Synthesis of Desthiobiotin from 7,8-Diaminopenelargonic Acid in Biotin Auxotrophs of *Escherichia coli* K-12i,2

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Received for publication 7 April 1969

The synthesis of desthiobiotin from 7,8-diaminopenelargonic acid (DAP) was demonstrated in resting cell suspensions of *Escherichia coli* K-12 bioA mutants under conditions in which the biotin locus was derepressed. The biosynthetically formed desthiobiotin was identified by chromatography, electrophoresis, and by its ability to support the growth of yeast and those *E. coli* biotin auxotrophs that are blocked earlier in the biotin pathway. Optimal conditions for desthiobiotin synthesis were determined. Desthiobiotin synthetase activity was repressed 67% when partially derepressed resting cells were incubated in the presence of 3 ng of biotin per ml. Serine, bicine, bicarbonate, and glucose stimulated desthiobiotin synthesis apparently by acting as sources of CO₂. The results of this study are consistent with an earlier postulated pathway for biotin biosynthesis in *E. coli*: pimelic acid → 7-oxo-8-aminopenelargonic acid → DAP → desthiobiotin → biotin.

In a previous study by Rolfe and Eisenberg (9), the following sequence of reactions was proposed for the synthesis of biotin based on genetic and biochemical evidence: pimelic acid → 7-oxo-8-pelargonic acid (7-KAP) → 7,8-diaminopenelargonic acid (DAP) → desthiobiotin → biotin, where each roman numeral designates the reaction blocked for the respective mutant group. The conversion of desthiobiotin to biotin has been amply documented in both resting and growing cells (6, 8, 11). More recently, the formation of 7-KAP from pimely-CoA and alanine was demonstrated in cell-free extracts of *Escherichia coli* (3). Although feeding experiments and accumulation studies with *E. coli* mutants suggested DAP as a possible intermediate, its direct conversion to desthiobiotin remained to be demonstrated. This study will show the formation of desthiobiotin from DAP in resting cells of various *E. coli* mutants.

1 This work is taken from a dissertation to be submitted by K. Krell in partial fulfillment of the requirements for the Ph.D. degree, under the Joint Committee on Graduate Instruction, Columbia University.


MATERIALS AND METHODS

**Bacterial strains.** The biotin-requiring mutants *(bioA105, bioA302, and bioA310)* used in this study had been isolated from cultures of *E. coli* K-12 strains Y10-1 and HfH and have been described elsewhere (9).

**Media.** Penassay Broth and nutrient agar were Difco products. The basal medium contained per liter of glass-distilled water: 6.8 g of KH₂PO₄, 1.0 g of (NH₄)₂SO₄, 0.1 g of MgSO₄·7 H₂O, 10 mg of Ca(NO₃)₂·4 H₂O, and 0.5 mg of FeSO₄·7 H₂O. The solution was adjusted to pH 7.0 with NaOH and sterilized in the autoclave. The following were separately filter-sterilized and added aseptically to each liter: 2.0 g of d-glucose, 0.1 g of L-leucine, and 5.0 mg of thiamine hydrochloride.

**Resting cells.** All cultures were incubated at 37 °C with continuous shaking (120 oscillations/min) in an incubator-shaker (New Brunswick Scientific Co., New Brunswick, N.J.). Penassay Broth (2 ml) was inoculated from a nutrient-agar-slant stock culture and incubated for 18 hr. The cells were harvested by centrifugation, washed twice with 0.9% saline, and suspended in 5 ml of saline. The cell concentration was determined turbidimetrically with a Klett photoelectric colorimeter using a no. 66 filter. A 2-ml portion of basal medium containing 0.1 ng of biotin per ml (minimal growth medium) was inoculated to give a final concentration of 1.5 × 10⁶ cells per ml. A second tube containing only basal medium was also inoculated, and it served as a control for reversion of the
inoculum. After incubation for 18 hr, the biotin-containing culture was inoculated into 100 ml of minimal medium. If larger quantities of cells were required, the 100-ml cultures were used as inocula for 1-liter volumes. When the stationary phase of growth had been reached, the cells were harvested by centrifugation in the cold. These cells were either washed twice with saline and used immediately or derepressed prior to use.

Derepressed cells were prepared by resuspending resting cells in the same volume of basal medium and incubating them for 2 hr at 37 C with shaking. Under these conditions, the turbidity usually doubled. After derepression, the cells were harvested, washed, and suspended to the desired concentration in saline.

Repression. For repression studies, cells were grown in minimal growth medium, harvested by centrifugation, and washed in saline. The cells were distributed equally among ten 100-ml portions of basal medium contained in 300-ml Erlenmeyer flasks. Sufficient sterile biotin was added to the flasks to give a final concentration varying from 0 to 5 ng per ml. The flasks were incubated for 2 hr, harvested by centrifugation, washed twice with saline, and then assayed for desthiobiotin-synthesizing activity.

Desthiobiotin synthetase activity. Desthiobiotin-synthesizing capacity was determined in a 1-ml reaction mixture containing the following: 2 mg of cells (dry weight), 150 μmoles of potassium phosphate buffer (pH 7.5), 200 nmoles of dl-7,8-Diaminopelargonic acid hydrochloride, and other additions as noted. The reaction mixture was incubated in a New Brunswick water bath-shaker at 37 C with continuous agitation. The reaction was started by adding the cells and was terminated by centrifuging at 4 C. The supernatant fluid was decanted, and a sample was analyzed for desthiobiotin content by microbiological assay.

Determination of desthiobiotin. Desthiobiotin levels were determined by a bioassay procedure using a group III mutant, bioA302. Members of this mutant group have been shown to grow only in the presence of biotin or desthiobiotin (9). Quantitation of the growth response of this organism was accomplished by a modification of the turbidity assay of Snell (10), by using basal medium, or by the disc assay of Genghof, Partridge, and Carpenter (4) by using basal medium containing 2% agar and 0.001% 2,3,5-triphenyl-2H-tetrazolium chloride. Authentic samples of desthiobiotin were used as standards. The two assay procedures agreed closely and were used interchangeably.

Chromatography and electrophoresis. Ascending chromatography was carried out with either Whatman no. 3MM paper or thin-layer silicic acid-coated polyester sheets by using a solvent system consisting of n-butyl alcohol-acetic acid-water at a ratio of 60:15:25. Paper electrophoresis was performed with a Spinco-Durrum cell at 300 v for 3 hr by using 0.025 M sodium citrate buffer (pH 3.0). For the chromatograms, the position of biotin vitamers was revealed by bioautography with bioA302 or yeast as the responsive organism, whereas migration sites of biotin vitamers on electropherograms were demon-
bavidn, respectively. The concentration of the third component was too low to support the growth of yeast, the assay organism.

Further evidence of the identity of the newly synthesized vitamer with desthiobiotin was obtained by paper electrophoresis at pH 3.0. Figure 1 shows the migration of the components present in the reaction mixture and in the control mixture containing no cells. To obtain a high order of bioautographic resolution, the punch-out technique (2) was used to determine the mobility of the vitamers. In the control, only one biologically active compound was observed, migrating 8.8 cm toward the cathode. In the reaction mixture, two components were present which migrated 1.0 and 10.2 cm toward the cathode. These values are not corrected for electrodosmosis. Under similar conditions, an authentic sample of desthiobiotin remains near the origin, whereas DAP migrates 9.3 to 10.8 cm toward the cathode.

Optimal conditions for desthiobiotin synthesis. For these studies, a derepressed culture of bioA105 resting cells was used. Figure 2A shows the time-course of desthiobiotin synthesis from DAP. The rate was approximately linear up to the first hour, and then it gradually decreased. Figure 2B illustrates the direct proportionality of desthiobiotin formation with increasing DAP concentration over the range 0 to 100 nmoles per ml. The system was saturated above 150 nmoles per ml, at which point desthiobiotin formation represented 2% conversion of the substrate, based on the concentration of the d-enantiomer. There was no evidence of substrate inhibition at twice the saturating concentration of DAP. By varying cell concentration in the presence of 200 nmoles of DAP per ml and 2 mg (dry weight) of cells per ml, except where variable (A) as a function of time, (B) with DAP concentration, (C) as a function of concentration of cells, and (D) as a function of pH.

![Bioautography of paper electropherograms of supernatant-fluid samples from reaction mixtures in the presence of (○) and in the absence (△) of resting cells.](http://jb.asm.org/)

**Fig. 1.** Bioautography of paper electropherograms of supernatant-fluid samples from reaction mixtures in the presence of (○) and in the absence (△) of resting cells.

**Fig. 2.** Desthiobiotin synthesis with bioA-105-derepressed resting cells in the presence of 200 nmoles of DAP per ml and 2 mg (dry weight) of cells per ml, except where variable (A) as a function of time, (B) with DAP concentration, (C) as a function of concentration of cells, and (D) as a function of pH.
presence of increasing concentrations of biotin. A similar observation was made with E. coli biotin mutants capable of synthesizing 7-KAP in cell-free extracts (3). When bioA105 resting cells suspended in basal medium containing varying concentrations of biotin were incubated at 37°C with shaking for 2 hr, a decrease in desthiobiotin-synthesizing activity was observed. Over the range 0 to 3 ng of biotin per ml, there was a 67% decrease in activity (Fig. 3). When 50 μg of chloramphenicol was added to the reaction mixture to inhibit protein synthesis during the relatively long incubation time, identical results were obtained. These data strongly suggest that biotin acts as a corepressor in the control of desthiobiotin synthesis.

Effect of various carbon sources. In an attempt to stimulate the rate of desthiobiotin synthesis and possibly to elucidate the origin of the ureido carbonyl group, various carbon sources were added to the basic reaction mixture. The compounds added and their effect on desthiobiotin accumulation are shown in Table 1. Histidine, glycine, and formate, possible "C₄" donors, gave no stimulation. On the other hand, serine gave a 1.5-fold stimulation. To determine whether a C₄ donor in conjunction with a folate system was operative, 50 μg of amethopterin was added to the basic reaction mixture, i.e., with DAP alone. No inhibition of desthiobiotin formation was observed, suggesting that a C₄ donor may not be involved.

Lezius et al. (5) had previously shown that radioactive bicarbonate was incorporated into the ureido carbonyl group of biotin and suggested that carbamyl phosphate was the intermediary donor. Whereas carbamyl phosphate has little effect in our system, HCO₃⁻ gave the same degree of enhancement as did serine. It would appear, therefore, that serine may exert its effect by virtue of its ability to supply CO₂ via pyruvic acid. The addition of glucose was even more effective, giving a 2.5-fold stimulation. The presence of 50 μg of chloramphenicol per ml failed to cause any inhibition, suggesting that the glucose effect was not due to new enzyme synthesis during the assay.

DISCUSSION

This study has demonstrated the conversion of DAP to desthiobiotin by resting cells of E. coli bioA mutants that normally accumulate desthiobiotin in the growth medium. This biotin vitamer was identified by chromatography, electrophoresis, and its ability to support the growth of yeast and those E. coli biotin auxotrophs that are blocked earlier in the biotin pathway. The same cells were also able to form desthiobiotin from 7-KAP to about 20% the extent of the DAP-to-desthiobiotin conversion (unpublished data), thus suggesting that the latter reaction is not the rate-limiting step in the overall sequence.

Further support for DAP as an intermediate in the biotin biosynthetic pathway was provided by the repression studies with various concentrations of biotin. Whereas the degree of repression is less than that observed previously for 7-KAP synthetase activity (3), the conditions were not identical in the two studies. The repression by biotin of the enzymes responsible for three steps in the biotin pathway in E. coli has now been demonstrated (3, 8). The fact that the degree of repression by biotin is approximately the same in the three enzyme systems studied and the fact that they are all part of the same operon suggest that coordinate repression is involved.

![Fig. 3. Effect of biotin concentration on desthiobiotin-synthesizing activity.](http://jb.asm.org/)

**Table 1. Rate of accumulation of desthiobiotin in the presence of various additions**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concen (μmoles/ml)</th>
<th>Desthiobiotin synthesis (μmoles/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>2.94</td>
</tr>
<tr>
<td>L-Serine</td>
<td>26</td>
<td>4.11</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>25</td>
<td>2.71</td>
</tr>
<tr>
<td>Glycine</td>
<td>26</td>
<td>2.48</td>
</tr>
<tr>
<td>Sodium formate</td>
<td>25</td>
<td>2.48</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>25</td>
<td>4.11</td>
</tr>
<tr>
<td>Carbamyl phosphate</td>
<td>23</td>
<td>2.15</td>
</tr>
<tr>
<td>Glucose</td>
<td>28</td>
<td>6.87</td>
</tr>
</tbody>
</table>
similar conclusion was reached in previous studies with *Phycomyces blakesleeanus* (2).

The stimulatory effects of glucose, serine, and bicarbonate on desthiobiocin synthesis have as a common denominator, bicarbonate. This finding supports the isotopic evidence of Lezius et al. (5) that CO₃⁻ is incorporated into the ureido carbonyl group of biotin. Whether bicarbonate enters via carbamyl phosphate, as suggested by these investigators, cannot be unequivocally determined in resting cells. Whereas carbamyl phosphate did not enhance desthiobiocin synthesis, the impermeability of the cells to this compound cannot be excluded. The greater enhancement of desthiobiocin synthetase activity in the presence of glucose, compared with bicarbonate or serine, suggests that glucose is acting as an energy source as well as a bicarbonate donor. Preliminary experiments with cell-free extracts have shown a requirement for adenosine triphosphate, magnesium, and bicarbonate in desthiobiocin synthesis.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AM 04036 from the National Institute of Arthritis and Metabolic Diseases, by American Cancer Society grant 1983, and by a general research support grant from the National Institutes of Health.

Kenneth Krell was a predoctoral trainee under Public Health Service training grant TI-AM-5168-11.

LITERATURE CITED