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A role for alanine in the ammonium regulation of cephalosporin biosynthesis in *Streptomyces clavuligerus*

Suseela Kasarenini* and Arnold L. Demain

Fermentation Microbiology Laboratory, Biology Department, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

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SUMMARY

It is known that excess ammonium supply decreases cephalosporin production and represses cephalosporin synthases. We wondered whether an additional important effect could be inhibition of synthase action by alanine. We had previously shown that ammonium addition induced alanine dehydrogenase and increased intracellular alanine and that alanine could inhibit resting cell synthesis of cephalosporins. In the present work we confirm the alanine inhibition of antibiotic production by resting cells. We found L-alanine inhibited three of the four synthases tested: ACV synthetase, cyclase and expandase; the epimerase was not inhibited. These data suggest that interference in cephalosporin production by growth in ammonium salts involves synthase inhibition by intracellular alanine, in addition to the known role of ammonium in synthase repression.

INTRODUCTION

The nitrogen source contained in the growth medium has a great effect on the production of cephalosporins by fungi and actinomycetes [3,10,11]. Aharonowitz and Demain [1] first showed that ammonium ion negatively affects cephalosporin (including cephamycin C) biosynthesis by *Streptomyces clavuligerus*. Braña et al. [5] showed that ammonium ion exerted repression of certain enzymes of cephalosporin biosynthesis, i.e. isopenicillin N synthase ('cyclase') and deacetoxycephalosporin C synthase ('expandase'); isopenicillin N epimerase levels were unaffected or marginally decreased [6]. Ammonium inhibition of enzyme action does not occur as evidenced by: (i) the lack of a negative effect of NH_4^+ on resting cell antibiotic production in the absence of protein synthesis, and (ii) the lack of inhibition of cyclase, epimerase or expandase when NH_4^+ is added to cell-free assay systems. Later, Zhang et al. [12] found δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase ('ACVS') to be very sensitive to ammonium repression and rather insensitive to ammonium inhibition. In *Nocardia lactamdurans*, Castro et al. [7] found cyclase, epimerase and expandase to be repressed, but not inhibited, by NH_4^+.

Although NH_4^+ did not decrease the rate of cephamycin formation when added to resting cells of *S. clavuligerus*, alanine did [6]. The possible significance of an alanine effect was increased by the following observations: (i) Of the nitrogen assimilation enzymes of microorganisms, *S. clavuligerus* contains glutamine synthetase, glutamate synthase and alanine dehydrogenase, but not glutamate dehydrogenase or alanine-α-ketoglutarate aminotransferase [2,4]. (ii) Only the glutamine synthetase–glutamate synthase pathway functions in nitrogen assimilation, the alanine dehydrogenase acting as a dead-end route, presumably to detoxify excess NH_4^+ via conversion to alanine. Generally, in microorganisms, alanine dehydrogenases have high K_m values for ammonium and may only be important when high, possibly toxic, levels of ammonium are in the environment. (iii) Alanine dehydrogenase is induced by NH_4^+ (or alanine) in *S. clavuligerus*. Addition of 80 mM ammonium to a growth medium, already containing 15 mM asparagine as nitrogen source, raised alanine dehydrogenase by as much as 75-fold [4]. (iv) Alanine was one of only two amino acids whose internal concentration varied markedly with changes in the nitrogen source used for growth [4], addition of NH_4^+ elevating alanine levels 2- to 4-fold.

MATERIALS AND METHODS

Streptomyces clavuligerus NRRL 3585 was used in this study. Media, growth conditions, assay organisms, measurements of growth and antibiotic production, preparation of cell-free extracts, and enzyme and protein assays were those previously described [12]. Resting cell cultures were prepared as described by Braña et al. [5] with the exception that 10 mM L-lysine was included in the buffer to supply the limiting amino acid, α-aminoadipate, and 20 μg lincomycin ml^{-1} was used to inhibit protein synthesis. The carryover of

Correspondence to: A.L. Demain, Fermentation Microbiology Laboratory, Biology Department, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

*Present address: Textan Chemicals (P) Ltd, R & D Dept, PO Box 5034, Ashok Nagar, Madras 600083, India.

lincomycin into the bioassay system did not inhibit the *Escherichia coli* Ess assay organism. Cephalosporin assays refer to total cephalosporins including cephamycin C, as measured by a filter paper disc–agar diffusion assay using *E. coli* Ess (a mutant super-sensitive to β-lactam antibiotics) as assay organism.

RESULTS

Effects of amino acids on resting cell biosynthesis

Nineteen amino acids and NH_4Cl were tested at 10-mM concentration for their effect on cephalosporin biosynthesis by resting cells in the presence of lysine and lincomycin. In the absence of added lysine, the rate of cephamycin production was 20% lower. Most nitrogen sources had no effect, i.e. production was at 90–106% of the control value. However L-alanine, L-cysteine, L-threonine, L-serine and L-valine inhibited by 27–39%. Thus we were able to confirm the findings of Braña et al. [6] that alanine is inhibitory and NH_4^+ is not; however we could not confirm inhibitory activity for glutamic acid and glutamine.

A number of the nitrogen sources were tested next at higher levels. Figure 1 shows that of the compounds tested, L-alanine and L-threonine were the most inhibitory. Cysteine was not examined in this study because of its oxidation to insoluble cystine and possible artifactual effects.

We considered that the high inhibitory activity of L-threonine was due to its known concerted feedback inhibitory effect on aspartokinase in cooperation with L-lysine [9], thus limiting lysine production and formation of the limiting precursor of cephalosporin synthesis, L-α-aminoadipate. If so, added lysine should reverse inhibition by threonine. That this is the case is shown in Table 1 where increased lysine levels decreased threonine inhibition of resting cell biosynthesis from 36% down to 14%.

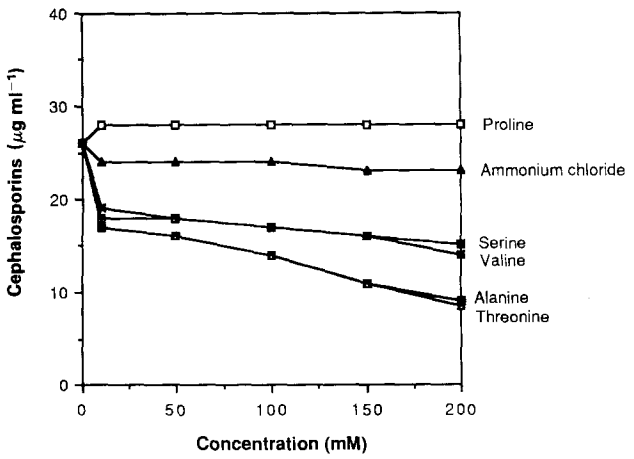

Fig. 1. Effect of certain amino acids and NH_4Cl on resting cell production of cephalosporins in the presence of 10 mM L-lysine and lincomycin at 20 μg ml^{-1}.

TABLE 1

Reversal of L-threonine inhibition of resting cell biosynthesis by L-lysine

Lysine (mM)	Threonine (mM)	Cephalosporin production	
		(μg ml^{-1})	(% inhibition)
0	0	25	
0	200	16	36
10	0	28	
10	200	18	36
50	0	32	
50	200	22	31
100	0	35	
100	200	28	20
200	0	37	
200	200	32	14

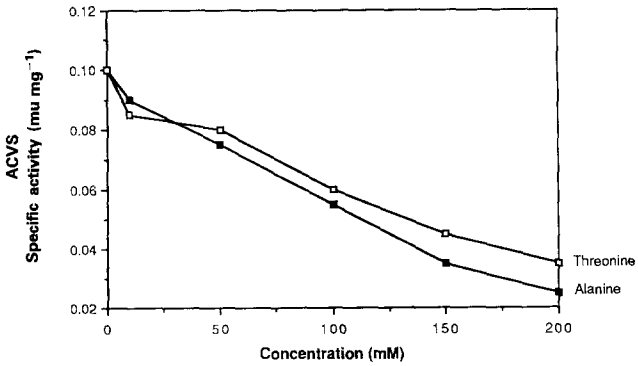

Fig. 2. Inhibition of ACVS activity by L-alanine and L-threonine.

Effect of L-alanine and L-threonine on enzyme activity

Figure 2 shows that the addition of L-alanine or L-threonine to the ACVS assay system inhibited activity. Fifty percent inhibition occurred at 125–150 mM of either amino acid. L-alanine also inhibited cyclase activity as shown in Fig. 3; 50% inhibition occurred at about 70 mM. Threonine

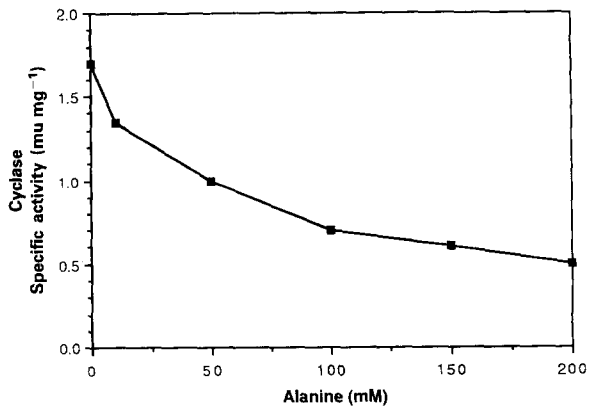

Fig. 3. Inhibition of cyclase activity by L-alanine.

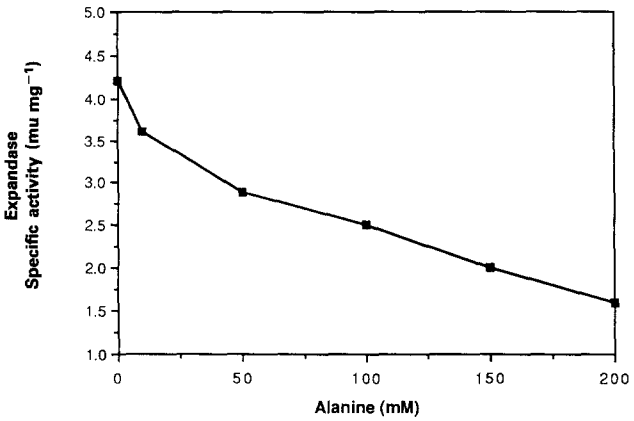

Fig. 4. Inhibition of expandase activity by L-alanine.

had no inhibitory activity (data not shown). Epimerase was not inhibited by either L-alanine or L-threonine, even at 200-mM concentrations (data not shown). Expandase, like cyclase, was inhibited by alanine (Fig. 4) but not by threonine (data not shown). Fifty percent inhibition occurred at 140 mM L-alanine.

DISCUSSION

Growth in high NH_4^+ leads to repression of many of the enzymes of cephalosporin biosynthesis in *S. clavuligerus* and hence decreased antibiotic formation. It is also clear that NH_4^+ itself does not inhibit cephalosporin biosynthesis by resting cells or the action of the biosynthetic enzymes which have been examined thus far. The question is whether the entire negative effect of growth in NH_4^+ can be attributed to enzyme repression; we think not. It is clear that growth in ammonia induces alanine dehydrogenase, increases the intracellular concentration of alanine, the latter being inactive as a source of nitrogen for cell growth. This internal buildup of alanine could inhibit the action of biosynthetic enzymes such as ACVS, cyclase and expandase. Admittedly, a high concentration of L-alanine, approximately 100 mM, is required to inhibit these enzymes by 50% when added to crude cell-free extracts. We have observed levels of intracellular alanine in wild-type *S. clavuligerus* as high as 143 nmol mg^{-1} protein (Braña and Demain, unpublished data). If one converts this figure to intracellular concentration, the value is approximately 40 mM. This calculation assumes that 1 mg dry cell weight (DCW) is equivalent to a specific cell volume of 2 μl [8] and that protein is 50% of DCW. Certainly 40 mM intracellular L-alanine would be expected to exert a significant inhibition of the action of ACVS (Fig. 2), cyclase (Fig. 3) and expandase (Fig. 4).

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