 0.25 mM DAPA; 40 mM NaH114CO3 (1298 dpm per nmole); and 2.5 µg of dethiobiotin synthetase. The reaction was started by the addition of the enzyme and was carried out for 10 min at 37°. After the reaction had been stopped by the addition of 0.5 ml of 12% trichloracetic acid, the supernatant was quantitatively transferred to a counting vial and lyophilized. To the residue were added 0.1 ml of water, 2 ml of absolute ethanol, and 10 ml of toluene liquid scintillation counting solution. Absolute radioactivity was calculated from observed radioactivity by the use of 14C-toluene internal standard.

<table>
<thead>
<tr>
<th>Deletion</th>
<th>Acid-stable radioactivity</th>
<th>Net 14CO2 incorporated</th>
<th>Dethiobiotin form mnoles/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1275</td>
<td>1.96</td>
<td>2.11</td>
</tr>
<tr>
<td>None</td>
<td>3950</td>
<td>1.96</td>
<td>2.11</td>
</tr>
<tr>
<td>DAPA</td>
<td>1330</td>
<td>1.96</td>
<td>2.11</td>
</tr>
<tr>
<td>Enzyme</td>
<td>1432</td>
<td>1.96</td>
<td>2.11</td>
</tr>
</tbody>
</table>

a Determined on a 10-µl sample by bioassay.

**Table III**

Stoichiometry of ADP formation

Reaction mixtures (1 ml) contained a final concentration of 0.15 M Tris chloride, pH 7.7; 0.05 mM DAPA; 40 mM NaH114CO3; 10 mM MgCl2; 0.05 mM 8-14C-ATP (1.06 x 10^9 dpm per nmole), and 15 µg of dethiobiotin synthetase. The reaction was started by the addition of enzyme and was carried out for 10 min at 37°. To each of the tubes was then added 0.5 mg each of carrier ATP, ADP, and AMP, and the tubes rapidly cooled to 0° in an ice bath. The reaction mixtures were applied to column of Bio-Rad AG 1-8X anion exchange resin in the chloride form. AMP, ADP, and ATP were eluted and the radioactivity determined as described under "Methods."

<table>
<thead>
<tr>
<th>Reaction conditions</th>
<th>14C-ADP present</th>
<th>Net ADP formed</th>
<th>Dethiobiotin form mnoles/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>12.2</td>
<td>11.5</td>
<td>8.2</td>
</tr>
<tr>
<td>-DAPA</td>
<td>3.5</td>
<td>3.3</td>
<td>0</td>
</tr>
<tr>
<td>-DAPA and enzyme</td>
<td>3.5</td>
<td>3.3</td>
<td>0</td>
</tr>
</tbody>
</table>

a Determined on a 10-µl sample by bioassay.
TABLE IV
* Nucleotide triphosphate requirement *

<table>
<thead>
<tr>
<th>Nucleotide triphosphate</th>
<th>Dethiobiotin</th>
<th>Activity relative to ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>2.53</td>
<td>100</td>
</tr>
<tr>
<td>CTP</td>
<td>1.40</td>
<td>55</td>
</tr>
<tr>
<td>GTP</td>
<td>0.22</td>
<td>9</td>
</tr>
<tr>
<td>UTP</td>
<td>0.12</td>
<td>5</td>
</tr>
<tr>
<td>GTP</td>
<td>0.04</td>
<td>2</td>
</tr>
</tbody>
</table>

TABLE V

* Carbamyl phosphate activity during purification of dethiobiotin synthetase *

<table>
<thead>
<tr>
<th>Sample</th>
<th>Carbamyl phosphate</th>
<th>HCO₃⁻ and ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.3 μM</td>
<td>5 μM</td>
</tr>
<tr>
<td></td>
<td>enzyme units/mg protein</td>
<td></td>
</tr>
<tr>
<td>Dialyzed cell-free extract</td>
<td>0.30</td>
<td>0.42</td>
</tr>
<tr>
<td>2nd (NH₄)₂SO₄ fraction</td>
<td>0</td>
<td>1.20</td>
</tr>
<tr>
<td>Hydroxylapatite chromato-</td>
<td>0.30</td>
<td>0</td>
</tr>
<tr>
<td>graphy</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

observed in resting cells may be attributed to a difference in permeability to the two substrates.

The ability of carbamyl phosphate to serve as a substrate for dethiobiotin synthetase has already been described in crude cell-free extracts (2). To investigate further its role in the reaction, carbamyl phosphate was tested with samples of the enzyme at various stages of purification to compare its activity as a substrate at two different concentrations with that of HCO₃⁻ plus ATP.

The results shown in Table V indicate that enzymatic activity with carbamyl phosphate does not co-purify with that for HCO₃⁻ plus ATP. Moreover, it does not serve as a substrate for the purest enzyme preparation.

In our previous study with dialyzed crude cell-free extracts, it was suggested that HCO₃⁻ may not be the actual substrate because of the relatively high K₅ value as compared to ATP and DAPA. To distinguish whether CO₂ or HCO₃⁻ is the actual substrate of the reaction, the procedure of Cooper et al. (18) was utilized. A solution of limiting amounts of either bicarbonate or CO₂ (acidified bicarbonate) was added to a reaction mixture containing DAPA, Mg²⁺, ATP, and enzyme. Samples of 10 μl were taken at 20-sec intervals and placed onto filter paper penicillin assay discs which had been previously moistened with trichloroacetic acid to inactivate the enzyme. The dethiobiotin formed was then assayed according to the standard bioassay procedure. It can be seen in Fig. 8 that more dethiobiotin is produced in the presence of CO₂ than HCO₃⁻, suggesting that CO₂ is the substrate. Upon the addition of

![Fig. 8. Kinetics of dethiobiotin synthetase activity when either HCO₃⁻ (○○○) or HCO₃⁻ plus 100 μg of carbonic anhydrase (●●●), or CO₂ (△△△), or CO₂ plus 100 μg of carbonic anhydrase (■■■) are present. Reaction mixtures of 1 ml volume contained a final concentration of 0.15 μM Tris chloride buffer, pH 7.7; 10 mM MgCl₂; 0.05 μM ATP; and either 10 mM HCO₃⁻ or 10 mM CO₂. The reaction was started by the addition of 15 μg of dethiobiotin synthetase and was carried out at 10°. When CO₂ was the substrate, 0.4 M HCl was added to an equal volume of 0.4 M NaHCO₃ and the solution was allowed to come to equilibrium.](http://www.jbc.org/)

Carbonic anhydrase, which promotes almost instantaneous equilibrium between CO₂ and HCO₃⁻, dethiobiotin formation with CO₂ is reduced, since the the CO₂ is converted to HCO₃⁻. Conversely, more dethiobiotin is produced when carbonic anhydrase is added to HCO₃⁻, again indicating that CO₂ is the actual substrate in the dethiobiotin synthetase reaction.

Inhibitors of Dethiobiotin Synthetase—The bioassay could not be used to investigate whether biotin or dethiobiotin acted as end product inhibitors of the dethiobiotin synthetase reaction. However, the use of the radiochemical assay, which involves the DAPA-dependent formation of acid-stable radioactivity when NaHCO₃ was the substrate, made this feasible. The results of such a study showed that concentrations of d-biotin up to 10 μM were not inhibitory. Dethiobiotin concentrations up to 1 μM showed no inhibition, but at a level of 10 μM, dethiobiotin was responsible for a 30% inhibition of enzyme activity.

Since ADP was found to be an end product of dethiobiotin synthesis, its possible role as an inhibitor of the reaction was studied with respect to ATP concentration. Lineweaver-Burk plots of the results of an experiment in which three concentrations of ADP were tested against increasing ATP concentrations indicated that ADP is a competitive inhibitor. Graphical estimation from a Dixon plot of 1/V against ADP concentration at varying levels of ATP gives a Kᵠ value of 0.23 μM for ADP.

Some enzymes which catalyze CO₂ fixation reactions, such as pyruvate carboxylase and propionyl-CoA carboxylase utilize biotin as the prosthetic group. It has been shown that avidin can inhibit the action of such enzymes, presumably by binding to the biotin moiety of the enzyme. In an experiment with dethiobiotin synthetase, avidin levels up to 1 unit per μg of en-
zyme were not inhibitory, suggesting the absence of a biotinyl prosthetic group.

**DISCUSSION**

The cell-free extracts of heat-induced cultures of strain N821 had increased levels of the enzyme dethiobiotin synthetase and thus permitted the isolation of a sufficient quantity of purified enzyme for its subsequent characterization. The final enzyme preparation had a purity of greater than 90% and was free of ATPase activity, as indicated by Table III. Throughout the entire purification study, it was observed that the enzyme activity behaved as though a single protein was involved. None of the chromatographic steps revealed more than one peak of activity. The extremely low yields obtained may have been due to the cold lability of the enzyme during the chromatography at low temperatures. The first evidence for cold lability of an enzyme was reported by Pullman et al. (20) for the mitochondrial ATPase and has since been established for a number of allosteric proteins. The serum lipoproteins have also been shown to be unstable at low temperatures by Oneley, Gurd, and Melin (21). The fact that a good recovery of enzyme activity could be attained on the hydroxylapitate column when employed at room temperature would indicate that this problem could be minimized by performing all chromatographic procedures at ambient temperature.

The molecular weight of the native dethiobiotin synthetase was determined to be approximately 42,000. On SDS electrophoresis, the molecular weight of the major component was estimated to be 24,500, indicating that the native enzyme is a dimer. The minor component with a molecular weight identical with the native protein may represent undissociated native protein rather than the minor impurity, since only 10 μg of protein were used for this determination. No conclusion can be drawn with respect to the subunits being identical in amino acid composition, since SDS gel electrophoresis is insensitive to charge distribution. However, the genetic evidence to date indicates that only one cistron is involved in dethiobiotin synthesis (3).

There was no indication of any change in the substrate requirements of the enzyme during the purification, other than the reduction of the Kₐ values for CO₂ and ATP. Specificity studies indicated that only CTP could replace ATP to any significant extent. One unique similarity between ATP and CTP which distinguishes them from the other nucleotides tested is the presence of the 6-amino group in these two compounds. It is possible that this functional group is necessary for interaction at the active site of the enzyme. Diaminobiotin proved to be about 37% as active as DAPA under conditions which were optimal for dethiobiotin synthesis from DAPA. The ability of dethiobiotin synthetase to catalyze the synthesis of biotin from diaminobiotin can explain the observation of Stokes and Gunning (22) that diaminobiotin but not dethiobiotin is biologically active for *Lactobacillus arabinosus* and *Lactobacillus casei*. These organisms appeared to lack the enzyme for the conversion of dethiobiotin to biotin and as a result behave like group IV mutants of *E. coli* (1). However, if dethiobiotin synthetase was present in these two organisms, then they can convert diaminobiotin to biotin and thus circumvent the genetic block.

The experiments carried out with CO₂ and HCO₃⁻ in the presence and absence of carbonic anhydrase suggest that CO₂ is the actual substrate in the dethiobiotin synthetase reaction. This finding is similar to the observations of Cooper et al. (18) in their study of ribulose diphosphate carboxylase. These investigators determined an apparent Kᵦ of approximately 20 μM for bicarbonate in this reaction and, with the aid of carbonic anhydrase, were able to show that the true substrate was CO₂ with a corrected Kᵦ of 0.45 μM. In the case of dethiobiotin synthetase, the apparent Kᵦ for bicarbonate is 2 μM. When a similar correction is applied, the result gives a Kᵦ value for CO₂ of approximately 40 μM. This value is only one order of magnitude greater than that of DAPA and 4-fold greater than that of ATP.

The possibility that carbamyl phosphate might be the direct donor in dethiobiotin synthesis has already been discussed (23). The activity of carbamyl phosphate as a co-substrate relative to that of CO₂ plus ATP was reduced to zero during the isolation of dethiobiotin synthetase. This evidence together with that presented earlier (3) does not support a rule for this compound in dethiobiotin synthesis, but rather suggests the direct incorporation of CO₂.

Previous studies on the biotin operon (3) indicated that the gene cluster is probably transcribed as a polycistronic message and that control of the pathway is through enzyme repression by biotin. The present experiments indicate that neither biotin nor dethiobiotin appear to control dethiobiotin synthesis by end product inhibition. Both biotin and dethiobiotin were found to be noninhibitory at concentrations up to 10⁸ and 10⁶ times their respective combined intracellular levels, as found in stationary cultures of *E. coli* (24). The low activity

![Fig. 9. Proposed reaction mechanism for dethiobiotin synthesis from DAPA.](http://www.jbc.org/)
observed with ADP in the absence of ATP could have been due to ATP contamination of this preparation. On the other hand, the dethiobiotin synthetase reaction was found to be competitively inhibited by ADP. The inhibition originally observed when ATP was added to crude enzymes may have been due to the presence of ATPase activity in these preparations which could have been removed on the first ammonium sulfate fractionation. Whether the action of ADP is part of a control mechanism is still to be determined.

The stoichiometric data obtained from incorporation studies have indicated that the following overall reaction is catalyzed by dethiobiotin synthetase.

\[ \text{DAPA + ATP + CO}_2 \rightarrow \text{dethiobiotin + ADP + P}_i \]

We have previously postulated a possible mechanism for dethiobiotin synthesis in which the formation of the monocarbamate of DAPA was the first step in the reaction. The second step was the activation of the monocarbamate by ATP to form an N-substituted carbamyl phosphate. The final step was the ring closure with loss of inorganic phosphate. The reaction mechanism shown in Fig. 9 is in accord with our observation that although two amido bonds are formed during the synthesis of dethiobiotin, only 1 molecule of ATP is consumed.

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