CYSTATHIONINE γ-LYASE ACTIVITY IN THE CEPHAMYCIN C PRODUCER *STREPTOMYCES LACTAMDURANS*

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Extracts of the cephamycin C producer *S. lactamdurans* were found to possess cystathionine γ-lyase activity (E.C. 4.4.1.1). This represents the first demonstration of this enzyme of the reverse transsulfuration pathway in a prokaryotic organism. A likely involvement of reverse transsulfuration in antibiotic synthesis is indicated by the fact that propargylglycine, a mechanism-based inhibitor of the γ-lyase, is a strong inhibitor of cephamycin C production.

Experiments employing cell-free systems have recently been quite successful in elucidating details of the unusual reactions by which the Arnstein tripeptide is converted into penicillins and cephalosporins[1~3]. By contrast, many aspects of the metabolic pathways leading to the incorporation of cysteine into β-lactams remain unclear. Since formation of the aminoadipoylcysteine dipeptide is presumably the first reaction in β-lactam synthesis[4], clarification of some of these points could be expected to provide important insights into the regulation of the initiation of antibiotic synthesis.

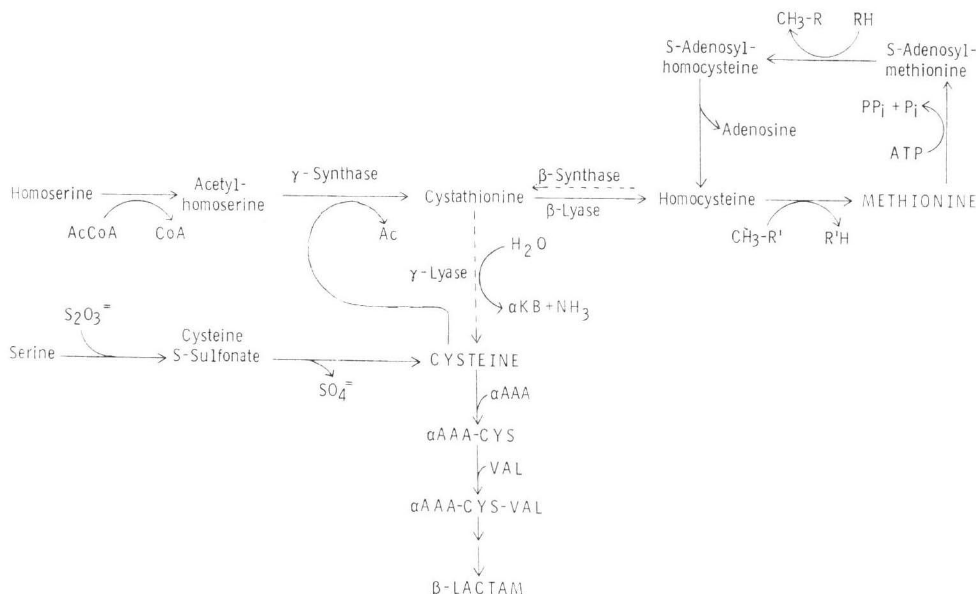
The cysteinyl moiety of the penicillins produced by *Penicillium chrysogenum* can be generated *via* both sulfate reduction and reverse transsulfuration[5,6] (Fig. 1). However, high-producing strains of *P. chrysogenum* are efficient in sulfate anabolism[7,8], and reverse transsulfuration has been thought to play only a minor role in penicillin synthesis. Cysteine for the production of cephalosporins by *Cephalosporium acremonium,* on the other hand, derives preferentially from methionine *via* reverse transsulfuration[9]. The role of methionine in this orgnism is complicated by the fact that it acts as an effector of antibiotic synthesis in addition to its role as a sulfur donor[10~12].

Recent research has further underscored the complexity of the role of methionine in cephalosporin fermentations. A mutant of *C. acremonium* lacking cystathionine γ-lyase was found to be impaired in antibiotic production and to be unresponsive to methionine additions which were stimulatory to its parent[13,14], suggesting that the γ-lyase is important and perhaps essential to cephalosporin synthesis. However, a second mutant with blocks in sulfate assimilation as well as in cystathionine synthesis has been found to be stimulated by cysteine-S-sulfonate, an immediate precursor of cysteine derived from the sulfate reduction pathway[15]. This result confirms the report of KOMATSU and KODAIRA[16] which indicated that certain mutants of *C. acremonium* can in fact derive cysteine for cephalosporin synthesis from sulfate. Thus, although reverse transsulfuration may be the preferred route for the incorporation of cysteine into cephalosporin in wild-type *C. acremonium,* the process is not essential, and mutants can be isolated which are able to utilize the sulfate reduction pathway.

In contrast to these extensive studies with *Penicillium* and *Cephalosporium,* the metabolism of cysteine and methionine by β-lactam-producing actinomycetes is a topic which has been virtually ignored. Perhaps most significantly, the present literature does not clarify whether these filamentous bacteria are able to transfer sulfur from methionine and homocysteine to cysteine. Prokaryotes are classically said to strictly lack this capacity[17,18], but the possible importance of reverse transsulfuration in bacterial

Fig. 1. Physiologically important reactions of cysteine, methionine, and their metabolites in fungi and bacteria.

The figure is not an exhaustive summary, but is meant to provide an overview of general patterns involved in sulfur metabolism. The steps indicated by broken arrows represent the two reactions of reverse transsulfuration, previously thought to occur only among eukaryotes.



antibiotic production finally prompted an investigation of the metabolism of methionine by the cephamycin C producer *Streptomyces lactamdurans**.

These studies with *S. lactamdurans* have produced the first demonstration of a bacterial cystathionine γ-lyase. Aspects of the production and regulation of the γ-lyase during the cephamycin C fermentation are presented in this paper. Furthermore, the specific importance of this enzyme in cephamycin C synthesis is investigated through the use of mechanism-based inhibitors of γ-elimination reactions.

Materials and Methods

Culture Conditions

The strain of *S. lactamdurans* used in these studies was MA4914. This organism is a high-producing, ultraviolet-induced mutant of MA2908 (NRRL 3802), the original soil isolate[19]. The defined medium used for both seed development and antibiotic production contained (per liter): sodium L-glutamate (4.25 g), glucose (10.0 g), NH_4Cl (1.00 g), inositol (0.20 g), K_2HPO_4 (2.0 g), NaCl (0.50 g), $MgSO_4 \cdot 7H_2O$ (0.50 g), $Na_2S_2O_3$ (0.25 g), $CaCO_3$ (0.25 g), $FeSO_4 \cdot 7H_2O$ (0.025 g), $ZnSO_4 \cdot 7H_2O$ (0.010 g), $MnSO_4 \cdot H_2O$ (0.005 g), and *p*-aminobenzoate (0.001 g) adjusted to pH 7.0. Seed cultures (40 ml of defined medium in 250-ml, three-baffle flasks) were grown on a gyratory shaker (220 rpm, 2-in. [*ca.* 5.1-cm] throw) at 28°C and used to inoculate production medium at 48 hours. The production cultures were grown in the defined medium under the same conditions, except that unbaffled flasks were used. Dry cell weight determinations showed that logarithmic growth in the production cultures terminated at 48 hours. "Sulfate-free" synthetic medium was prepared by substituting $MgCl_2 \cdot 2H_2O$ for $MgSO_4 \cdot 7H_2O$ and omitting $Na_2S_2O_3$.

* Preliminary biochemical data indicate that this organism may belong to the genus *Nocardia* (S. A. CURRIE, unpublished data).

Cephamycin C Assay

The level of cephamycin C in centrifuged culture broth was determined using an agar diffusion assay with *Vibrio percolans* ATCC 8451 as the test organism. Cephamycin C titers are reported in arbitrary units.

Cystathionine γ-Lyase Assay

Cells were usually harvested for enzyme assay after 40 ~ 48 hours of growth and then washed twice with water. The washed cells were resuspended in a solution made up of 20 ml of Tris-HCl buffer (0.20 M, pH 8.5), 2.5 ml of glycerol, 1.0 ml of pyridoxal phosphate stock (400 μg/ml), and 10 mg of dithiothreitol. The cells were broken by twice passing them through a cooled French Pressure Cell at 6,000 psi. Debris was removed by centrifuging 10 minutes at 12,000 *g*, and the remaining supernatant was used as the cell-free extract. Enzyme incubations consisted of 0.5 or 1.0 ml of cell-free extract (1 ~ 5 mg protein), 0.25 mg cystathionine, and 40 μg pyridoxal phosphate in a final volume of 2.0 ml. The incubations were run for 30 or 60 minutes at 30°C and were stopped by the addition of 1.0 ml of a 1 : 9 mixture of 50% trichloroacetic acid and ethanol. Precipitated proteins were removed *via* centrifugation, and the cysteine in 1.0 ml of supernatant was determined using the acid ninhydrin assay of GAITONDE[20]. This assay is highly specific and gives essentially no reaction with cystathionine, methionine, or homocysteine. The lack of color development with homocysteine implies that the assay is specific for γ-lyase activity and does not measure any β-lyase activity (which would generate homocysteine from cystathionine); a test such as the dithiobisnitrobenzoic acid assay for sulfhydryl groups lacks this specificity and does not distinguish between γ- and β-lyase activities. When working with crude cell-free extracts it was necessary to include an enzyme blank to correct for the time-dependent generation of cysteine from an endogenous substrate contained in the extract. That the enzyme catalyzing this formation of cysteine was not the γ-lyase was indicated by the fact that the temperature optimum of this enzyme was significantly higher than that of the γ-lyase. Furthermore, the interfering enzyme was not subject to inhibition by mechanism-based inhibitors of γ-elimination reactions.

The concentration of protein in the cell-free extract was determined using the procedure of LOWRY *et al.*[21], with bovine serum albumin as standard. The specific activity of the cystathionine γ-lyase was expressed in terms of μmoles of cysteine formed per mg of protein per hour.

Chemicals

The cystathionine used in these studies was purchased from the Sigma Chemical Company. It was 95 ~ 98% pure and believed to be a mixture of the L- and L-allo-stereoisomers. DL-Propargylglycine was also purchased from Sigma. 3,3,3-Trifluoroalanine was purchased from E. Merck (Darmstadt). Other chemicals used in these experiments were reagent grade and were commercially available.

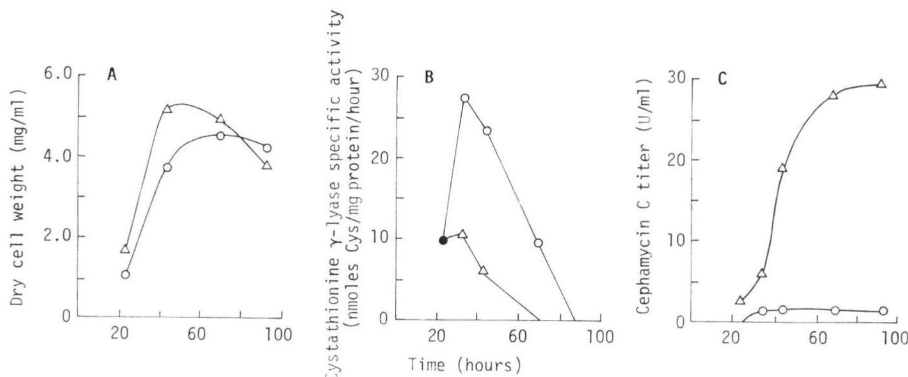
Results

S. lactamdurans MA4914 produces cystathionine γ-lyase activity when it is grown in synthetic medium under standard conditions. The specific activity profile indicates that the enzyme is present during the growth phase and during the period of rapid antibiotic synthesis (Figs. 2A, 2B, and 2C). The γ-lyase is absent from standard incubations after 70 hours.

In an attempt to increase enzyme titers for characterization experiments, *S. lactamdurans* MA4914 was grown under conditions of sulfur limitation. The cells grew somewhat slowly under these conditions (Fig. 2A), but cystathionine γ-lyase activity was increased 3-fold (Fig. 2B), indicating a significant derepression effect. Cells grown under sulfur limitation produced no cephamycin C, however.

Initial characterization of the γ-lyase in crude cell-free extracts showed it to have a temperature optimum of 30 ~ 40°C and a pH optimum of 8.5 ~ 9.0. The enzyme is inhibited by cysteine, suggesting that feedback inhibition may be an important regulatory mechanism *in vivo*. More thorough characterization was deferred until a partially purified sample of the γ-lyase becomes available.

Fig. 2. Growth (A), cystathionine γ-lyase (B), and cephamycin C (C) profiles in standard (△) and sulfur-starved (○) fermentations of *S. lactamdurans*.



The mechanism-based inhibitor propargylglycine has been used in detailed studies of the mechanism of the cystathionine γ-lyase reaction in rat liver[22,23]. Addition of propargylglycine (PGly) to incubations of the streptomycete enzyme showed a clear inhibitory effect (Table 1), indicating that the enzyme has a requirement for pyridoxal phosphate and that the mechanism of the bacterial γ-lyase reaction is fundamentally similar to that of the liver γ-lyase reaction. 3,3,3-Trifluoroalanine (TFA), another mechanism-based inhibitor[24], was shown to be a less potent inhibitor of the streptomycete γ-lyase than PGly (Table 1).

Table 1. Inhibition of *S. lactamdurans* cystathionine γ-lyase by propargylglycine and 3,3,3-trifluoroalanine.

The inhibitors were added to enzyme incubations at the levels stated, and a 5-minute pre-incubation period was observed before cystathionine was added to start the reaction.

Inhibitor (mM)	% Inhibition	
	Propargylglycine	Trifluoroalanine
0.00	0	0
0.18	18	0
0.36	27	0
0.72	56	11
2.2	93	41

The importance of the cystathionine γ-lyase reaction in cephamycin C synthesis was investigated by adding PGly to standard fermentations at the time of inoculation. Under these conditions growth was slowed slightly, but cells did grow to normal densities. Cephamycin C synthesis was found to be inhibited 75% by the addition of PGly to 2 mM (Fig. 3). Furthermore, addition of PGly during the phase of antibiotic production caused an immediate and complete inhibition of cephamycin C synthesis (Fig. 4). TFA, the less potent inhibitor of cystathionine γ-lyase, was also significantly less active as an inhibitor of cephamycin C synthesis (Figs. 3 and 4).

Discussion

All studies performed prior to these experiments indicated that prokaryotic organisms universally lack cystathionine γ-lyase activity[17,18]. The present demonstration of a cystathionine γ-lyase in the cephamycin C producer *Streptomyces lactamdurans* is the first report of this enzyme in a bacterium. Such a finding represents an important exception to the general patterns of sulfur metabolism classically described for prokaryotes and eukaryotes. Furthermore, it suggests that the metabolism of sulfur by actinomycetes may differ significantly from sulfur metabolism in other bacteria. The presence of a cystathionine β-synthase was not investigated in these experiments, but the possibility that *S. lactam-*

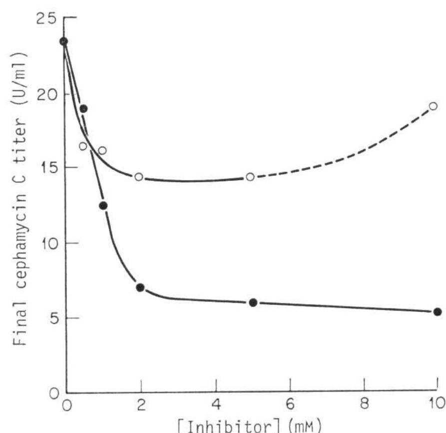


Fig. 3. Inhibition of cephamycin C production by propargylglycine (●) and 3,3,3-trifluoroalanine (○) added at inoculation.

The increase in bioactivity in the 10 mM TFA fermentation is apparently due to toxicity of a metabolite of TFA toward the assay bacterium.

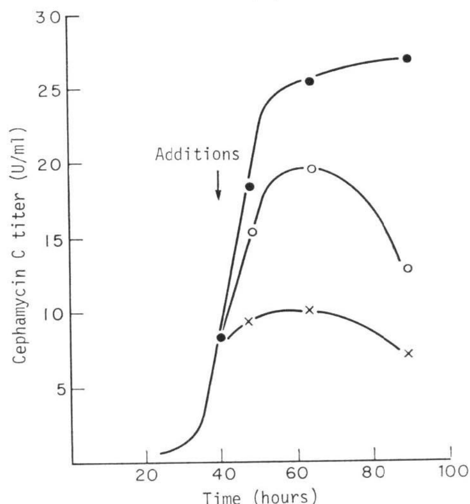


Fig. 4. Inhibition of cephamycin C synthesis by propargylglycine (×) and 3,3,3-trifluoroalanine (○) added to 5 mM during the phase of antibiotic production.

Control fermentations (●) received no additions.

durans produces this enzyme—and is therefore fully capable of performing reverse transsulfuration—should certainly be considered.

The potent inhibition of antibiotic production by low levels of PGly added at inoculation (Fig. 3) and the immediate and complete inhibitory effect of PGly added at 40 hours (Fig. 4) indicate that PGly inhibits an enzyme which is directly involved in cephamycin C synthesis. The dual activities of PGly as an inhibitor of cystathionine γ-lyase activity and cephamycin C synthesis suggest that the cysteinyl moiety of the antibiotic may be derived in large part *via* reverse transsulfuration. This hypothesis is further supported by the additional finding that the activities of PGly and TFA as inhibitors of cephamycin C synthesis mirror their relative activities as γ-lyase inhibitors. However, a possible second effect of PGly upon an enzyme in the sulfate reduction pathway has not as yet been ruled out, so that the relative importance of reverse transsulfuration in cephamycin C synthesis is not unequivocally established.

WHITNEY *et al.* reported preliminary findings that showed ^{35}S label from methionine to be incorporated into cephamycin C by *Streptomyces clavuligerus*[25]. This result is a further indication that reverse transsulfuration is probably operative in the actinomycetes and of some importance in β-lactam synthesis. However, lack of data on the efficiency of the incorporation again prevents a precise assessment of the relative importance of this pathway in antibiotic production.

The persistent production of low but significant levels of cephamycin C at relatively high levels of PGly (Fig. 3) appears anomalous to the above theory of the importance of reverse transsulfuration. This phenomenon may indicate, however, that *S. lactamdurans* is capable of deriving some cysteine for cephamycin C synthesis from the sulfate reduction pathway. The enzymes involved in sulfate reduction in this organism have not been studied in detail as yet, but label from thiosulfate is known to be efficiently incorporated into cephamycin C[26].

Recent experiments have further demonstrated cystathionine γ-lyase activity in *Streptomyces cattleya*, the producer of the unusual β-lactam antibiotic thienamycin (KERN & INAMINE, unpublished data). While the γ-lyase may in fact be common among the actinomycetes, a specific survey of other representative bacteria is required for a proper assessment of its distribution. Also, a comparison of the physical and biochemical properties of purified bacterial γ-lyase with those of the *Neurospora* enzyme would be interesting and could have important implications for theories concerning the evolution of sulfur metabolism in microorganisms.

To date there is a nearly complete dearth of information in the literature regarding the metabolism of sulfur-containing amino acids by filamentous bacteria. The experiments reported here must be extended in terms of both the enzymes and the organisms examined before conclusions can be drawn about the extent to which this area of actinomycete metabolism resembles that of the fungi. Certainly, though, the involvement of "fungal" types of enzyme activities in the incorporation of sulfur into cephamycin C would stand in sharp contrast to recent studies of the origin of the aminoadipoyl moiety; actinomycetes have been shown to generate this side-chain from L-lysine in a series of reactions demonstrated only in bacteria[27].

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