Bacterial Degradation of Biotin

CATABOLISM OF $^{14}$C BIOTIN AND ITS SULFOXIDES*

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SUMMARY

A biotin-degrading particulate system has been obtained by sonic rupture of a soil pseudomonad which grows on d-biotin as sole source of carbon, nitrogen, and sulfur. Investigation of the catabolism of $^{14}$C-biotin and its sulfoxides in this system has revealed the following.

Degradation of biotin to CO$_2$ is enhanced in the buffer-washed particulate preparations by addition of adenosine triphosphate, Mg$^{2+}$, nicotinamide adenine dinucleotide, and coenzyme A. Maximal activation is found with $10^{-5}$ M nicotinamide adenine dinucleotide.

Production of CO$_2$ from ureido carbonyl and aliphatic carboxyl portions of biotin and biotin d-sulfoxide is effectively inhibited by azide but is decreased only slightly by high concentrations of acetate or malonate. Oxybiotin, homobiotin, norbiotin, dethiobiotin, and the diaminocarboxylate from biotin moderately inhibit degradation of biotin and its d-sulfoxide. L-Cysteine is effective as an apparent inhibitor, but this amino acid is very actively metabolized in the particulate preparations.

Biotin and both $d$- and $l$-sulfoxides of biotin are efficiently catabolized by the particulate preparations and exhibit mutual competition as substrates. Production of CO$_2$ is most rapid from biotin $d$-sulfoxide, whereas the $l$-sulfoxide is somewhat less active than biotin. Very little degradation of biotin sulfone or diaminocarboxylate occurs. Oxybiotin, homobiotin, and norbiotin are not catabolized.

Several catabolites of biotin have been isolated from incubation mixtures by column and paper chromatography.

Although considerable information now is available on the biosynthesis and metabolic function of $d$-biotin, less is known concerning the catabolism of this vitamin. Numerous investigators have reported the presence of several natural biotin analogues in the culture media and cells from microorganisms grown with biotin or such compounds as enhance its formation (1). It was shown that certain biotin-requiring microorganisms activate excess biotin in the medium in which they are grown (2). Most of the radioactivity from a dose of carbonyl-labeled biotin injected in the rat was found to be excreted in the urine, but less than half as the unaltered $^{14}$C-biotin (3). Breakdown of carbonyl-labeled biotin was shown with slices of kidney cortex from guinea pigs (4). Extracts of liver and of a soil bacterium have been shown capable of catalyzing the activation of biotin to its adenylation and coenzyme A ester forms (5). Recent studies described the total degradation of carbonyl- and carbonyl-labeled biotin by whole cells and broken cell preparations from a pseudomonad which grows on biotin as a sole source of carbon, nitrogen, and sulfur (6).

In the present paper, we describe the activation, inhibition, and substrate specificity of a bacterial particulate system in which readily catabolizes biotin and its sulfoxides. Several unknown catabolites of biotin have been isolated and are being characterized.

EXPERIMENTAL PROCEDURE

Materials—The strain of bacterium used was described previously (6). Crystalline $d$-biotin, $dl$-dethiobiotin, L-cysteine, and L-methionine were purchased from Nutritional Biochemicals. The $l$-biotin was obtained as carbonyl-labeled (32.4 mC per mmole) from Nuclear-Chicago and as carboxyl-labeled (25.5 mC per mmole) from Hoffmann-La Roche (Basel). Oxybiotin, $d$-norbiotin, and $d$-homobiotin were also from Hoffmann-La Roche. $^{14}$C-Phosgene was from Nuclear Research Chemicals. ATP was from Sigma; NAD was from Pabst Laboratories; CoA was from Biochemical-Biocroniger, malonic acid was from Matheson. Hydroxide of Hyamine 10X was purchased from Packard.

$^{14}$C-Compounds Synthesized—The $d$- and $l$-sulfoxides of carbonyl- and of carbonyl-labeled biotin were made by treatment of the $^{14}$C-biotins with equimolar H$_2$O$_2$ essentially following the method of Melville (7). After reaction with 500 mg of biotin, most of the solvent (glacial acetic acid) was evaporated and the mixture was adjusted to pH 6.5 with 5 N NaOH. The solution was poured over a column, 2.5 × 35 cm, of 100 to 200 mesh Dowex 1-X8 (formate) and the $^{14}$C-compounds were eluted with a linear gradient from 1 liter of water to 1 liter of 1 M ammonium
**Effects of cofactors on degradation of biotin**

Particulate preparations (unwashed and washed three times) from 5 mg of original cells were added to incubation mixtures containing 30 mmoles of each cofactor, 90 mmoles of d-carboxyl-labeled biotin, and 0.2 mmoles of potassium phosphate buffer (pH 6) in a total volume of 3 ml.

<table>
<thead>
<tr>
<th>System</th>
<th>$^{14}$CO$_2$ from biotin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particles</td>
<td>2850</td>
</tr>
<tr>
<td>Washed particles</td>
<td>1434</td>
</tr>
<tr>
<td>A</td>
<td>2660</td>
</tr>
<tr>
<td>Minus ATP</td>
<td>2300</td>
</tr>
<tr>
<td>Minus Mg$^{2+}$</td>
<td>2230</td>
</tr>
<tr>
<td>Minus NAD</td>
<td>2050</td>
</tr>
<tr>
<td>Minus CoA</td>
<td>1880</td>
</tr>
</tbody>
</table>

* Relative to unwashed particles without added cofactors.

Preparations derived from 5 mg of original cells with 30 or 90 mmoles of substrate, 30 mmoles each of ATP, Mg$^{2+}$, NAD, and CoA, and 0.2 mmoles of potassium phosphate buffer (pH 6 or 6.2) in a total volume of 3 ml. The contents were contained in a 50-ml Erlenmeyer flask which was stoppered with a rubber septum to which was appended a polypropylene center well containing a wick of Whatman No. 1 filter paper. Ten minutes prior to terminating the reaction, 0.5 ml of a 1 N solution of Hyamine in methanol was injected into the center well. The reactions were terminated, usually after 1 hour at 30°C, by a second injection of 1 ml of 20% trichloroacetic acid into the incubation mixture. The flasks were swirled for 15 min to assure complete absorption of $^{14}$CO$_2$ by the Hyamine solution on the paper wick. The rubber septum was removed and the center well together with paper wick was dropped into a counting vial containing 10 ml of a toluene solution of scintillators. Radioactivity as disintegrations per min was determined with the use of $^{14}$C-benzoate as an internal standard in a Packard Tri-Carb liquid scintillation spectrometer.

**Chromatography**—Catabolites and remaining substrates were isolated from 20-fold increased reaction mixtures (60 ml) which were incubated for 2 hours at 30°C. The contents were cooled to 0°C and subjected to sonic oscillation for 10 min. The mixtures were centrifuged for 20 min at 25,000 × g, and the supernatant solutions were applied to a column, 2.5 × 35 cm, of 100 to 200 mesh Dowex 1-X8 (formate) for elution with a linear gradient from 1 liter of water to 1 liter of 1 M ammonium formate. Fractions of 100 ml were collected and radioactivity was determined in 0.5-ml aliquots added to Bray's solution for scintillation counting (11). Combined fractions were lyophilized, applied to a column, 3.5 × 30 cm, of Dowex 50-X12 (H$^+$) for removal of ammonium ions, and the $^{14}$C-material was eluted with water and lyophilized.

Aliquots were applied to sheets of Whatman No. 1 paper and the chromatograms were developed in descending butanol-acetic acid-water (2:1:1:1, v/v/v/v). The $^{14}$C-compounds were detected by cutting the paper into 21 equal segments and counting the radioactivity of segments in 10 ml of Bray's solution (11).

**RESULTS**

**Activators**—Effects of added cofactors on the degradation of carboxyl-labeled biotin by particulate preparations from the bacteria are shown by the data in Table I. Approximately 40% of the carbonyl carbon of $^{14}$C-biotin was converted to $^{14}$CO$_2$ during incubation at 30°C for 1 hour with the unwashed particulate preparation to which no cofactors were added. Particulate preparations which were washed three times by suspending and centrifuging from phosphate buffer were only half as active. Even less activity was measured after further washings. All such washed particles were relatively unstable when stored frozen and thawed for use. Restoration of activity of the particles washed three times was nearly complete when ATP, Mg$^{2+}$, NAD, and CoA were added at 10$^{-5}$ M concentrations. The apparent $K_m$ for NAD is less than 10$^{-6}$ M. Little or no additional stimulation was found with similar concentrations of pyridoxal 5-phosphate, thiamine pyrophosphate, lipoate, FMN, or FAD.

**Inhibitors**—Apparent inhibitory actions of unlabeled and non-degradable compounds against loss of $^{14}$CO$_2$ from carboxyl- and carboxyl-labeled biotin and biotin d-sulfoxide are shown by the...
data in Table II. Azide effectively inhibits degradation of biotin and the d-sulfoxide, whereas relatively high concentrations of acetate or malonate cause only small decreases in production of $^{14}$CO$_2$ from either carbonyl- or carboxyl-labeled substrates. Oxybiotin, homobiotin, norbiotin, dethiobiotin, and the diaminocarboxylate from biotin moderately inhibit degradation of biotin and its $d$-sulfoxide when the inhibitors are added at concentrations which are over 10-fold that of substrate. Unlabeled L-cysteine is reasonably effective as an apparent inhibitor; however, it is very actively metabolized in the particle system. L-Methionine is considerably less active as an inhibitor.

**Substrates**—Cross-competition of biotin with $d$- and $l$-sulfoxides as substrates with $^{14}$C label in either carbonyl or carboxyl position is shown by the data in Table III. The quantity of $^{14}$CO$_2$ derived from either carbonyl- or carboxyl labeled biotin is decreased by the presence of unlabeled $d$- or $l$-sulfoxide of biotin. Biotin $d$-sulfoxide is more effective as a competitive substrate than the $l$-sulfoxide. Even greater suppression of $^{14}$CO$_2$ production from either carbonyl or carboxyl position of $d$- or $l$-sulfoxide occurs in the presence of unlabeled biotin. Biotin is more effective as a competitive substrate against the $l$-sulfoxide. The magnitudes for competition appear similar between carbonyl- and carboxyl-labeled substrates.

Rates of appearance of $^{14}$CO$_2$ from degradations of carboxyl- and carbonyl-labeled biotin and $d$-sulfoxide are illustrated in Fig. 1. The sulfoxide is more rapidly and extensively degraded than biotin.

Relative substrate behaviors of biotin and analogues with $^{14}$C label in either carbonyl or carboxyl position are shown by the data in Table IV. The most active substrate with the particulate system used is biotin $d$-sulfoxide. The $l$-sulfoxide is somewhat less active than biotin. Very little degradation of sulfone occurs in either carbonyl or carboxyl position, as is also the case with the carboxyl labeled diaminocarboxylate from biotin. Replacement of the biotin sulfur atom with oxygen as in oxybiotin and increasing or decreasing the length of the aliphatic chain as in homobiotin and norbiotin, respectively, yield compounds which are not degraded at the carbonyl position.

**Catobolites**—Elution patterns from column chromatography of carboxyl-labeled biotin and catobolites on Dowex 1 (formate) are presented in Fig. 2. Commercial preparations of $^{14}$C-biotin are usually contaminated with small amounts of biotin sulfoxide which, as shown in Fig. 2A, is eluted at lower concentrations of ammonium formate than biotin. Similar columns with known radioactive analogues also separate biotin and its sulfoxides from sulfone. The latter compound is eluted subsequent to sulfoxide and prior to biotin under these conditions. Following a 2-hour
in incubation of the carbonyl-labeled biotin with an active particulate fraction, most of the biotin has disappeared and three 14C-containing fractions (I, II, and III) appear in the effluent from column chromatograms. The principal catabolite in Fraction I is neither biotin nor the sulfone, since additions of these compounds as carriers and continued recrystallizations from water caused extensive loss of radioactivity.

Yet another principal catabolite which is eluted between sulfoxide and biotin appears in the chromatographic pattern from a similar incubation of carboxyl-labeled biotin.

Mobilities of the 14C-catabolites from carbonyl-labeled biotin and appropriate standards on paper chromatograms are shown by the data in Table V. The RF values for the principal catabolites in Fractions I and II are similar to that of biotin sulfone in the butanol-acetic acid-water system and slightly larger in the butanol-methanol benzene-water system. When an aliquot from Fraction I was made 0.1 M in HCl and heated at 100° for 10 min, the RF value of radioactive material increased in the butanol-methanol-benzene-water system. The principal component in Fraction III migrates like biotin in both solvent systems.

**DISCUSSION**

The enhancement of biotin degrading activity which occurs upon addition of ATP, Mg2+, NAD, and CoA to washed particulate preparations from bacteria must reflect, at least in part, dependence on these cofactors for activation and degradation of the aliphatic carboxylate portion of the molecule. The formation of biotin 5'-adenylate and biotinyl-CoA has been shown with extracts from a similar soil microorganism (5) and the ready utilization of biotin-CoA found with broken cell preparations from the particular organism used in the present study (6). The present observation that loss of the carbonyl carbon from biotin or biotin d-sulfoxide. Inhibitions caused extensive loss of radioactivity.

Inhibition of the degradation of biotin and its d-sulfoxide by azide reflects the probable participation of terminal electron transport to molecular oxygen. Quantitatively unlike the system found in kidney cortex (4), acetate and especially malonate, a classic inhibitor of the Krebs cycle, are nearly ineffective in inhibiting the loss of carbonyl carbon with the bacterial preparation. Malonate is only weakly effective in suppressing loss of carbonyl carbon from biotin or biotin d-sulfoxide. Inhibitions found with biotin analogues in the present system are moderate,
but again less than reported for homobiotin, dethiobiotin, and the diamino carbocylate from biotin with the kidney system (4).

The strong apparent inhibition caused by cysteine, which itself is actively catabolized by the bacterial particles, may indicate that this amino acid or a metabolite can overload part of the system which degrades the same or a similar compound derived from biotin. However, another explanation for the behavior of cysteine may be related to a real inhibition similar to that recently reported for oxidation of S-methyl groups by rat tissues (12).

Cross-competitions observed with labeled and unlabeled biotin and biotin sulfoxides indicate the similar substrate behavior of these compounds. The present findings that d-sulfoxide appears more effective than the l form in competing with biotin as substrate in the particulate system, and the somewhat greater rate and extent of degradation of biotin d-sulfoxide when compared with biotin or l-sulfoxide may suggest the natural participation of the d-sulfoxide in the degradative process. In this connection, it is interesting that the d-sulfoxide predominates from chemical synthesis (7) and is generally more active in replacing biotin for growth of microorganisms (13), but the l-sulfoxide has been found more widely occurring in culture filtrates from the pseudomonad used in the present study (14) and from other microorganisms (15–17). It is possible that biotin d-sulfoxide is more readily formed and degraded by such organisms, wherein less reactive l-sulfoxide accumulates.

The very poor reactivity of biotin sulfone as substrate indicates that this compound probably is not an intermediate in the direct pathway for total catabolism of biotin in the pseudomonad. The stability of sulfone and similar inactivity of oxybiotin reflect considerable specificity toward the sulfur end of the molecule. Inertness of carboxyl-labeled diamino carbocylate from biotin also points to specificity toward the carbonyl function. Thus, structural requirements in the bicyclic ring system are quite narrow. Inactivities of carboxyl-labeled homobiotin and norbiotin may suggest a direct dependence of ring cleavage on length of the aliphatic side chain, or that catabolites derived from oxidation of side chains of the homo and nor analogues, e.g. 2-carbon chain acid via \( \beta \)-oxidation, cannot undergo subsequent oxidation to the 1-carbon chain acid derived from biotin.

Definitive conclusions on the pathway of biotin catabolism must await identification of such unknown catabolites as the present study has shown to accumulate in the bacterial system. However, it appears likely that \( \beta \)-oxidation of the valerate side chain may proceed through loss of two acetate units and that peroxidative attack of the sulfur with formation of d-sulfoxide may be prerequisite to ring cleavage.

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REFERENCES

Bacterial Degradation of Biotin: CATABOLISM OF 14C-BIOTIN AND ITS SULFOXIDES
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