Production of Cephalosporin C by Single and Double Sulfur Auxotrophic Mutants of *Cephalosporium acremonium*¹

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An early blocked sulfur amino acid auxotroph, *Cephalosporium acremonium* mutant 274-1 (which could be satisfied by methionine or cysteine), utilized organic sulfur compounds for cephalosporin C production in the following order of decreasing effectiveness; methionine > cystathionine > cysteine, despite the fact that cysteine is considered to be the immediate precursor of the antibiotic. When a genetic block was added to mutant 274-1 in the transsulfuration pathway from cysteine to methionine, the double mutant 11-8 (which grows on methionine but not cysteine) failed to produce cephalosporin C from cysteine even though enough methionine was added to support normal growth. Addition of the non-sulfur analogue, norleucine, resulted in antibiotic production from cysteine in the double mutant. These facts support the hypothesis that methionine stimulation of cephalosporin C production is due to a role of methionine other than that of sulfur donation.

The probable pathway of sulfur metabolism in *Cephalosporium acremonium* is shown in Fig. 1. Although the immediate donor of sulfur to cephalosporin C is cysteine, this amino acid exerts little to no stimulatory effect on antibiotic production in a sulfate-containing medium. Methionine, on the other hand, is an excellent stimulator (for review, see reference 2). We suspect that the effect of methionine is a regulatory one. We have recently shown (6) that methionine has an obligatory role in stimulation of cephalosporin C production from sulfate even though the shortest path from sulfate to cephalosporin C (Fig. 1) does not include methionine. Using a methionine auxotroph (S-1), presumably blocked in transsulfuration between cystathionine and homocysteine, we found that limitation of methionine in the presence of excess sulfate inhibited antibiotic production but not growth; i.e., the mutant produced no cephalosporin C from sulfate whereas its parent did produce the antibiotic. Although our calculations indicated that the small amount of methionine added for growth of mutant S-1 would not be expected to shut off sulfate assimilation, we felt that the known depressive effect of methionine on sulfate uptake (1) constituted an unne-

cessary complication. The present work was undertaken to determine whether methionine was also obligatory for the conversion of cysteine to cephalosporin C using mutants blocked so that they cannot utilize sulfate as a source of sulfur.

MATERIALS AND METHODS

Cultures. *C. acremonium* CW19 is a prototrophic strain obtained from Eli Lilly and Co. As described below, mutant 274-1 was obtained after ultraviolet irradiation of strain CW19 and is presumably blocked in conversion of sulfate to cysteine. It does not grow on sulfate as the sole sulfur source but does grow on cysteine, cystathionine, homocysteine, or methionine. Mutant 11-8 was isolated after ultraviolet irradiation of mutant 274-1. It responds only to homocysteine or methionine and is presumably a double mutant blocked both in conversion of sulfate to cysteine and in conversion of cysteine to methionine.

Media and cultural conditions. Growth media and culture growth conditions have been previously described (6).

Cephalosporin C and dry cell weight determinations. Antibiotic titer and dry cell weight were determined as described in a previous paper (6).

Mutagenesis. *C. acremonium* CW19 after growth for 7 days on slants of the complete sporulation agar of Lemke and Nash (7) was used for mutagenesis. After adding 10 ml of 0.1 M phosphate buffer, pH 7.4, conidia and other morphological forms were washed off the surface of several agar slants by vigorously agitating the tubes. After centrifugation and washing of the pooled preparation, the pellet was resuspended

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FIG. 1. Probable pathway of sulfur metabolism in *Cephalosporium acremonium*. Mutant 274-1 is thought to be blocked in one of the steps between SO_4^{--} and cysteine and thus will grow on cysteine, methionine, cystathionine, or homocysteine. Mutant 11-8 has the same early block as its parent (274-1) but in addition is thought to be blocked in the reaction from cystathionine to homocysteine in the transsulfuration path. It cannot make methionine from cysteine or cystathionine and thus can only grow on methionine or homocysteine. SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

in 5 ml of 0.85% sodium chloride and the cell concentrations were determined microscopically. The suspensions were irradiated with ultraviolet light (General Electric lamp G8T5; 258 nm) at a rate of 18 ergs/min and a distance of 30 cm. The cells were agitated during irradiation by vibrating the petri plate containing the suspension with a vibrator platform (Syntron Jogger, model PJ-4, Syntron Co., Homer City, Pa.). The cells were incubated for 1 h in the dark after irradiation. Viable counts were determined by plating 0.1 ml of appropriately diluted samples on complete sporulation agar. Colonies from complete sporulation agar were picked with sterile toothpicks and transferred to the sulfate-containing minimal sporulation agar plates (7) and to the minimal medium supplemented with either L-cysteine, DL-cystathionine, DL-homocysteine (derived from DL-homocysteine-thiolactone [8]) or DL-methionine at a level of 0.1% (wt/vol). Mutant 274-1 was isolated as an isolate which grows well on all the supplemented minimal media but not on minimal medium.

Mutant 274-1 was grown and treated with ultraviolet light as above. Survivors were tested for sulfur requirements. Mutant 11-8 was obtained as a culture which grows well on minimal agar plates supplemented with homocysteine or methionine but does not grow on minimal agar in the presence or absence of sulfate, cysteine, or cystathionine.

Chemicals. Cephalosporin C was the gift of C. A. Claridge of Bristol Laboratories (Syracuse, N.Y.). DL-Homocysteine-thiolactone was purchased from Aldrich Chemical Co., Milwaukee, Wis.

RESULTS

Cephalosporin C production by mutant 274-1. Mutant 274-1 did not grow with sulfate

as sulfur source but did respond to cysteine, cystathionine, homocysteine, or methionine. It is thought to be blocked between sulfate and cysteine in the sulfate reduction pathway (Fig. 1). It was of considerable interest to determine the ability of these sulfur sources to support cephalosporin C production. Although in previous studies (1, 3, 4, 9, 10), cysteine, cystathionine, and homocysteine failed to duplicate the marked stimulation of methionine, these earlier studies were done with prototrophic cultures in which sulfate would be competing with the organic sulfur compounds. The isolation of mutant 274-1 thus afforded the opportunity to test sulfur sources without competition from sulfate.

In chemically defined liquid medium (6), DL-homocysteine supported a limited amount of growth at a concentration of 1 g/liter but higher levels were toxic to growth. Its effect on antibiotic synthesis thus could not be ascertained.

The experiment described in Fig. 2 compares L-cysteine at three concentrations with DL-methionine at 5 mg/ml. Antibiotic production was much poorer with cysteine than with methionine. Although growth in cysteine was slower than that in methionine, the extent of growth

FIG. 2. Growth and production of cephalosporin C by mutant 274-1 in the presence of DL-methionine (5 mg/ml or 34 mM) or L-cysteine (1, 3, and 5 mg/ml or 8.3, 25, and 41 mM, respectively).

was similar for the two amino acids. It is thus clear that cysteine is much poorer than methionine as a sulfur source for antibiotic production. Cysteine was also examined at 0.3 g/liter, but growth and cephalosporin C production were poorer than at the higher concentrations shown in Fig. 2.

Unlike cysteine, cystathionine supported rates and extents of growth as high as observed with methionine (Fig. 3). Despite nearly identical growth, cephalosporin C production in cystathionine was slower and somewhat less extensive than that in methionine. A lower concentration of cystathionine (not shown) was less effective.

It was evident that as the sulfur-containing amino acids increased in metabolic distance from methionine and got closer to cephalosporin C (Fig. 1), they became less able to stimulate antibiotic synthesis. These data supported the concept that methionine is more than a mere antibiotic precursor, i.e., sulfur.

Studies with double mutant 11-8. The data above suggested that sulfur intermediates must first be converted to methionine before they can stimulate antibiotic production. A test of this hypothesis became possible with the isolation of mutant 11-8, blocked in the path from cysteine

to methionine and (like its parent) in the early pathway from sulfate to cysteine (Fig. 1). We knew that the parent culture (274-1) could grow on cysteine and produce cephalosporin C. The question we now asked was whether growth in excess cysteine leads to antibiotic production when the path from cysteine to methionine is genetically blocked, i.e., in mutant 11-8.

Before using mutant 11-8 to answer the above question, one complication had to be eliminated. Our experimental plan was to supply methionine (the growth requirement of mutant 11-8) at a level low enough to support optimum growth but not antibiotic production. In addition, cysteine would be added to provide sulfur for antibiotic biosynthesis. However it is known (J. Nüesch, personal communication) that cysteine represses and inhibits methionine uptake in *C. acremonium*. Indeed, we found (Fig. 4) that addition of L-cysteine to the medium at the start of a fermentation inhibits growth of mutant 11-8. This problem was solved by intermittently adding small amounts (0.3 g/liter) of cysteine at regular intervals starting 1.5 to 3 days after inoculation. Such a procedure resulted in no inhibition of growth.

The minimum methionine level which would just support optimum growth of mutant 11-8 was found to be 0.15 g of DL-methionine per liter (Fig. 5). Mutant 11-8 and its parent, strain 274-1, were then tested for antibiotic synthesis in the presence of excess cysteine. Mutant 11-8

FIG. 3. Growth and production of cephalosporin C by mutant 274-1 in the presence of DL-methionine (3 mg/ml or 20 mM) or DL-cystathionine (1, 3, and 5 mg/ml or 4.5, 14, and 23 mM, respectively).

FIG. 4. Inhibition of growth of mutant 11-8 (met-) in methionine by L-cysteine. Duration of experiment: 5 days.

FIG. 5. Growth of mutant 11-8 in increasing concentrations of methionine. No cysteine was added. Duration of experiment: 4.5 days.

was tested at the low level of methionine (0.15 g/liter) as well as at a high concentration (3 mg/ml) of methionine.

Figure 6 shows that fairly good cephalosporin C production by parent 274-1 from cysteine occurred without exogenous methionine. However, mutant 11-8 could not produce cephalosporin C unless a high level of methionine was added. Since the parent produced respectable amounts of cephalosporin C in cysteine but the mutant could not, it is clear that blocking the path from cysteine to methionine virtually eliminated cephalosporin C production from cysteine. This demonstration of the essentiality of excess methionine for antibiotic synthesis from cysteine supports the hypothesis that methionine has a nonprecursor role.

Replacement of required methionine by norleucine. Our earlier studies with prototrophic strain CW19 (5) showed that methionine stimulation of cephalosporin C biosynthesis was duplicated by norleucine, a structural analogue of methionine which does not contain a sulfur atom. With mutant 11-8, we could now ask the following question. If we limit internal

methionine but supply excess sulfur in the form of cysteine for antibiotic synthesis, can we now bring about cephalosporin C synthesis with norleucine? This question was answered by growing mutant 11-8 at a low concentration of DL-methionine with intermittent feeding of L-cysteine to a total concentration of 3.0 g/liter beginning 1.5 days after inoculation. Inadvertently, a higher level of methionine (0.4 g/liter) than the minimum sufficient concentration (0.15 g/liter) was used in this experiment resulting in some production in the absence of norleucine supplementation. However the results are clear. The DL-norleucine dosage-response curve (Fig. 7) shows a direct relationship between cephalosporin C synthesis and the dose of norleucine. This finding further supports the hypothesis that methionine's control of antibiotic synthesis in the presence of excess cysteine is divorced from its role as a precursor of cephalosporin sulfur.

DISCUSSION

There is no doubt that methionine contributes sulfur to the cephalosporin C molecule (1, 9). The controversial question is whether this donation of sulfur is responsible for stimulation of cephalosporin C biosynthesis. One would imagine that, if sulfur donation were important for stimulation, intermediates on the sulfur path between methionine and cephalosporin C could replace methionine as a stimulator of antibiotic formation. However prototrophic cultures of *C. acremonium* are only

FIG. 6. Specific production of cephalosporin C from L-cysteine (3 mg/ml) by mutant 11-8 in the presence of DL-methionine at a high (3 mg/ml) and low (0.15 mg/ml) level and by its parent 274-1 in the absence of methionine. Rates and extents of growth were normal in all cases. The L-cysteine was added to all flasks in increments after 3 days.

FIG. 7. Stimulation of cephalosporin C production by DL-norleucine in mutant 11-8 grown with a low concentration of DL-methionine (0.4 g/liter) and a higher concentration of L-cysteine (3 g/liter fed intermittently after 1.5 days of growth).

mildly stimulated or not stimulated at all by homocysteine, cystathionine, or cysteine (1, 3, 4, 9, 10). In these earlier experiments, one has to be concerned with the possible complicating effect of sulfate assimilation; i.e., there was no guarantee that the organic sulfur source would be taken up and used in the presence of sulfate. The present experiments with mutant 274-1, blocked between sulfate and cysteine, eliminated this possible interference since good growth on an organic sulfur source insured that the compound had been taken up and used. Our studies with mutant 274-1 conclusively demonstrate that intermediates between methionine and cephalosporin C are unable to fully replace methionine for stimulation of antibiotic synthesis. The fact that the stimulatory activity of the sulfur amino acids decrease as they become metabolically more distant from methionine and closer to cephalosporin C indicates the relative unimportance of sulfur donation to the phenomenon of stimulation of cephalosporin C formation.

Our approach to studying the stimulatory role of methionine has been to limit intracellular methionine levels by mutation to methionine auxotrophy. Intracellular methionine concentrations are thus restricted by limiting the exogenous supply of methionine fed to methionine auxotrophs used in this study and in a previous one (6). This technique has revealed that cephalosporin C biosynthesis is strictly

dependent on the presence of methionine in excess of the amount required for normal growth. The significance of this observation is amplified by the fact that under these conditions sulfur is available for antibiotic synthesis in the form of either excess sulfate (mutant S-1 in reference 6) or excess cysteine (mutant 11-8 in the present study). Furthermore, in the presence of limited methionine and excess sulfur for antibiotic synthesis in the form of cysteine, the concentration of cephalosporin C produced is directly proportional to the concentration of added norleucine. Thus, the probable reason that cystathionine is more active than cysteine for mutant 274-1 is that it is metabolically closer to methionine than is cysteine and thus is more easily converted to the "inducer" of cephalosporin biosynthesis.

Nüesch et al. (9) obtained a mutant (8650 *slp*) with nutritional requirements similar to our mutant 274-1. They found that cysteine, cystathionine, and homocysteine supported even faster and more extensive growth than methionine but failed to replace methionine with respect to cephalosporin C production. A further observation (9) that they made was that this sulfate nonutilizing mutant produced considerably more antibiotic in the presence of methionine than did its prototrophic parent, i.e., 800 to 900 µg/ml versus 100 µg/ml. We were able to confirm this finding in that mutant 274-1 produced 800 to 1,100 µg of cephalosporin C per ml whereas prototrophic CW19 formed only about 400 µg/ml. Nüesch and co-workers propose (J. Nüesch, personal communication) that higher cephalosporin C production by sulfate nonutilizing mutants is due to inability to form cysteine which is a repressor and inhibitor of methionine permease.

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