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Reverse Transsulfuration and Its Relationship to Thienamycin Biosynthesis in *Streptomyces cattleya*

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Cystathionine γ-lyase (EC 4.4.1.1) was purified from *Streptomyces cattleya*, an actinomycete which produces the unusual β-lactam antibiotic thienamycin. The enzyme displays broad substrate specificity and is similar to γ-lyases purified from other microorganisms. That the γ-lyase functions in vivo to provide cysteine for antibiotic synthesis was shown by two types of experiments. First, cystathionine and methionine, as well as cysteine itself, are efficiently utilized by *S. cattleya* for thienamycin biosynthesis. Second, propargylglycine, a mechanism-based inactivator of cystathionine γ-lyase in vitro, inhibits the synthesis of thienamycin in vivo. This inhibition can be substantially reversed by providing the cells with another source of cysteine, such as cystine.

Reverse transsulfuration is the term that has been applied to the reactions by which the sulfur atom of homocysteine and the carbon chain of serine are combined to form cysteine (5). This pathway and some additional reactions of sulfur metabolism are diagrammed in Fig. 1. A key enzyme of this pathway is cystathionine γ-lyase (EC 4.4.1.1), which catalyzes the breakdown of cystathionine to cysteine, α-ketobutyrate, and ammonia. Early studies have shown that enteric bacteria such as *Escherichia coli* and *Salmonella typhimurium* lack cystathionine γ-lyase and therefore do not carry out reverse transsulfuration (3). More recently, Nagasawa et al. (16) have extended these studies to include 93 strains belonging to 21 genera and have found no evidence for γ-lyase activity in nonfilamentous bacteria. Previous studies from this laboratory have shown, however, that the filamentous bacterium *Nocardia lactamdurans*, which is used for the commercial production of cephamycin C, contains γ-lyase activity (10). Based on these results, Nagasawa et al. (16) examined 50 actinomycetes for γ-lyase activity and found the enzyme to be present in different amounts in all of them. Thus, unlike other types of bacteria, the ability to carry out reverse transsulfuration appears to be a general feature of sulfur metabolism in the actinomycetes.

The metabolism of methionine and cysteine has been extensively studied in *Penicillium chrysogenum* and *Cephalosporium acremonium* because of the importance of these compounds as precursors of the β-lactam antibiotics (17). In *Penicillium* spp., reverse transsulfuration appears to play a minor role in the provision of cysteine for penicillin biosynthesis because high-producing strains are very efficient in sulfate anabolism (19, 22). In wild-type *Cephalosporium* spp., reverse transsulfuration is apparently the preferred route for the incorporation of cysteine into cephalosporins (2). Treichler et al. (24, 25) have demonstrated that a mutant strain of *C. acremonium* lacking cystathionine γ-lyase is impaired in antibiotic production, a result which suggests that the enzyme may be essential for cephalosporin synthesis. However, it is clear from studies with other strains that *C. acremonium* can also use the sulfate reduction pathway when the transsulfuration pathway is blocked by mutation (11, 21). In contrast with the large amount of data available on sulfur metabolism of the antibiotic-producing fungi, little work has been published on the metabolism of cysteine and methionine by actinomycetes, even though this class of microorganisms is very important to the fermentation industry. Kern and Inamine (10) provided the first evidence that β-lactam-producing actinomycetes also use the reverse transsulfuration pathway for antibiotic biosynthesis, with the finding that 3, that propargylglycine and 3,3,3,-trifluoroalanine, mechanism-based inactivators of cystathionine γ-lyase, are inhibitors of cephamycin C synthesis by *N. lactamdurans*.

In the studies reported here, we have extended our observations to another actinomycete, *Streptomyces cattleya*, which produces the unusual β-lactam antibiotic thienamycin (Fig. 2) and also penicillin N and cephamycin C (9). We wished to determine whether cystathionine γ-lyase plays a more general role in the provision of cysteine for β-lactam biosynthesis. Furthermore, we have partially purified and characterized the γ-lyase from this organism to compare its properties to those of γ-lyases derived from different sources. Cystathionine γ-lyase has been purified from two other microbial sources, *Neurospora crassa* (4) and, more recently, to homogeneity from *Streptomyces phaeochromogenes* (16). The enzyme has also been obtained in homogeneous form from rat liver (14).

(A preliminary report of this work has appeared elsewhere [J. M. Williamson, R. Meyer, and E. Inamine, Fed. Proc. **42**:2210, 1983].)

MATERIALS AND METHODS

Materials. The strains of *S. cattleya* used in these studies were MA 4297 (NRRL 8057) and MA 5617, a derivative of MA 4297, the original soil isolate (9). Thienamycin and cephamycin C are products of Merck Sharp & Dohme Research Laboratories, Rahway, NJ. Electrophoresis grade chemicals, Dowex AG1X2 (50/100 mesh; chloride form), Dowex AG50WX2 (50/100 mesh; hydrogen form), and hydroxylapatite, were obtained from Bio-Rad Laboratories,

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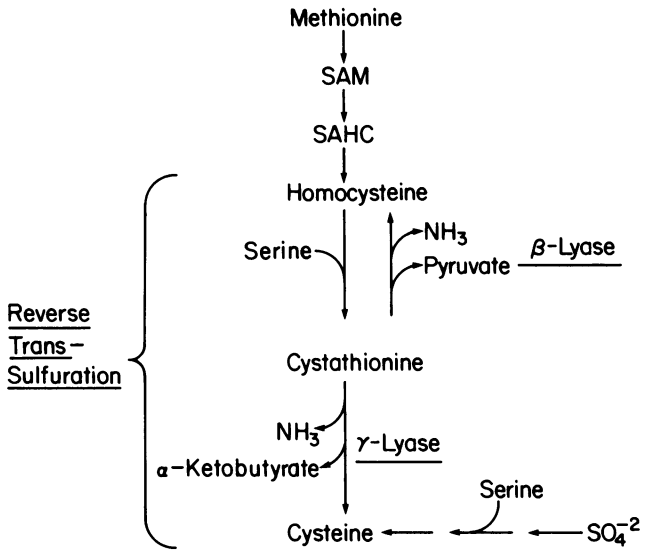


FIG. 1. Some reactions of sulfur amino acid metabolism.

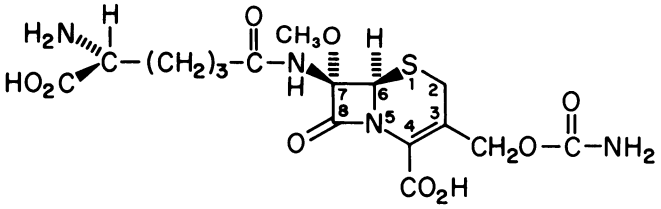
Richmond, Calif. Molecular weight standards for gel filtration and Sephacryl S-300 were obtained from Pharmacia, Inc., Piscataway, N.J. L-[^{35}S]cystathionine and [^{35}S]sulfate were from Amersham Corp., Arlington Heights, Ill. L-[^{35}S]methionine was from New England Nuclear Corp., Boston, Mass. Enzyme grade ammonium sulfate and sucrose were from Schwartz/Mann, Orangeburg, N.Y. The following were obtained from Sigma Chemical Co., St. Louis, Mo.: DEAE–Sepharose CL-6B, Sepharose 4B, disodium EDTA, pyridoxal phosphate, 5,5'-dithiobis(2-nitrobenzoic acid), L-(+)-cystathionine, 2,4-dinitrophenylhydrazine, sodium 2-ketobutyrate, sodium pyruvate, lactate dehydrogenase, NADH, ninhydrin, dithiothreitol, DL-homoserine, *O-succinyl*-L-homoserine, *O-acetyl*-DL-homoserine, L-cystine, L-djenkolic acid, DL- and *meso*-lanthionine, *S*-(carboxymethyl)-L-cysteine, β-chloro-DL-alanine, DL-homocysteine, L-methionine, L-threonine, L-serine, (*O*-phospho)-DL-threonine, cystamine, L-serine-*O*-sulfate, DL-propargylglycine, and 2-(*N*-morpholino)ethanesulfonic acid (MES). Diaion HP-20 was from Mitsubishi Chemical Industries, Ltd. Dowex 1X2 (chloride form) was converted to the bicarbonate form by the procedure of Treiber et al. (23). Ingredients for the culture media were obtained from the following sources: corn gluten meal and corn steep liquor from Corn Products Co.; solulac from Grain Processing Corp.; ardamine YEP from Yeast Products Co.; proflo from Trader's Protein Division; polypropylene glycol-2000 from Dow Chemical Co., Midland, Mich.; isoleucine and 2-(*N*-morpholino)propanesulfonic acid (MOPS) from Sigma; and monosodium glutamate monohydrate from Fisher Scientific Co., Pittsburgh, Pa.

Culture media. All media were prepared with distilled water. The complex seed medium contained the following (grams per liter): sucrose (30.0), solulac (15.0), ardamine YEP (5.0), and corn gluten meal (5.0). The pH was adjusted to 7.5 by the addition of NaOH before sterilization. The semidefined seed medium contained (per liter): monosodium glutamate monohydrate (5.0 g), NH_4Cl (1.5 g), K_2HPO_4 (2.0 g), inositol (0.4 g), NaCl (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.025 g), $ZnSO_4 \cdot 7H_2O$ (0.01 g), *p*-aminobenzoic acid (0.1 mg), $CoCl_2 \cdot 6H_2O$ (0.01 g), ardamine YEP (10.0), glycerol (10.0 g), and $CaCO_3$ (0.25 g). The pH was adjusted to 7.0 by the addition of NaOH before sterilization. The complex production medium contained the following (per liter): glycerol (30.0 g), glycine (1.3 g), corn steep liquor (25.0 g), solulac (10.0 g), proflo (5.0 g), $CoCl_2 \cdot 6H_2O$ (0.01 g), polypropylene glycol-2000 (1.25 ml), and MOPS buffer (10.5 g). The pH was adjusted to 6.8 by the addition of NaOH before sterilization. Two types of chemically defined production media were used. The first defined medium A contained (grams per liter): monosodium glutamate monohydrate (3.75), L-isoleucine (2.4), NH_4Cl (0.75), K_2HPO_4 (1.0), $CoCl_2 \cdot 6H_2O$ (0.01), $MgSO_4 \cdot 7H_2O$ (0.5), $FeSO_4 \cdot 7H_2O$ (0.025), glycerol (10.0), and MES buffer (14.6). The pH was adjusted to 7.0 by the addition of NaOH before sterilization. Defined medium B (low-sulfate medium) contained $MgCl_2 \cdot 2H_2O$ in place of $MgSo_4 \cdot 7H_2O$. Incubation conditions for the fermentation were as described previously (28).

Preparation of resting cells. Resting cells were prepared as described previously (28). The cells were isolated by centrifugation and washed three times with either 50 mM sodium MES buffer (pH 6.5) or 50 mM sodium phosphate buffer (pH 6.5). Phosphate buffer was used when the thienamycin synthesized by the resting cells was to be purified; MES buffer was used for the other types of experiments. The washed cells were resuspended in the same buffer in a final volume equivalent to one-fourth of the initial volume and used for resting cell experiments.

When the fermentation was carried out in complex production medium, the procedure for the growth of the cells was the same as that described for the preparation of resting cells, except complex seed medium was used for the development of both stages of the seed cultures and complex production medium was substituted for defined production medium.

Purification of thienamycin. Thienamycin was purified by the method of Treiber et al. (23), which involves chromatography on Dowex AG1X2 (bicarbonate form; column size, 2.5 by 30 cm), Dowex AG1X2 (chloride form; column size, 1.5 by 24 cm), and Diaion HP-20 resin (column size, 1.4 by 46 cm). Between chromatographic steps, the partially purified thienamycin was concentrated by rotary evaporation.



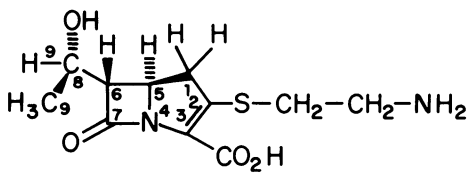


FIG. 2. β-Lactam antibiotics produced by *S. cattleya*.

Assay procedures for cystathionine γ-lyase. A number of different, complementary methods were used to quantitate cystathionine γ-lyase activity.

Method A involved determination of the rate of formation of mercaptoamino acids by reaction with 5,5′-dithiobis(2-nitrobenzoic acid). The procedure used was adapted from that of Flavin and Slaughter (6). A typical reaction mixture contained (in a total volume of 1.0 ml): 0.1 M potassium phosphate buffer (pH 8.0), 2.0 mM disodium EDTA, 0.6 mM 5,5′-dithiobis(2-nitrobenzoic acid), 10 μM pyridoxal phosphate, 4.0 mM HCl, 4.0 mM L-(+)-cystathionine, and γ-lyase. The final pH of the reaction mixture was 7.6. The rate of change of absorbance at 412 nm and 37°C was monitored continuously with a Cary 219 spectrophotometer.

Meter B involved determination of the amount of keto acids formed by reaction with 2,4-dinitrophenylhydrazine by the procedure of Shizuta and Tokushige (20). A typical reaction mixture contained (in a total volume of 0.5 ml): 0.1 M potassium phosphate buffer (pH 8.0), 2 mM disodium EDTA, 4.0 mM L-(+)-cystathionine, 4.0 mM HCl, 10 μM pyridoxal phosphate, and γ-lyase. Reaction mixtures were incubated at 37°C for 20 to 60 min. The reaction was quenched by the addition of 1 M HCl (0.5 ml). 2,4-Dinitrophenylhydrazine (0.1% in 2 M HCl; 0.15 ml) was then added. The mixtures were incubated at 37°C for 10 min. After incubation, NaOH (2 M; 1.0 ml) was added, and the absorbance at 416 nm was determined.

Method C involved determination of the rate of formation of keto acids by the use of a coupled assay with lactate dehydrogenase. A typical reaction mixture contained (in a total volume of 1.0 ml): 0.1 M potassium phosphate buffer (pH 8.0), 2.0 mM disodium EDTA, 4.0 mM L-(+)-cystathionine, 4.0 mM HCl, 10 μM pyridoxal phosphate, 0.3 mM NADH, 20 U of lactate dehydrogenase, and γ-lyase. The rate of change of absorbance at 340 nm and 37°C was monitored continuously with a Cary 219 spectrophotometer.

Method D involved reaction of the cysteine formed with ninhydrin by the procedure of Gaitonde (7). A typical reaction mixture contained the following (in a total volume of 2.0 ml): 0.1 M potassium phosphate buffer, 10 mM dithiothreitol, 10 μM pyridoxal phosphate, 4.0 mM L-(+)-cystathionine, 4.0 mM HCl, 2.0 mM disodium EDTA, and γ-lyase. The mixtures were incubated at 37°C for 30 to 60 min. The reaction was quenched by the addition of 1.0 ml of 50% trichloroacetic acid-ethanol (1:9). Insoluble material was removed by centrifugation. Portions (1.0 ml) of the supernatant solutions of each reaction mixture were added to individual tubes containing glacial acetic acid (1.0 ml) and ninhydrin reagent (1.0 ml; formed by dissolving 1.5 g of ninhydrin in 36 ml of glacial acetic acid and 24 ml of concentrated HCl). The tubes were capped, and the mixtures were boiled for 10 min. After the reaction mixtures had been boiled, they were cooled in an ice bath. Ethanol (2.0 ml) was added, and the absorbance at 560 nm of each mixture was determined. The amount of cysteine formed was determined from a standard curve. This assay method is highly specific and gives no reaction with cystathionine, methionine, or homocysteine.

Method A was used to quantitate recovery of γ-lyase activity during purification of the protein. Methods B and, occasionally, D were used to locate the enzymatic activity in column fractions. Of the assay methods used, only method D is absolutely specific for cystathionine γ-lyase activity. A unit of activity is the amount of enzyme needed for the production of 1 nmol of product per minute under standard assay conditions.

High-pressure liquid chromatographic methods. A Varian model 5000 liquid chromatograph equipped with a Vista 401 automatic data station was used. Thienamycin and cephamycin C were determined as described previously (28).

Pyruvate and α-ketobutyrate were determined with an HPX-87H Aminex ion exclusion column (Bio-Rad; 300 by 7.8 mm) at the following settings: mobile phase, 2.25 mM sulfuric acid; flow rate, 0.8 ml/min; column temperature, 55°C; injection volume, 0.01 ml; detection by absorbance at 215 nm; and absorbance units, full scale, of 0 to 1.0.

Miscellaneous methods. Electrophoresis in the presence of sodium dodecyl sulfate was carried out in slab gels by the procedure of Laemmli (12). The developed gels were first washed in a solution containing 10% methanol and 10% acetic acid to remove the sodium dodecyl sulfate. They were then stained with Coomassie blue and destained with the methanol-acetic acid solution. Radioactivity was determined by liquid scintillation counting with Scintiverse I (Fisher Scientific) as scintillant. Protein was determined by the method of Lowry et al. (13) with bovine serum albumin as standard.

Enzyme purification. All steps were carried out at 4°C. Buffer A contained 0.2 M potassium phosphate (pH 7.0). Buffer B contained 50 mM potassium phosphate (pH 7.0), 0.5 mM disodium EDTA, and 50 μM pyridoxal phosphate. Buffer C was similar in composition to buffer B except that the phosphate concentration was reduced to 5 mM.

(i) Step 1. *S. cattleya* was grown in defined medium B for 22 h as described above. The cells were isolated by centrifugation and washed once with H_2O. The washed cells (93 g, wet weight) were frozen and ruptured in an X-Press (obtained from Tekmar). The resulting frozen material was allowed to thaw in 60 ml of buffer A. Insoluble material was removed by centrifugation at 31,000 × *g* for 1 h. This step yielded 136.0 ml of crude extract. The specific activity of the crude extract ranged from 3 to 10 U/ml in a number of trials.

(ii) Step 2. Solid ammonium sulfate (16.4 g/100 ml) was added to the crude extract with stirring (30% of saturation). The precipitate that formed was isolated by centrifugation and discarded. Additional solid ammonium sulfate (24.9 g/100 ml) was added to the 30% supernatant solution to raise the concentration of the salt to 70% of saturation. The precipitate that formed was isolated by centrifugation and dissolved in buffer B (20 ml). The enzyme solution was dialyzed for 12 h against two changes of 1.5 liters each of buffer B.

(iii) Step 3. The dialyzed ammonium sulfate fraction (31.0 ml; 0.65 g of protein) was applied to a column (2.5 by 25 cm) of DEAE–Sepharose CL-6B equilibrated with buffer B. The column was washed with 300 ml of buffer B and then with a linear gradient established between 275 ml of buffer B and 275 ml of buffer B containing 0.5 M KCl. The enzyme eluted in a single peak near the end of the gradient. The fractions containing activity were combined, and the protein was precipitated by the addition of solid ammonium sulfate to 70% of saturation with the salt. The precipitate was collected by centrifugation and dissolved in buffer B.

(iv) Step 4. The enzyme solution (8.7 ml; 0.12 g of protein) was applied to a column (2.5 by 110 cm) of Sepharose 4B equilibrated and eluted with buffer B. Fractions containing activity were combined and dialyzed for 12 h against two changes of 1.5 liters each of buffer C.

(v) Step 5. The enzyme solution obtained in step 4 (52.8 ml; 0.06 g of protein) was loaded onto a column (2.0 by 19.5 cm) of hydroxylapatite equilibrated with buffer C. The column was washed with 50 ml of buffer C. The enzyme was eluted

TABLE 1. Purification of cystathionine γ-lyase from *S. cattleya*

Step	Procedure	Total activity (U)	Sp act (U/mg)	Recovery (%)	Relative sp act (U/mg)
1	Crude extract	2,530	3.2	100	1.0
2	$(NH_4)_2SO_4$ fractionation	2,230	3.4	88	1.1
3	Chromatography on DEAE–Sepharose	1,880	15.9	74	5.0
4	Chromatography on Sepharose-4B	1,190	20.5	49	6.4
5	Chromatography on hydroxylapatite	630	88.0	25	27.5

from the column by washing with buffer containing 25 mM potassium phosphate.

(vi) Step 6. The fractions containing activity were combined and concentrated by dialysis against buffer B containing 50% sucrose (wt/vol). The sucrose was removed by dialysis against two changes of 0.6 liters each of buffer B. The final volume of the purified γ-lyase solution was 17.8 ml (7.1 mg of protein). A summary of the results of the purification procedure is shown in Table 1.

RESULTS

Properties of cystathionine γ-lyase from *S. cattleya*. (i) Purification. Previous work with *N. crassa* (5) and *N. lactamdurans* (10) has shown that the specific activity of cystathionine γ-lyase was increased markedly by the growth of either of these organisms on sulfate-limited media. When this type of experiment was carried out with *S. cattleya*, a similar result was obtained: growth of the cells on sulfate-limited defined medium (as described above) gave a 2.5-fold increase in the specific activity of the enzyme (assay methods A or D gave similar results). Cells grown on sulfate-limited medium were therefore used as the starting material for purification of the γ-lyase from *S. cattleya*. The details of the purification procedure are given in Materials and Methods, and the results are summarized in Table 1. The specific activity of the purified γ-lyase was found to vary from batch to batch in the range of 0.1 to 0.8 μmol/min per mg of protein. The reason for this variation is unknown. Irrespective of the final specific activity, analysis of the purified protein by gel filtration on a column of Sephacryl S-300 showed a single peak of material absorbing at 280 nm coincident with the peak of enzymatic activity; analysis of the purified protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed the presence of two major bands of protein (M_r, 100,000 and 40,000), along with a number of minor bands.

(ii) Physical and catalytic properties of the enzyme. The K_m of the purified γ-lyase for L-(+)-cystathionine is 0.45 mM (assay method A). The pH optimum for the action of the enzyme is rather broad, 7.5 to 8.6 (assay method C). The temperature optimum for enzyme activity is 55°C or higher (assay method A).

TABLE 2. Substrate specificity of cystathionine γ-lyase from *S. cattleya*[a]

Substrate	Relative velocity
L-(+)-Cystathionine	1.00
L- or DL-Homoserine	0.03
(*O*-Succinyl)-L-homoserine	1.09
(*O*-Acetyl)-DL-homoserine	0
L-Cystine	1.80
L-Djenkolic acid	0.58
DL- and *meso*-Lanthionine	0.93
S-Carboxymethyl-L-cysteine	0.02
β-Chloro-DL-alanine	0.61

[a] Each reaction mixture contained (in a total volume of 0.5 ml): 0.12 M potassium phosphate buffer (pH 8.0), 0.01 mM pyridoxal phosphate, 2 mM substrate (if single isomer) or 4 mM substrate (if racemate), 0.75 mM disodium EDTA, 4.0 mM HCl, and purified γ-lyase (0.02 mg of protein). When compounds of limited solubility were used as substrates (such as cystine, djenkolate, and lanthionine), the final concentrations were 0.2 or 0.4 mM, and the results were compared with those obtained with cystathionine at the same concentration. The reaction mixtures were incubated at 37°C for 32 min. The amount of keto acid formed during the incubation was determined by reaction with 2,4-dinitrophenylhydrazine as described in Materials and Methods.

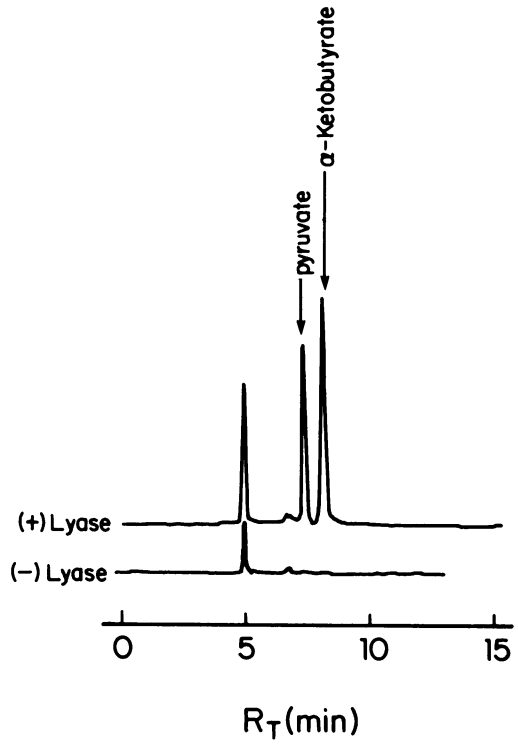


FIG. 3. Stoichiometry of cleavage of cystathionine by *S. cattleya* cystathionine γ-lyase. The reaction mixture contained (in a total volume of 0.4 ml): 0.15 M potassium phosphate (pH 8.0), 5 mM L-(+)-cystathionine, 0.75 M disodium EDTA, 10 μM pyridoxal phosphate, and purified γ-lyase (10 U). The mixtures were incubated for 92 min at 37°C. The reaction was quenched by boiling for 5 min. The mixtures were then cooled in an ice bath, and 0.08 ml of 0.5 M sulfuric acid was added to each. Insoluble material was removed by centrifugation, and the supernatant solutions were analyzed for pyruvate and α-ketobutyrate by high-pressure liquid chromatography as described in Materials and Methods.

A number of compounds were found to serve as substrates of the γ-lyase in addition to cystathionine. These results are summarized in Table 2. The following compounds were also tested but gave no activity as substrates: DL-homocysteine, L-methionine, L-threonine, L-serine, *O*-phospho-DL-threonine, cystamine, and L-serine-*O*-sulfate.

The molecular weight of the native form of cystathionine γ-lyase from *S. cattleya* was determined as 280,000 by gel filtration on a column of Sephacryl S-300. Thyroglobulin (M_r, 669,000), ferritin (M_r, 440,000), and catalase (M_r, 232,000) were used as standards.

The γ-lyase can be totally inactivated by treatment with either sodium borohydride or neutralized hydroxylamine (8.0 mM $NaBH_4$ or NH_2OH; 1.5 h at 25°C). The enzyme inactivated with hydroxylamine can be reactivated by the addition of pyridoxal phosphate (after dialysis to remove the excess hydroxylamine). In contrast, dialyzed enzyme that had been inactivated by sodium borohydride was not reactivated by pyridoxal phosphate.

(iii) Stoichiometry of cystathionine cleavage. A preliminary experiment, in which purified γ-lyase was incubated with cystathionine and the amounts of cysteine (assay method D) and α-keto acid produced (assay method B) were determined, showed that only about half as much cysteine was formed as α-keto acid. This result suggested that the enzyme was carrying out β- and γ-elimination with cystathionine. This suggestion was confirmed by an experiment in which the α-keto acids produced by the action of the enzyme on cystathionine were separated by high-pressure liquid chromatography and quantitated (Fig. 3). The ratio of γ-/β-elimination was found to be 1.6.

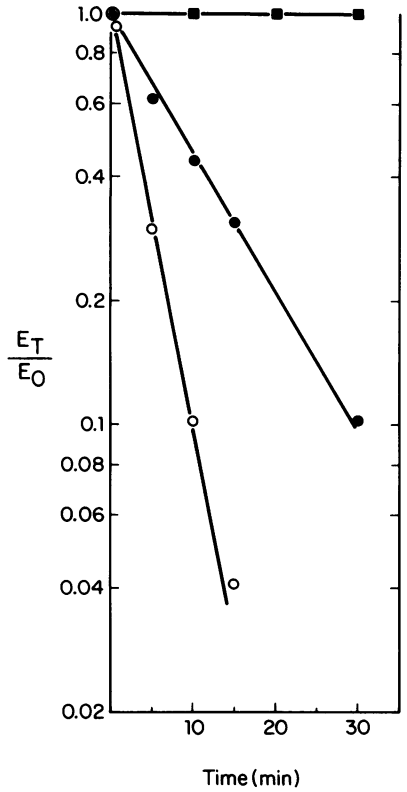


FIG. 4. Inactivation of cystathionine γ-lyase by propargylglycine. Inactivation (○) of the γ-lyase was carried out in mixtures containing (in a total volume of 0.3 ml): 50 mM potassium phosphate (pH 7.5), 50 μM pyridoxal phosphate, 0.5 mM disodium EDTA, purified γ-lyase (1.1 mg of protein), and 0.13 mM DL-propargylglycine. In one experiment, no propargylglycine was added (■). In another experiment, DL-propargylglycine (0.13 mM) and *O*-succinyl-L-homoserine (4.0 mM) were added (●). Portions (0.03 ml) were removed at the indicated times and added directly to assay mixtures. Assay method A was used (see Materials and Methods). E_T, Activity of the treated sample at time, *t*; E_O, activity of the control sample.

TABLE 3. Precursors of the cysteaminyl side chain of thienamycin[a]

Compound	Concn (mM)	Molar specific incorporation of thienamycin[b]
L-[^{35}S]cystathionine	1.0	0.74
L-[^{35}S]methionine	0.7	0.35
[^{35}S]sulfate	4.0	0.09

[a] Resting cells were prepared as described previously (28). Incubation (18 to 22 h) was carried out in 250-ml Erlenmeyer flasks (15 to 26 flasks per experiment) on a gyratory shaker (220 rpm; 5.1-cm throw) at 27°C. Each flask contained (in a total volume of 40 ml): cells (0.6 g dry weight), 20 mM sodium phosphate buffer (pH 6.5), and ^{35}S-substrate at the concentration indicated. The specific radioactivities of the substrates used were as follows: L-[^{35}S]methionine, 69.0 μCi/mmol; L-[^{35}S]cystathionine, 35.9 μCi/mmol; and [^{35}S]sulfate, 4.6 μCi/mmol. After incubation, the contents of the flasks were pooled and the cells were removed by centrifugation. Thienamycin was purified from the supernatant solution as described in Materials and Metholds and its specific radioactivity was determined.

[b] Molar specific incorporation is expressed as the specific radioactivity of the purified thienamycin divided by the specific radioactivity of the precursor.

(iv) Inactivation by DL-propargylglycine. Abeles and Walsh (1) first showed that cystathionine γ-lyase from rat liver is inactivated by incubation with propargylglycine (DL-2-amino-4-pentynoic acid). Their data indicated that this compound reacts at the active site of the enzyme and is thus a mechanism-based inactivator or suicide substrate for the γ-lyase. Figure 4 shows the results of an experiment in which purified cystathionine γ-lyase from *S. cattleya* was incubated with DL-propargylglycine. It can be seen that this treatment results in a time-dependent loss of γ-lyase activity. Furthermore, the addition of a substrate of the enzyme *O*-succinyl-L-homoserine (Fig. 4) substantially reduces the rate of inactivation by propargylglycine. Similar results were obtained when cystathionine was used as protectant. When the rate of inactivation of the enzyme as a function of the concentration (0.04 to 0.3 mM) of propargylglycine was examined, a linear relationship was found. This result suggests that the K_i of propargylglycine for the γ-lyase is considerably higher than 0.3 mM. Extensive dialysis of γ-lyase inactivated by treatment with propargylglycine (to 5% of its initial activity) did not restore enzymatic activity.

Function of cystathionine γ-lyase in vivo. (i) Sulfur donors for thienamycin biosynthesis. Previous results from this laboratory have established that cysteine is the precursor of the cysteaminyl side chain of thienamycin (28). To assess the importance of the reverse transsulfuration pathway in antibiotic biosynthesis, a number of sulfur compounds were tested for their ability to serve as precursors of thienamycin in resting cells of *S. cattleya* (Table 3). It can be seen that these compounds may be ranked as sulfur donors for thienamycin synthesis as follows: cystathionine > methionine >> sulfate. The molar-specific incorporation obtained with cystathionine is 74% of the theoretical maximum, a result similar to that obtained previously with ^{14}C- or ^{35}S-labeled cystine (28).

TABLE 4. Inhibition of resting cell synthesis of thienamycin by propargylglycine[a]

Compound	Inhibition of thienamycin synthesis (% of control without propargylglycine)
None	15
L-Cystine (0.6 mM)	56
L-Methionine (0.7 mM)	52
L-Leucine (0.6 mM)	23
L-Cystathionine (0.6 mM)	23

[a] Resting cells were prepared as described previously (28). Incubation was carried out in culture tubes (25 by 150 mm) on a gyratory shaker (220 rpm; 5.1-cm throw) at 27°C for 24 h. Each tube contained the following (in a total volume of 4.0 ml): cells (0.05 g), 50 mM sodium MES buffer (pH 6.5), 4.4 mM DL-propargylglycine, and amino acid at the concentrations indicated. The control for each condition contained the amino acid, but not propargylglycine, to correct for the effect of some of the amino acids themselves on thienamycin synthesis. After incubation, the cells were removed by centrifugation. The presence of thienamycin in the supernatant solution was determined by high-pressure liquid chromatography as described previously (28).

(ii) Inhibition of antibiotic synthesis in vivo by propargylglycine. Addition of DL-propargylglycine (9 mM) to complex production medium at the time of inoculation led to inhibition of both thienamycin (89% inhibition) and cephamycin C synthesis (86% inhibition) by *S. cattleya* after 96 h of incubation under standard fermentation conditions. There was no effect of propargylglycine on the growth of the cells at the concentration used in this experiment. Addition of propargylglycine to resting cells of *S. cattleya* also gave inhibition of theinamycin synthesis (Table 4). Inhibition of thienamycin synthesis by propargylglycine was substantially reduced by incubation with small amounts of cystine or methionine, whereas leucine and cystathionine were relatively ineffective.

DISCUSSION

Cystathionine γ-lyase, purified from *S. cattleya*, exhibits the broad substrate specificity characteristic of enzymes of this class. *S. cattleya* γ-lyase has an affinity for cystathionine (K_m <1 mM) similar to that of the enzymes from *S. phaeochromogenes* (16) and *N. crassa* (4) and differing from that of the rat liver enzyme (K_m, 3 mM [15]). Homoserine and *O*-acetyl-L-homoserine are poor substrates for the *N. crassa* and *S. cattleya* enzymes, whereas *O*-succinyl-L-homoserine is a good substrate for the enzymes from both sources (5). *S. phaeochromogenes* γ-lyase has a low affinity for, but high maximal velocity with, homoserine as substrate; neither *O*-acetyl- nor *O*-succinyl-L-homoserine were apparently tested as substrates for the enzyme (16). In contrast with the results with all of the enzymes from microbial sources, rat liver cystathionine γ-lyase shows the greatest activity with homoserine as substrate (15). All of the γ-lyases reported to date carry out elimination reactions with cystine, djenkolic acid, and DL-lanthionine as substrates (5, 15, 16). With cystathionine as substrate, however, rat liver γ-lyase carries out γ-elimination exclusively (8, 15). The enzymes from *N. crassa* (6) and *S. phaeochromogenes* (16) catalyze about 15% β-elimination from cystathionine in addition to γ-elimination. Cystathionine γ-lyase from *S. cattleya* carries out 62% γ-elimination and 38% γ-elimination. Because the enzyme preparation from *S. cattleya* is not homogeneous, it could be argued that at least a portion of the β-elimination is catalyzed by a contaminating β-lyase. This seems unlikely, however, since propargylglycine, which inactivates enzymes that act on the γ-carbon of their substrates (26), inactivates *S. cattleya* cystathionine γ-lyase to greater than 95% with monophasic kinetics. The assay method used in these studies, reaction of the sulfhydryl products of cystathionine cleavage with 5,5′-dithiobis(2-nitrobenzoic acid), would have allowed the detection of residual β-lyase activity. It is tempting to speculate that the ability of the microbial cystathionine γ-lyases to carry out some β-elimination as well as γ-elimination reflects their origin as mutated forms of the β-lyase.

Both the *S. cattleya* and *S. phaeochromogenes* cystathionine γ-lyases are large, multisubunit, pyridoxal phosphate-dependent enzymes. The temperature optima of the two actinomycetal enzymes are high, on the order of 50°C; stability problems with the *S. cattleya* γ-lyase at temperatures in excess of 55°C prevented a more precise determination of its temperature optimum. The pH optimum of the *S. cattleya* enzyme is somewhat lower than that reported for the *S. phaeochromogenes* enzymes (9.0 [16]). In addition, the fact that cystathionine γ-lyase activity is increased in *S. cattleya* by growth of the cells under conditions of sulfate limitation suggests that the enzyme is under some sort of metabolic control. Similar observations were also made with the *N. crassa* (5) and *N. lactamdurans* γ-lyases (10).

That the γ-lyase of *S. cattleya* and, therefore, the reverse transsulfuration pathway actually function in vivo for antibiotic biosynthesis is shown by two types of experiments. First, both cystathionine and methionine, as well as cysteine itself, can serve as efficient sulfur donors for thienamycin biosynthesis. In contrast, sulfate is a poor source of sulfur for thienamycin synthesis. Second, propargylglycine, an inactivator of cystathionine γ-lyase in vitro, inhibits to a similar extent the synthesis of both thienamycin and cephamycin C by *S. cattleya*. These β-lactam antibiotics share cysteine as a common precursor (27, 28). The fact that the addition of propargylglycine leads to inhibition of the biosynthesis of both of these antibiotics suggests that the reverse transsulfuration pathway is the major pathway used to provide cysteine in *S. cattleya*. This conclusion is reinforced by the finding that the addition of small amounts of cystine, the product of γ-lyase action, substantially reverses the inhibition of resting cell synthesis of thienamycin by propargylglycine. Since 3.7 times more of a single isomer of propargylglycine than of cystine was added, it seems unlikely that the ability of cystine to reverse propargylglycine inhibition is due simply to interference with uptake of the inhibitor. Cystathionine, the substrate of the γ-lyase, did not reverse propargylglycine inhibition of thienamycin synthesis. This result is reasonable since reversal of inhibition by cystathionine would be expected only if a sufficiently high concentration of the substrate could be continuously maintained in vivo to prevent inactivation of the enzyme by propargylglycine. Leucine, a compound reported to reverse the antibacterial effects of propargylglycine in other organisms (18), did not reverse inhibition of thienamycin biosynthesis by resting cells. This result supports the conclusion that propargylglycine inhibits thienamycin biosynthesis because of its effect on sulfur metabolism and not because of effects on other, unrelated enzyme systems. Finally, the ability of methionine to reverse propargylglycine inhibition of thienamycin biosynthesis is more difficult to explain. Since both methionine and cysteine are required for thienamycin biosynthesis (28), it seems possible that methionine could reverse the inhibition by sparing the small endogenous pool of cysteine for antibiotic biosynthesis. The validity of this hypothesis, however, rests on the assumption that *S.*

cattleya can convert cysteine-sulfur to methionine-sulfur via direct transsulfuration. This has not yet been demonstrated experimentally.

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