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Mode of Action of the Biotin Antimetabolites Actithiazic Acid and α-Methyldethiobiotin

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Actithiazic acid and α-methyldethiobiotin inhibited the conversion of dethiobiotin to biotin in resting-cell suspensions of *Escherichia coli*. The concentrations which effected 50% inhibition were 0.45 and 1.1 μM for actithiazic acid and α-methyldethiobiotin, respectively. Cells grown in low concentrations of the two biotin antimetabolites showed derepression of the biotin A operon, as evidenced by the enhanced levels of the enzymes 7,8-diaminopelargonic acid aminotransferase and dethiobiotin synthetase. Derepression was not due to any direct regulatory effect of the antibiotics but was the consequence of the inhibition of the biotin synthetase enzyme; this inhibition prevented the intracellular concentration of biotin from reaching the levels required for normal regulation of the biotin A operon.

Actithiazic acid has been isolated from culture filtrates of *Streptomyces* spp. and shown to inhibit specifically the in vitro growth of mycobacteria (7, 11, 13, 19). Addition of minute amounts of biotin can readily reverse the inhibitory effects of the antibiotic. Since biotin is not a growth requirement for the organism, it has been suggested that the antibiotic may interfere with biotin synthesis (7, 20). A similar suggestion was proposed by Pittillo and Foster (17), who observed that in addition to inhibiting the growth of *Aerobacter aerogenes*, actithiazic acid decreases the biotin concentration within the cell.

Dhyse and Hertz (2) observed an increase in the excretion of biotin vitamers in culture filtrates of *Escherichia coli* grown in the presence of actithiazic acid. The vitamers (compounds capable of replacing the biotin requirement of an organism) were identified only by their ability to bind to the protein avidin. Ogata et al. (15), in an extensive study of the vitamer excretion patterns of the culture filtrates of various yeasts, molds, and bacteria grown in the presence of actithiazic acid, showed that in all instances, the total vitamer excretion rate was markedly enhanced (2- to 20-fold), whereas the true biotin levels decreased. The term total vitamers includes biotin and all vitamers capable of supporting the growth of *Saccharomyces cerevisiae* (3). Since the vitamer appearing in the highest concentration has been identified as dethiobiotin, it has been suggested that the antibiotic may be acting competitively at some site in the conversion of the dethiobiotin to biotin. In addition, Ogata et al. (15) also observed elevated levels of the total biotin vitamers in the culture filtrates of *Bacillus sphaericus* grown in the presence of both actithiazic acid and concentrations of biotin, which normally repress total biotin vitamer accumulation.

The antibiotic α-methyldethiobiotin was isolated by Martin et al. (12) along with α-methylbiotin from the fermentation liquor of *Streptomyces lydicus*, which had previously been observed to yield α-dehydrobiotin (8). In a study of the biological properties of α-methyldethiobiotin, Hanka et al. (9) found a pattern similar to that observed for actithiazic acid: the antibiotic was most effective against several strains of mycobacteria in a synthetic medium and ineffective against a variety of organisms in a complex medium. The growth of *E. coli* and *Bacillus subtilis*, however, was inhibited in a defined medium, and the inhibition could be readily overcome by trace amounts of biotin. Dethiobiotin was somewhat less effective in overcoming the inhibition.

In the present study, evidence is presented that shows that the two biotin antimetabolites were inhibitors of the enzymatic conversion of dethiobiotin to biotin in resting-cell preparations. The consequences of this inhibition were the derepression of the biotin A operon, owing to the decreased intracellular biotin levels, and the concomitant excretion of biotin vitamers into the culture medium.

MATERIALS AND METHODS

Bacterial strains. Resting-cell suspensions of *E. coli* K-12 strain ABD313-136 were used for the kinetic studies. This organism is a regulatory mutant (*bioR*), and therefore, all the biotin-synthesizing enzymes are

derepressed (5). The role of the two antibiotics in the regulation of the biotin operon was studied with *E. coli* K-12 strain Y10-1, a biotin prototroph, and strain ME105, a biotin auxotroph (*bioB*). The latter strain, a derivative of strain Y10-1, cannot convert dethiobiotin to biotin and hence will grow only on biotin or biotin-*d*-sulfoxide. This biotin auxotroph was also used to determine the true biotin concentrations of the culture medium. Strain ME125, which is unable to synthesize 7-keto-8-aminopelargonic acid (*bioF*), was the assay organism for total biotin vitamer determinations. Strains ME109 and ME302 were used to assay 7,8-diaminopelargonic acid aminotransferase (*bioA*) and dethiobiotin synthetase (*bioD*), respectively. The reaction sequence for the biosynthesis of biotin and the genes coding for the enzymes involved in each step are as follows: pimeloyl coenzyme A + alanine $\xrightarrow{(bioF)}$ 7-keto-8-aminopelargonic acid $\xrightarrow{(bioA)}$ 7,8-diaminopelargonic acid $\xrightarrow{(bioD)}$ dethiobiotin $\xrightarrow{(bioB)}$ biotin.

Culture conditions. For the kinetic experiments, strain ABD313-136 was grown overnight at 37°C in Vogel-Bonner medium (21) with vigorous shaking. The cells were harvested by centrifugation at 8,000 rpm and washed once with 0.05 M sodium phosphate buffer, pH 7.0. The cells were suspended in a small volume of buffer to give a final cell concentration equivalent to 6 or 20 mg (dry weight)/ml. Sulfate-starved cells were prepared by suspending the overnight culture in the minimal medium, in which $MgSO_4$ was replaced by $MgCl_2$. The cells were shaken for 1 h at 37°C and then harvested, washed, and concentrated as described above. The Vogel-Bonner minimal medium supplemented with 0.25% biotin-free casein hydrolysate (Nutritional Biochemicals Corp.) was the growth medium for strains Y10-1 and ME105. Biotin at a concentration of 0.2 ng/ml was included in the minimal medium for the growth of all biotin auxotrophs.

Enzyme assays. For the biotin synthetase activity measurements, the reaction mixture contained (in a volume of 1 ml): 50 mM sodium phosphate buffer, pH 7.0; 28 mM glucose; 1 mM $MgCl_2$; 0.5 mM Na_2SO_4 or $(NH_4)_2S$; 100 μg of chloramphenicol; and cells equivalent to 5 mg (dry weight). The concentration of *dl*-dethiobiotin was 10 mM except where indicated. The reaction mixture was incubated at 37°C for 20 or 30 min with shaking, and the reaction was terminated by the addition of 0.2 ml of 24% trichloracetic acid. After centrifugation, the supernatant fluid was assayed for true biotin by the bioassay procedure; strain ME105 was used as the assay organism (5). The 7,8-diaminopelargonic acid aminotransferase activity of the crude dialyzed extracts was assayed by the method of Eisenberg and Stoner (6); ME109 was used as the assay organism. The dethiobiotin synthetase activity measurements were carried out by the procedure of Eisenberg and Krell (4); ME105 was used as the assay organism.

Regulation studies. Strain Y10-1 was used to ascertain the role of the antimetabolites in the regulation of the biotin A operon. A number of flasks containing various concentrations of the antibiotics in the minimal casein medium were inoculated with an 18-h culture. The flasks were shaken at 37°C, and the cells were grown to mid-log phase (absorbance at 650 nm = 0.5). After being harvested by centrifugation, the cells were suspended in a small volume of 0.05 M sodium phosphate buffer, pH 7.0, containing 3 mM 2-mercaptoethanol and disrupted by sonication (Heat Systems-Ultrasonics Inc.). The suspension was centrifuged at 20,000 rpm for 30 min (Beckman type-30 rotor), and the supernatant fluid was dialyzed overnight in the breakage buffer and then assayed for the aminotransferase and dethiobiotin synthetase activities.

For determining if actithiazic acid could overcome the biotin repression of the biotin A operon, strain ME105 was first repressed by growing the culture to mid-log phase in the minimal casein medium containing 5 ng of biotin per ml. This concentration of biotin was previously shown to repress the biotin A operon almost completely (4, 5). The cells were harvested by centrifugation, washed in the minimal casein medium, and distributed among a number of flasks, each containing 40 ml of fresh medium and 5 ng of biotin per ml. Various amounts of actithiazic acid were added to the flasks to cover a wide range of actithiazic acid/biotin ratios. Each flask was shaken for 1 h at 37°C. The cells were harvested by centrifugation, and the crude extracts, prepared as indicated above, were assayed for aminotransferase and dethiobiotin synthetase activities.

Paper chromatography. Ascending paper chromatography was carried out on Whatman 3MM filter paper; butanol-acetic acid-water (65:15:35) was used as the solvent system. After being dried, the paper was placed onto the surface of a minimal agar plate inoculated with ME125 for the auxanographic identification of biotin vitamers.

Reagents. Actithiazic acid was a gift from A. C. Finlay of Pfizer Inc., and α-methyldethiobiotin was kindly furnished by L. J. Hanka of The Upjohn Co. The preparations of 7-keto-8-aminopelargonic acid and 7,8-diaminopelargonic acid were previously described (4, 6). *dl*-Dethiobiotin and *d*-biotin were products of Sigma Chemical Co., and all other chemicals were of the highest purity.

RESULTS

Biotin synthetase activity. The conditions for optimal enzyme activity were explored with both SO_4-starved and nonstarved cells, and only the data for the latter are shown in Fig. 1. Biotin formation was linear for about 1 h and for the cell concentration range of 1 to 6 mg (dry weight)/ml. *d*-Dethiobiotin was saturating at about 5 μM, and the average K_m and V_{max} were 1 μM and 15.5 pmol/ml per min, respectively. The pH optimum for the reaction was 7.0 to 7.2, and the pH of the solution dropped an average of one unit after 60 min of incubation. The sulfate-starved cells showed a similar pattern except that *d*-dethiobiotin was saturating between 2 to 3 μM. It was possible to show a sulfur requirement only for the starved cells (Table 1). There was a 20- to 25-fold increase in activity with ammonium sulfate, sodium sulfate, and glutathione, whereas cysteine and methionine were less effective. The activity of the nonstarved cells in the absence of a sulfur donor was about 30-fold greater than the activity of the starved cells. Further addition of a sulfur donor to these cells

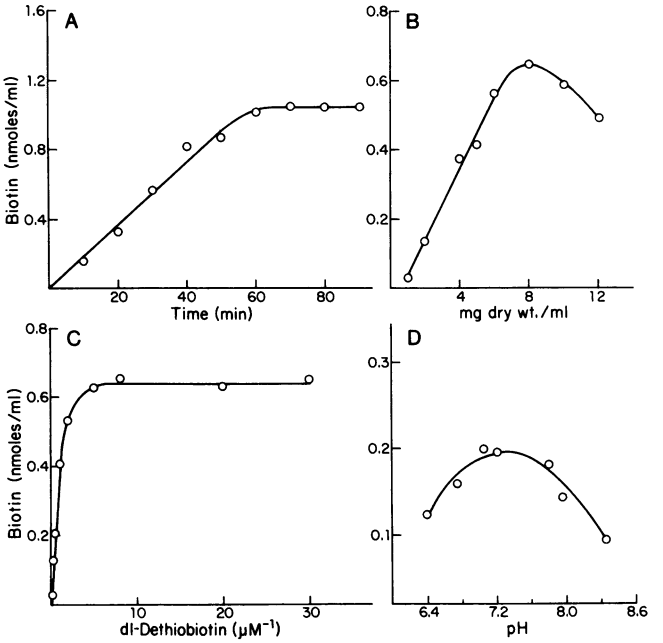

FIG. 1. Optimal conditions for the biotin synthetase reaction in resting cells of *E. coli* strain ABD313-136. (A) Time study. (B) Effect of cell concentration. (C) Effect of dethiobiotin concentration. (D) Effect of pH. Other components of the reaction mixture are indicated in the text. The cell concentration was 5 mg (dry weight)/ml.

did not substantially enhance the synthesis of biotin, suggesting that the intracellular concentration of the donor was adequate under the assay conditions used. Cysteine and 2-mercaptoethanol proved to be inhibitory in this system. As has been previously shown for other systems (15), a source of energy is also essential, and of a number of carbon sources tried, such as succinate, malate, and lactate, glucose fulfilled this role best. Iodoacetic acid inhibited the reaction completely.

Inhibition studies. When the biotin prototroph strain Y10-1 was grown in the minimal medium containing 19 μg of actithiazic acid per ml, there was a 27% increase in the generation time, whereas the same concentration of α-methyldethiobiotin gave only a 17% increase (data not shown). Paper chromatography of the culture medium showed that in the presence of actithiazic acid, only dethiobiotin could be found when the assay organism ME125, which responds to all intermediates in the biotin pathway, was used. No biotin could be detected with ME105, which responds only to biotin and biotin-*d*-sulfoxide. The increase in dethiobiotin concentration as measured with ME302 was about 10-fold. With α-methyldethiobiotin, we observed both dethiobiotin and 7-keto-8-aminopelargonic acid. A small amount of biotin was also observed in the control when assayed with ME105. Actithiazic acid also proved to be more effective than α-methyldethiobiotin in inhibiting the regulatory mutant ABD313-136 (Table 2). It is evident that both antibiotics also inhibited biotin formation from two of the intermediates in the biotin pathway, 7-keto-8-aminopelargonic acid and 7,8-diaminopelargonic acid; again, actithiazic acid was the more-effective inhibitor. Figure 2 shows that inhibition of the conversion of dethiobiotin to biotin increased with increasing concentrations of the two antibiotics. The concentrations of the two antibiotics which effected 50% inhibition were 0.45 and 1.1 μM, respectively.

TABLE 1. Effect of sulfur donors on biotin synthetase activity in sulfate-starved and nonstarved resting cells of *E. coli* strain ABD313-136

Sulfur source (mM)	Biotin formed (nmol/ml)[a]	
	Starved	Nonstarved
None	0.03	0.90
$(NH_4)_2S$ (0.5)	0.66	1.03
Na_2SO_4 (0.5)	0.66	1.03
Methionine (5.0)	0.14	0.93
Glutathione (5.0)	0.74	1.12
Cysteine (5.0)	0.34	0.48
2-Mercaptoethanol (5.0)	0.03	0.46

[a] The components of the reaction mixture are indicated in the text. Incubation time, 30 min.

Regulatory aspects. Since the excretion of high

TABLE 2. Comparison of the inhibitory activities of actithiazic acid and α-methyldethiobiotin on biotin biosynthesis from intermediates in the biotin pathway

Intermediate (μM)	% Inhibition[a]	
	Actithiazic acid	α-Methyldethiobiotin
dl-Dethiobiotin (10)	77	33
7-Keto-8-aminopelargonic acid (20)	77	28
7,8-Diaminopelargonic acid (100)	37	23

[a] The components of the reaction mixture are indicated in the text, and the strain was *E. coli* ABD313-136. The concentration of each antibiotic was 3.9 μM.

concentrations of biotin intermediates has usually been associated with derepression of the biotin operon (5), a study of the activities of two of the enzymes in the biotin biosynthetic pathway, 7,8-diaminopelargonic acid aminotransferase and dethiobiotin synthetase, was undertaken with strain Y10-1 grown in the presence of various concentrations of the two inhibitors. The results shown in Fig. 3 indicate a marked increase in the activities of both enzymes; the enzyme activities reached maximum levels at about 1 μg/ml with actithiazic acid and about twice that concentration with α-methyldethiobiotin.

Ogata et al. (15) suggested a possible direct regulatory role for actithiazic acid in the biosynthesis of biotin. This was based in part on the observation that actithiazic acid can overcome the inhibition of the total vitamer excretion in the growth medium by a concentration of biotin that is almost completely inhibitory for *B. sphaericus*. The actithiazic acid/biotin ratio was 2,000:1, and at high concentrations of biotin, this effect of actithiazic acid was reversed. Table 3 shows the results of a repression study with strain ME105 in the presence of a concentration of biotin which completely represses the biotin A operon in *E. coli*. In the absence of biotin or in the presence of actithiazic acid alone, derepressed levels of the two biotin-biosynthetic enzymes were obtained. The addition of 5 ng of biotin per ml repressed the synthesis of both enzymes, but the combination of both actithiazic acid and biotin did not overcome biotin repression when the ratios were varied over the range of 1,000:1 to 5,000:1.

DISCUSSION

The optimum conditions for the biotin synthetase reaction, conversion of dethiobiotin to biotin, have been determined for resting cells of *E. coli*. The two antibiotics, actithiazic acid and α-methyldethiobiotin, inhibited this reaction. The true biotin levels decreased rapidly with increasing concentrations of the antibiotics, whereas the intermediates, specifically dethiobiotin, show marked elevation in concentration, especially with actithiazic acid. Elevation of 7-keto-8-aminopelargonic acid was also observed with α-methyldethiobiotin. Actithiazic acid is the more effective of the two antibiotics, inhibiting biotin synthesis 50% at 0.45 μM, as compared with 1.1 μM for α-methyldethiobiotin. The mechanism for this inhibition is unknown. We had previously suggested on the basis of preliminary experiments (3) that the antibiotics were competitive inhibitors with dethiobiotin. However, repeated kinetic experiments with the intact cells gave variable results; thus, no definitive conclusion could be drawn as to the nature of the mechanism of inhibition. Certainly the structural similarities with α-methyldethiobiotin and the structural features of actithiazic acid required for antibacterial activity (1, 10, 14, 16) are compatible with a mechanism of competitive inhibition. The resolution of this question will have to await the preparation of an active cell-free extract, which has not been successfully attained to date.

It is recognized that the interpretation of data

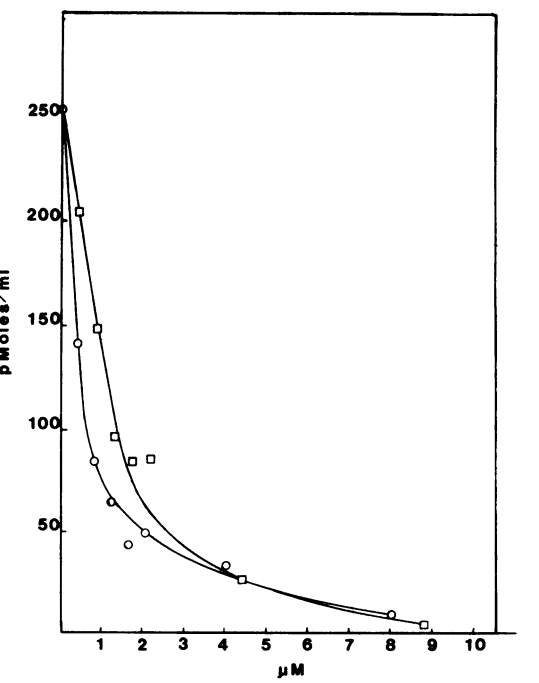

FIG. 2. Inhibition of biotin synthesis from dethiobiotin as a function of antibiotic concentration. (○) Actithiazic acid; (□) α-methyldethiobiotin. The components of the reaction mixture are indicated in the text. The concentration of *dl*-dethiobiotin was 4 μM, and the reaction time was 20 min.

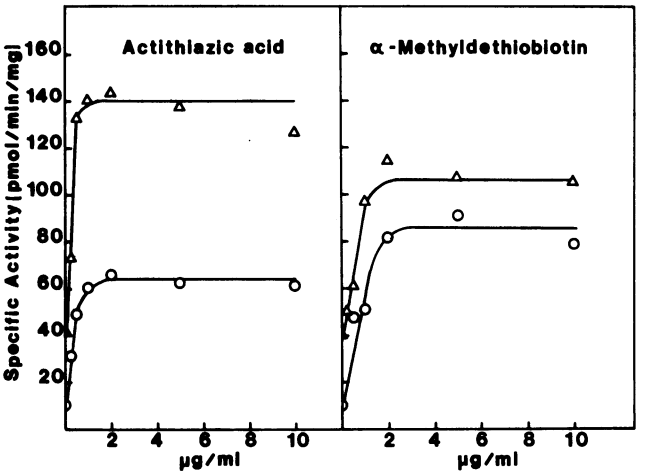

FIG. 3. Derepression of the biotin A operon of *E. coli* strain ME105 by actithiazic acid and α-methyldethiobiotin. Crude extracts were prepared from cells grown to mid-log phase and assayed for aminotransferase (△) and dethiobiotin synthetase (○) activities.

on whole cells is open to question, because the results may be influenced by activities at the cell membrane rather than those within the cell. However, the data obtained for the two intermediates of the biotin biosynthetic pathway, 7-keto-8-aminopelargonic acid and 7,8-diaminopelargonic acid, argue against the antibiotic activity being primarily a membrane phenomenon. Both intermediates were converted into biotin by resting and growing cells, and both antimetabolites inhibited this conversion. Nothing is known at present about the ability of cells to be permeated by dethiobiotin per se, but previous studies (18) showed that dethiobiotin can inhibit biotin transport, suggesting that the two compounds may utilize the same carrier system. Neither of the two intermediates inhibited biotin transport, nor did actithiazic acid at 100 times the concentration of biotin. Under the same conditions, α-methyldethiobiotin inhibited biotin transport only 12%, whereas dethiobiotin was 90% inhibitory (O. Prakash, unpublished data). In our kinetic experiments, only a sevenfold excess of antibiotic over dethiobiotin was used. It would therefore appear more likely that the antibiotics were acting intracellularly at the enzyme level.

TABLE 3. Effect of actithiazic acid on the biotin repression of the biotin A operon in strain ME105

Biotin (ng/ml)	Actithiazic acid (μg/ml)	Aminotransferase sp act[a]	Dethiobiotin synthetase sp act[b]
0	0	122	45
5	0	12	1
5	5	12	1
5	10	11	1
5	25	17	1
0	5	135	43
0	10	120	41
0	25	140	48

[a] Expressed as picomoles of 7,8-diaminopelargonic acid per minute per milligram.

[b] Expressed as picomoles of dethiobiotin per minute per milligram.

The derepression of the biotin A operon in the presence of the two antibiotics is the result of the inhibition of the biotin synthetase reaction, i.e., the conversion of dethiobiotin to biotin. With increasing concentrations of antibiotic, the internal concentration of biotin decreased to below the level required to repress the biotin A operon. The reaction of the antibiotics in regulation is therefore indirect, as initially suggested by the experiment of Ogata et al. (15), in which the total biotin synthesized by resting cells grown in the presence of actithiazic acid was markedly enhanced. However, it has not been possible to show a direct regulatory role for actithiazic acid, as was suggested by these investigators. Under conditions similar to those used in the growth experiments of Ogata et al. (15), actithiazic acid was unable to reverse the repression of the two biotin-biosynthetic enzymes by biotin. It is difficult to reconcile these opposing results except by pointing out that the conditions were quite different in the two experiments. Pimelic acid is required to observe the actithiazic effect with *B. sphaericus*, which is grown in culture for 4 days. During this interval, other reactions may come into play which are unrelated to the action of actithiazic acid. The fact that increasing concentrations of biotin can reverse this effect suggests that biotin is being

oxidized to biotin sulfoxide or biotin sulfone during this interval, thereby releasing the biotin A operon from repression. In the presence of actithiazic acid, this would result in an increase in total biotin vitamers.

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