Journal of Industrial Microbiology, 2 (1987) 251–255
Elsevier

SIM 00092

Methionine induction of ACV synthetase in *Cephalosporium acremonium*

Jin-you Zhang[a], Gerald Banko[a,*], Saul Wolfe[b] and Arnold L. Demain[a]

[a]*Fermentation Microbiology Laboratory, Department of Applied Biological Sciences, Massachusetts Institute of Technology, Cambridge, MA, U.S.A. and* [b]*Department of Chemistry, Queens University, Kingston, Ontario, Canada*

Received 18 May 1987
Revised 27 July 1987
Accepted 3 August 1987

Key words: Methionine effect; ACV accumulation; β-Lactam production

SUMMARY

Methionine markedly stimulates the biosynthesis of penicillin N and cephalosporin C in *Cephalosporium acremonium*. Examination of intra- and extracellular ACV tripeptide in non-producing mutant N-2 showed that growth in the presence of methionine increased ACV accumulation. Direct measurement of ACV synthetase activity in a cell-free system indicated that the methionine effect was mainly due to induction of this first enzyme of the β-lactam biosynthetic pathway, resulting in a corresponding increase in β-lactam production in both a low-producing strain and a high-producing mutant.

INTRODUCTION

Methionine stimulates the production of cephalosporin C in *Cephalosporium acremonium* (for review, see Ref. 4). Although the amino acid contributes its sulfur atom to the antibiotic, this is not the reason for its stimulatory effect. Stimulation is due to an effect of methionine on the morphology of the cells and the increase in specific activities of certain enzymes of the cephalosporin biosynthetic pathway. We have shown that methionine causes fragmentation of the mycelia [5] and an induction of two of the enzymes tested so far, i.e., isopenicillin N synthetase (cyclase) and deacetoxycephalosporin C synthetase (expandase) [10]. In the present study, we show that growth in the presence of methionine leads to an even greater increase in the production of the initial enzyme of the pathway, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase.

MATERIALS AND METHODS

Cultures

The cultures of *C. acremonium* used were the high-producing C-10 strain (*Acremonium chrysogenum* ATCC 48272), the low-producing CW-19 strain (*A. chrysogenum* ATCC 36225) and strain N-2, a non-producing mutant blocked after the formation of ACV [12]. Assay organisms were *Escher-*

* Present address: Chemie Linz AG, A-4020 Linz, Austria. Correspondence: Dr. Arnold L. Demain, Department of Applied Biological Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, U.S.A.

ichia coli ESS for expandase and *Micrococcus luteus* ATCC 381 for cyclase.

Media and culture conditions

C. acremonium CW-19 and C-10 were grown on the previously described slant medium, seed medium (No. 1) and chemically-defined production medium [11], using the same culture conditions. When methionine was present, it was added before autoclaving at a final concentration of 3.2 mg/ml of the DL-form. Non-producing mutant N-2 was grown in the same seed medium as used above but the fermentation medium was a complex medium containing sucrose, soybean meal and $CaCO_3$ [12] with or without 5 g/l DL-methionine.

Assays

Growth was estimated by optical density in the Klett-Summerson colorimeter (red filter) after dilution of the broth to below 150 Klett units. Conversion to mg mycelial wet weight/ml was accomplished by multiplying the observed Klett units by the dilution factor and dividing by 42 [9]. Protein in cell-free extracts was measured by the Bradford procedure [3] using bovine serum albumin as standard. β-Lactam antibiotics were determined by the chemical assay of Lübbe et al. [7] using cephalosporin C as standard. Note that both C-10 and CW-19 make predominantly cephalosporin C and smaller amounts of deacetylcephalosporin C and penicillin N. ACV accumulation by the N-2 mutant was determined as follows. At various times, a fermentation flask (50 ml broth) was sacrificed and the mycelia and supernatant fluid were separated by centrifugation. For determination of intracellular ACV, the mycelia were washed twice with cold Tris-HCl buffer (50 mM, pH 7.5). Two cell volumes of cold Tris-HCl buffer were added to the pellets and the suspension was sonicated by four 20 s treatments with a 1 min interval between each (Branson Sonifier, Cell Disruptor 200, output setting of 6, with microtips). After centrifugation, 0.5 ml of the supernatant fluid was treated with 0.5 ml methanol to precipitate the protein. After centrifugation, 0.5 ml of the supernatant fluid was treated with 2.5 mM dithiothreitol (DTT) and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and analyzed for ACV by HPLC [1,2]. For determination of extracellular ACV, culture supernatant fluids were assayed in a similar way omitting the sonication step. For the enzyme assays, the mycelia were disrupted in a French Press in the presence of glycerol [2]. After desalting the crude cell-free extract, cyclase and expandase assays were carried out as described earlier [6]; authentic isopenicillin N and cephalosporin C were used as standards, respectively. One unit of enzyme activity is that which produces 1 μg of isopenicillin N or cephalosporin C equivalent per min, respectively. ACV synthetase activity was estimated by measuring the amount of ACV pro-

Table 1

Effect of methionine on accumulation of ACV by mutant N-2[a]

Fermentation time (h)	Packed cell volume (ml)		Extracellular ACV (mg)		Intracellular ACV (mg)		Total ACV (mg)	
	−met	+met	−met	+met	−met	+met	−met	+met
24	2.2	1.9	–[b]	–	1.8	0.8	–	–
48	2.7	3.8	0.4	0.5	5.6	9.4	6.0	9.9
72	8.6	6.2	0.5	2.1	11.1	51.2	11.6	53.3
96	10.2	6.5	1.5	8.0	11.7	61.9	13.2	69.9
120	8.9	7.0	1.8	10.2	18.4	45.9	20.2	56.1

[a] All data are based on 50 ml of broth.

[b] Not done.

Table 2

Methionine stimulation of β-lactam biosynthesis in *C. acremonium* CW-19[a]

Medium	Maximum growth (mg wet cells/ml)	Maximum β-lactam (μg/ml)	Maximum specific β-lactam (μg/mg wet cells)	Maximum ACV synthetase (units/mg protein)	Maximum cyclase (units/mg protein)
Without DL-methionine	183	220	1.2	9.9	0.25
With 0.32% DL-methionine	175	450	2.6	21.0	0.48

[a] The data listed here were the maximum values among the six time points of a 7-day fermentation.

duced in the cell-free reaction, using HPLC as described earlier [1,2]. One unit of ACV synthetase activity is that which produces 1 pmol ACV per min.

RESULTS

Effect of methionine on ACV accumulation

Mutant N-2 is apparently blocked at the ACV stage, since it accumulates the dimer of ACV [12] and has been reported to lack cyclase, epimerase and expandase activities [8]. At the time our work with mutant N-2 was done, we had not yet successfully developed our ACV synthetase assay method. It was thought that the mutant could provide information concerning the possibility that methionine induces the first enzyme. Table 1 clearly shows that growth of strain N-2 with methionine leads to a marked increase (ca. 4-fold) in ACV production.

Effect of methionine on ACV synthetase

Since the above data on mutant N-2 indicated that methionine increases ACV accumulation, we tested the effect of growth with methionine directly on the level of ACV synthetase in the mycelia after our development of the ACV synthetase assay [1]. In the case of the low-producing culture (CW-19), methionine markedly stimulated the production of ACV synthetase (Table 2). As expected from our previous study [10], growth in methionine also increased the production of β-lactam antibiotics as well as the specific activity of cyclase.

Of particular interest to us was the effect of

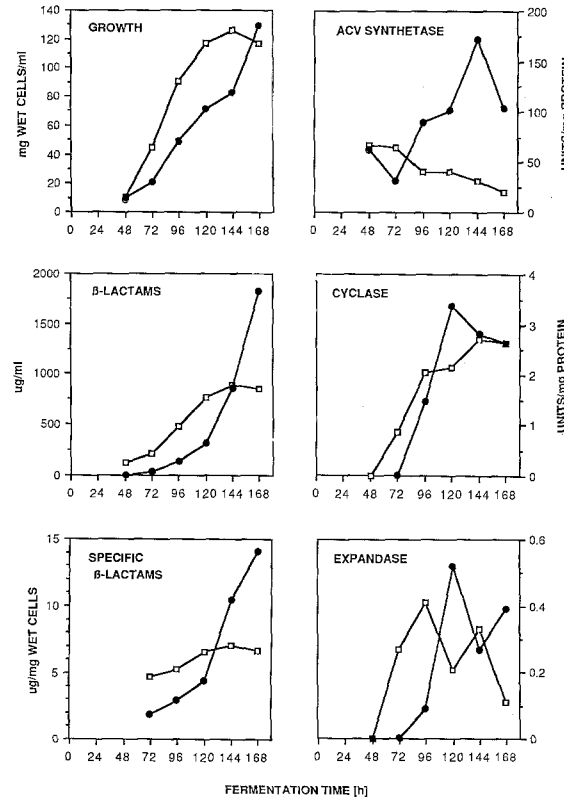

Fig. 1. Effect of methionine on growth, β-lactam biosynthesis and formation of cephalosporin synthetases by *C. acremonium* C-10. ●, 0.32% DL-methionine; □, no methionine.

methionine on cephalosporin production by the high-producing strain C-10. We had earlier found this strain to be somewhat deregulated with respect to glucose repression [11] but had not examined the methionine effect. Fig. 1 shows that methionine is fairly inhibitory to the growth of C-10 but nevertheless stimulates antibiotic production once growth ensues. Of the three enzymes studied here, ACV synthetase is apparently the main target of methionine induction, its increase in activity being followed by an increase in rate of β-lactam production. Indeed, the effect of methionine on the formation of cyclase and expandase in this strain is rather marginal. A second experiment on C-10 confirmed the data of Fig. 1.

DISCUSSION

The experiments presented here point strongly to ACV synthetase as the major site of the methionine effect in *C. acremonium*. In an early blocked mutant, in a low-producing strain and in a high-producing mutant derived by a series of mutations from the low producer, the main effects were seen on either ACV accumulation or ACV synthetase activity. In the high-producing mutant, regulation of cyclase and expandase by methionine appears to have been lost but the induction of the initial enzyme unique to production of all penicillins and cephalosporins remains.

The relative levels of the enzymes of β-lactam synthesis in the low and high producer are of interest. We had reported earlier [11] that cyclase and expandase specific activities are higher in strain C-10 than in CW-19. At least for cyclase, this was confirmed in the present work (up to a 6-fold difference is seen by comparing Table 2 and Fig. 1). An even larger difference can be seen for ACV synthetase, i.e., C-10 produces about 8–9-times more than does CW-19. Ramos et al. [8] recently reported that isopenicillin N epimerase is also much higher in specific activity in C-10 than in CW-19.

ACKNOWLEDGEMENTS

The work at MIT was supported by the National Science Foundation. The Queens University activities were funded by the Natural Sciences and Engineering Research Council of Canada. J.-Y. Z. acknowledges support from the Educational Committee of the People's Republic of China. G. B. was funded by Chemie Linz AG. We thank Daniel Liberman and Nadine A. Solomon for encouragement and advice. We also acknowledge the assistance of Y. Nakao and R. Baxter in providing us with cultures of *C. acremonium* strain N-2.

REFERENCES

1 Banko, G., A.L. Demain and S. Wolfe. 1987. δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine synthetase: a multi-functional enzyme with broad substrate specificity for synthesis of penicillin and cephalosporin precursors. J. Am. Chem. Soc., in the press.

2 Banko, G., S. Wolfe and A.L. Demain. 1986. Cell-free synthesis of δ-(L-α-aminoadipyl)-L-cysteine, the first intermediate of penicillin and cephalosporin biosynthesis. Biochem. Biophys. Res. Commun. 137:528–535.

3 Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein dye binding. Anal. Biochem. 12:248–254.

4 Demain, A.L. and S. Wolfe. 1987. Biosynthesis of cephalosporins. Dev. Ind. Microbiol. 27:175–182.

5 Drew, S.W., D.J. Winstanley and A.L. Demain. 1976. Effect of norleucine on mycelial fragmentation in *Cephalosporium acremonium*. Appl. Environ. Microbiol. 31:153–145.

6 Kupka, J., Y.-Q. Shen, S. Wolfe and A.L. Demain. 1983. Studies on the ring-cyclization and ring-expansion enzymes of β-lactam biosynthesis in *Cephalosporium acremonium*. Can. J. Microbiol. 29:488–496.

7 Lübbe, C., Y.-Q. Shen and A.L. Demain. 1986. A simple and rapid chemical method for the determination of cephalosporins. Appl. Biochem. Biotechnol. 12:31–35.

8 Ramos, F.R., M.J. Lopez-Nieto and J.F. Martin. 1986. Coordinate increase of isopenicillin N synthetase, isopenicillin N epimerase and deacetoxycephalosporin C synthetase in a high cephalosporin-producing mutant of *Acremonium chrysogenum* and simultaneous loss of the three enzymes in a non-producing mutant. FEMS Microbiol. Lett. 35:123–127.

9 Sawada, Y., J.E. Baldwin, P.D. Singh, N.A. Solomon and A.L. Demain. 1980. Cell-free cyclization of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine to isopenicillin N. Antimicrob. Agents Chemother. 18:465–470.

10 Sawada, Y., T. Konomi, N.A. Solomon and A.L. Demain. 1980. Increase in activity of β-lactam synthetases after growth of *Cephalosporium acremonium* with methionine or norleucine. FEMS Microbiol. Lett. 9:281–284.

11 Shen, Y.-Q., S. Wolfe and A.L. Demain. 1986. Levels of isopenicillin N synthetase and deacetoxycephalosporin C synthetase in *Cephalosporium acremonium* producing high and low levels of cephalosporin C. Bio/Technology 4:61–63.

12 Shirafuji, H., Y. Fujisawa, M. Kida, T. Kanzaki and M. Yoneda. 1979. Accumulation of tripeptide derivatives by mutants of *Cephalosporium acremonium*. Agric. Biol. Chem. 43:155–160.

13 Alonso, M.J. and J.M. Luengo. 1987. Antimicrob. Agents Chemother. 31: 357–359.

NOTE ADDED IN PROOF

(Received 2 September 1987)

M.J. Alonso and J.M. Luengo [13] recently reported that methionine stimulates the formation of ACV in *Cephalosporium acremonium* strain CW-19.