

STIMULATION OF CEPHALOSPORIN PRODUCTION BY METHIONINE PEPTIDES IN A MUTANT BLOCKED IN REVERSE TRANSSULFURATION

STEPHEN W. DREW* and ARNOLD L. DEMAIN

Department of Nutrition and Food Science, Massachusetts,
Institute of Technology, Cambridge, Mass. 02139, U.S.A.

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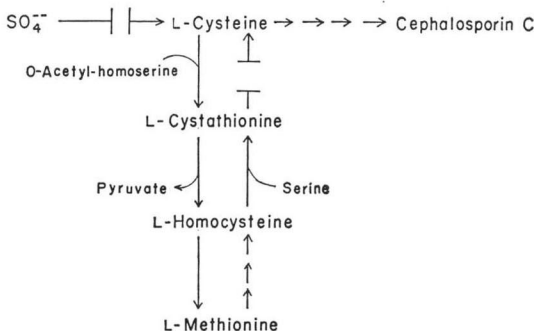
The previously reported¹²⁾ inability of methionine to stimulate cephalosporin C production in a cysteine auxotroph is due to cysteine interference with methionine uptake. In such a case, "illicit transport" of alanylmethionine can be used to demonstrate the efficacy of methionine in such mutants blocked in the path from methionine to cysteine. This result supports the hypothesis that the stimulatory activity of methionine is not due to its ability to donate sulfur to the cephalosporin C molecule.

Cysteine is the immediate precursor of cephalosporin sulfur, but methionine can also contribute its sulfur atom^{8,14)}. Although the passage of sulfur from methionine to cephalosporin C most probably goes through cysteine (Fig. 1), methionine is much more effective than cysteine as a stimulator of cephalosporin formation. Our hypothesis is that methionine does not stimulate by sulfur donation but by some effect on regulation and/or differentiation. We have recently presented data obtained with sulfur auxotrophs^{7,9)} showing that *Cephalosporium acremonium* has an obligatory requirement for methionine for cephalosporin C formation even though pathways were open from sulfate or cysteine to cephalosporin C. We interpret this to mean that methionine must be made or fed in concentrations over and above that required for growth to turn on antibiotic production. Our data⁹⁾ showing that norleucine, which contains no sulfur, can substitute for methionine as an effector further supports our hypothesis.

A consequence of the effector hypothesis is that methionine should be able to stimulate antibiotic synthesis in a mutant blocked in reverse transsulfuration *i.e.* in a mutant which can not convert methionine to cysteine. In a recent publication, NÜESCH and coworkers¹²⁾ reported

Fig. 1. Probable pathway of sulfur in *Cephalosporium acremonium*.

The double bars indicate probable sites of genetic blocks. The early block in one of the reactions between sulfate and cysteine is present in both parent strain 274-1 and its mutant H. The block between cystathionine and cysteine is present only in mutant H.



Contribution No. 2691 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Mass. 02139, U.S.A.

* Present address: Department of Chemical Engineering, Virginia Polytechnic Institute and State University, Blacksburg, Va. 24061, U.S.A.

that in such a mutant, methionine has no effect. Although this result appeared to dispute our hypothesis, we were concerned with the technical aspects of the experiment. The mutant is a cysteine auxotroph and must be fed cysteine along with the methionine being tested. Since cysteine is known to interfere with methionine uptake in *C. acremonium* (NÜESCH, personal communication), we wondered whether methionine even entered the cells in the above experiment. Since the mutant was not available to us, we prepared a similar auxotroph and studied its response to methionine. The present paper shows that the inactivity of methionine is due to cysteine interference, which can be bypassed by using the technique of "illicit transport" of methionine peptides; with such a manipulation, methionine stimulates cephalosporin formation in the blocked mutant.

Materials and Methods

Cultures and culture maintenance

Cephalosporium acremonium strain CW19 was obtained from Eli Lilly and Co. This strain is prototrophic and capable of producing about 1,500 μg of cephalosporin C per ml of medium when grown in a complex medium or about 400 $\mu\text{g}/\text{ml}$ when grown in a defined medium. Mutant 274-1 was obtained after UV irradiation of strain CW19 and is presumably blocked in conversion of sulfate to cysteine as previously described.⁸⁾ It will not grow on sulfate as the sole sulfur source but will grow on cysteine, cystathionine, homocysteine or methionine. Mutant H was obtained after 5-fluorodeoxyuridine enrichment of survivors of mutant 274-1 irradiated with UV. Mutant H, an obligate cysteine auxotroph, is a double mutant presumably blocked in conversion of sulfate to cysteine and in conversion of methionine to cysteine.

Strain CW19 was maintained on agar slants containing 1/10 strength LEPAGE-CAMPBELL medium^{11,13)}. The 1/10 strength LEPAGE-CAMPBELL maintenance slopes were modified to support growth of the auxotrophs by addition of 1.0 g/liter of either L-methionine (274-1) or L-cysteine (H).

Fermentations

Seed development and fermentations were conducted as previously described⁷⁾. The basal medium used for fermentation was that of DREW and DEMAINE⁷⁾ less methionine. In the present work, the concentrations of the amino acids added to this medium are given in the figure legends.

Microscopic examinations of fermentations were routinely performed. The contents of a particular flask were examined at $970\times$ magnification for bacterial contamination at the time of collection of each datum point. In addition to optical checks for microbial contamination, the auxotrophic cultures were checked for reversion by nutritional response on the pertinent agar media. These reversion checks were performed using mycelia from flasks sacrificed for data points.

Auxotrophic culture H proved very susceptible to spontaneous reversion. In this situation, many seed flasks were inoculated with culture H and their contents tested after two days for reversion. By the end of the third day of seed growth, it was possible to identify those flasks likely to contain revertants. Only those seed cultures free of revertants were used to inoculate the production media.

Isolation of mutant H by fluorodeoxyuridine selection

We found it quite difficult to obtain a mutant blocked in reverse transsulfuration. Many attempts were made by replica plating after mutagenesis to isolate a mutant responding only to cysteine from parent 274-1, which grows on either methionine or cysteine. A method devised by C.H. NASH (personal communication) and suggested by him finally led to success. Conidial preparations from slants of mutant 274-1 were obtained and subjected to UV irradiation.

tion as previously described⁸). The conidia were then transferred to a swelling and germination medium containing 0.1% L-methionine. This medium contains (per liter) 0.1 g glucose, 3.0 g ammonium sulfate, 9.0 mg magnesium sulfate, 2.0 g monobasic potassium phosphate, 1 g L-methionine, adjusted to pH 4.5. The conidia were allowed to swell for 12 hours without shaking (room temperature). After incubation, the pH was raised to 7.5 and 1 mg 5-fluorodeoxyuridine per ml was added. The cells were incubated for 6~8 hours to allow germination and then plated onto complete sporulation agar¹⁰) after appropriate dilution. This technique exerts a strong selection pressure for mutants which cannot metabolize methionine to cysteine since germination of other phenotypes leads to death by incorporation of fluorodeoxyuridine.

Assays

Determinations of cephalosporin C and dry cell weight were as previously described⁷).

Results

Lack of Methionine Response by Mutant H

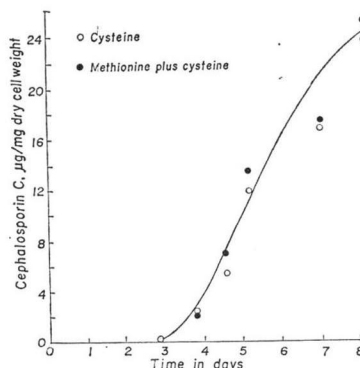
Throughout our studies of methionine stimulation of cephalosporin C biosynthesis, we have strived to distinguish between methionine's effect as a precursor of cephalosporin sulfur and its probable role as a regulator of antibiotic synthesis. The most critical question asked in the present studies is the following: can methionine stimulate cephalosporin C production in a culture which cannot convert methionine to cysteine, the latter being the direct precursor of cephalosporin sulfur? Culture H was obtained as a single step mutant from its parent strain 274-1 which is blocked between sulfate and cysteine. Mutant H is thus a double mutant blocked both in conversion of sulfate to cysteine (like 274-1) and in conversion of methionine to cysteine *i.e.* in reverse transsulfuration (Fig. 1). Mutant H will not grow on methionine, homocysteine or cystathionine, but will grow on cysteine; the parent grows on any of the four sulfur sources.

Our first fermentation experiment with mutant H confirmed the work of NÜESCH *et al.*¹²) with their mutant which has a similar phenotype. As expected (Fig. 2), cephalosporin C production by mutant H was not stimulated by methionine. In this experiment, 3 g of L-cysteine per liter were added at the beginning to satisfy the cysteine requirement for growth. We were concerned with the possibility that the presence of cysteine eliminated the uptake of methionine in both our experiment and that of NÜESCH *et al.*¹²) It is known (J. NÜESCH, personal communication) that cysteine represses and inhibits methionine uptake in *C. acremonium* and we have shown⁸) that cysteine inhibits the growth of a methionine auxotroph.

To prove that the ineffectiveness of methionine was due to cysteine interference with me-

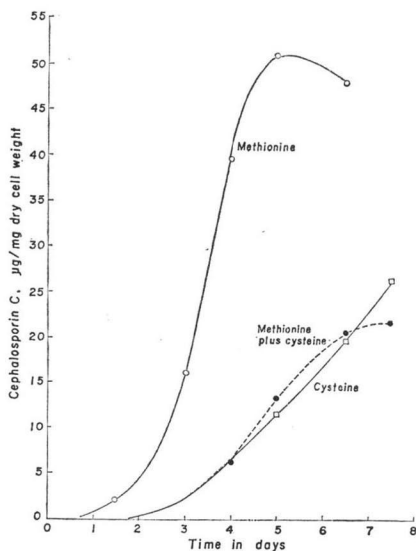
Fig. 2. Lack of methionine stimulation of cephalosporin C production by mutant H in the presence of cysteine.

The basal medium was the chemically defined medium of DREW and DEMAIN⁷) less methionine. Concentrations were DL-methionine, 3.0 g/liter; L-cysteine, 3.0 g/liter.



thionine uptake, we went back to culture 274-1, the parent of mutant H. We had earlier shown that 274-1 produces cephalosporin C when growing on methionine or cysteine (although methionine is much more effective). In the present experiment, we combined both and found that cephalosporin C production was identical in cysteine and in cysteine plus methionine, and lower than production than production in methionine alone (Fig. 3). It is clear then that the effect of methionine

Fig. 3. Lack of methionine stimulation of cephalosporin C production by parent culture 274-1 in the presence of cysteine. Details as in Fig. 2.



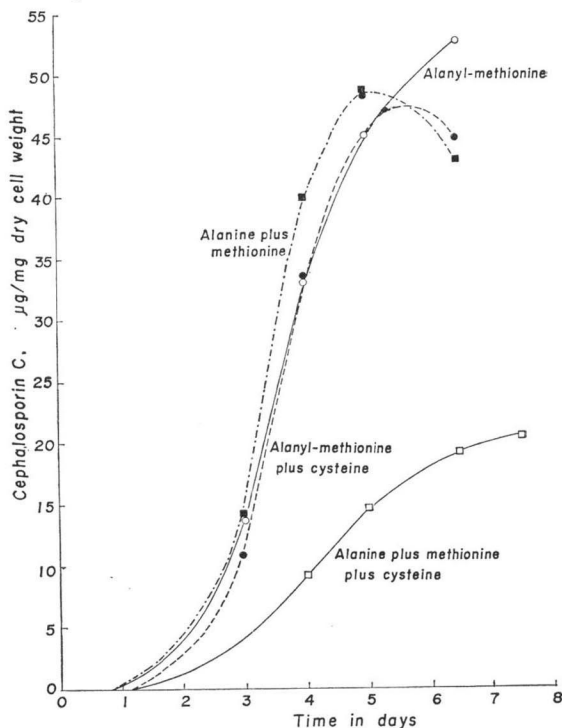
cannot be detected in the presence of cysteine.

Response to Methionine Peptides

Since mutant H requires cysteine for growth, we were presented with the difficult task of maintaining cysteine at a high enough level to permit normal growth but low enough, if possible, to permit unrestricted uptake of methionine. We felt that an easier solution to our problem might lie in the use of peptides of methionine. In some cases, amino acids which are not taken up by cells can be "smuggled" past the permeability barrier by using a dipeptide containing the amino acid in question. This "illicit transport" approach has recently been used with success by several workers^{1,9}. Before testing this approach with mutant H, we checked it on the parent, 274-1. We were pleased to find that alanylmethionine stimulated cephalosporin C synthesis in the presence of cysteine to the same level as observed with methionine in the absence of cysteine (Fig. 4). We also checked the effect of alanine; it had no complicating effect. This shows that the effect of alanylmethionine is due to its being a dipeptide of

Fig. 4. Alanylmethionine stimulation of cephalosporin C production by parent culture 274-1 in the presence of cysteine.

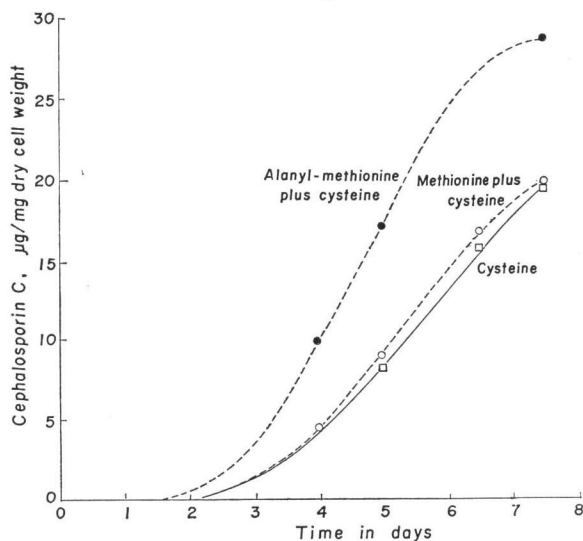
Concentrations of amino acids were DL-methionine, 3.0 g/liter; L-cysteine, 3.0 g/liter; DL-alanine, 1.8 g/liter; DL-alanyl-DL-methionine, 4.8 g/liter.



methionine, not to its content of alanine. Thus, alanylmethionine uptake, unlike that of methionine, is not restricted by cysteine.

We next examined the response of mutant H to the dipeptide. DL-Alanyl-DL-methionine was added to defined fermentation medium containing 3.0 g/liter L-cysteine and examined for its ability to stimulate antibiotic production by mutant H. The experimental data with mutant H is shown in Fig. 5. Alanylmethionine overcame the control of transport by cysteine and

Fig. 5. Alanylmethionine stimulation of cephalosporin C production by mutant H. Concentration of amino acids as in Fig. 4.



stimulated production of cephalosporin C. Once again free DL-methionine was unable to stimulate antibiotic production in the presence of cysteine. Furthermore the presence of free DL-alanine did not alter the pattern of antibiotic synthesis. Glycyl-DL-methionine was also tested for its ability to stimulate cephalosporin C biosynthesis in mutant H; the stimulation obtained was identical to that observed with alanylmethionine. Since alanylmethionine acts as a source of internal methionine in parent culture 274-1, it is logical that the stimulation of cephalosporin synthesis observed in culture H must be due to an increased internal methionine concentration. Since methionine cannot be metabolized to cysteine (the direct precursor of cephalosporin sulfur) in this mutant, the stimulating role of methionine in *C. acremonium* is definitely not due to its role as a precursor of antibiotic sulfur.

Discussion

The role of methionine in stimulating cephalosporin C biosynthesis in *Cephalosporium* has been an elusive topic of study for many years. Certainly the sulfur of methionine is transferred to cysteine, the immediate precursor of cephalosporin sulfur. In fact, studies with *Cephalosporium* and other fungi suggest that methionine is preferred over sulfate as a sulfur source.^{2,3,12,15,16} It is thus not surprising that several workers have concluded that methionine's role in stimulation of antibiotic formation is that of a donor of sulfur to cephalosporin C. Our work how-

ever indicates that methionine has a stimulating effect on cephalosporin synthesis that is divorced from its role as a precursor of sulfur. The ultimate test of this hypothesis had to involve a way of separating the known precursor role of methionine from any other effects. The present experiments with mutant H, an obligate cysteine auxotroph, accomplishes this objective by eliminating methionine's role as a donor of sulfur for antibiotic synthesis. The experimental data clearly show a unique, non-precursor role for methionine stimulation of cephalosporin C biosynthesis.

NÜESCH and coworkers¹²⁾ presented data indicating that in a mutant obtained by them which could not metabolize methionine to cysteine, cephalosporin C formation did not respond to added methionine. They suggested that methionine acted only as a precursor of cephalosporin sulfur but were puzzled by the ineffectiveness of cysteine, the immediate sulfur precursor. Since these workers have subsequently shown that cysteine both inhibits and represses methionine uptake by *C. acremonium*, it is evident that methionine did not enter the mycelium of their cysteine auxotroph and therefore they were unable to observe methionine stimulation of cephalosporin C biosynthesis. The use of methionine-containing dipeptides in our experiments with mutant strain H eliminated this problem and resulted in our observation of a definite methionine-mediated stimulation.

Although methionine serves at least two roles in antibiotic production by *Cephalosporium*, the exact nature of the non-precursor role is still unknown. The hypothesis that methionine is a regulator of antibiotic synthesis is supported by the stimulatory effect of its structural analog, norleucine⁵⁾. If indeed methionine acts as a regulator, then stimulation of antibiotic synthesis should be dependent on internal methionine concentration rather than the amount of methionine metabolized by the cell. In this situation, genetic or environmental alterations which increase the internal methionine concentration should also increase antibiotic production. Experiments to accomplish this goal are now underway.

Acknowledgements

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