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STIMULATION OF THE CONVERSION OF PENICILLIN N TO CEPHALOSPORIN BY ASCORBIC ACID, α-KETOGLUTARATE, AND FERROUS IONS IN CELL-FREE EXTRACTS OF STRAINS OF Cephalosporium acremonium

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SUMMARY

A cell-free system of the cephalosporin C fungus, Cephalosporium acremonium, has been described which converts penicillin N to a cephalosporin-like material which was biologically-active upon treatment with penicillinase with subsequent loss of activity when treated with cephalosporinase. We have confirmed this conversion independently in our laboratories and have shown that the activity was lost upon boiling, indicating the reaction is enzymatic in nature. Furthermore, we have shown that the production of the cephalosporin-like material was stimulated by the addition of ascorbic acid (3.8 μM) and ferrous ions (0.075 μM) to the reaction mixture, components which are co-factors in monooxygenase and dioxygenase reactions.

INTRODUCTION

Kohsaka and Demain (1) have described a cell-free system from Cephalosporium acremonium CW19 which converts penicillin N to a penicillinase-resistant, cephalosporinase-sensitive material. A recent report by Yoshida et al (2) indicated that the biologically-active material obtained in the conversion appeared to be deacetoxycephalosporin C. These reports have generated renewed interest in the relationship of penicillin N to cephalosporin C biosynthesis and provide further evidence in support of a biological ring expansion of a penicillin to a cephalosporin-like compound(s).

This report describes the confirmation in our laboratories of the activity of cell-free extracts of Cephalosporium acremonium strains CW19 and M-0198 in synthesizing a penicillinase-resistant, cephalosporinase-sensitive material when penicillin N is added to cell-free extracts. We further describe the effect of various co-factors on the cell-free conversion.

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MATERIALS AND METHODS

The media used and conditions for growing C. acremonium, strains CW19 and M-0198, have been described (3). The cultures were incubated at 25 C in 250-ml Erlenmeyer flasks containing 40 ml of chemically defined medium (3) and shaken on a New Brunswick Model G-53 rotary shaker at 200 rpm. The mycelia were harvested between 66 and 96 hrs. after inoculation.

The mycelial solids from six flasks were pooled and the mycelia were harvested by centrifugation (800 g for 10 min.), followed by washing twice with chilled sterile distilled water. The mycelia were then filtered and again washed twice with distilled water followed by filtration. The mycelia were resuspended in 40 ml of 0.05M McIlvaine's buffer (pH 7.2) containing 0.01M dithiothreitol. The suspension was inoculated for 1 hr. at 28 C with shaking at 200 rpm. The mycelia were centrifuged, washed twice with sterile distilled water and resuspended in 40 ml of 0.05M McIlvaine's buffer (pH 7.2) with 1.0M NaCl, 0.2M $MgSO_4$, 160 mg Cytophaga lytic enzyme L_1 (Gallard-Schlessinger Chemicals, Carle Place, New York) and 160 mg Arthrobacter zymolyase (Kirin Brewery, Tokyo, Japan). The suspension was incubated at 28 C for 3 hrs. with shaking (200 rpm). The resulting protoplast suspension was centrifuged at 3000g for 10 min. The resulting pellet was washed twice with 0.05M Tris buffer, pH 7.2, containing 0.01M KCl, 0.01M $MgSO_4$ and 1M sucrose. After the second wash the pellet was resuspended in 10^4ml of 0.05M Tris buffer, pH 7.2, containing 0.01M KCL and 0.01M $MgSO_4$ and frozen at -20 C. After thawing, the suspension was centrifuged at 7000g for 10 min. The supernatant was the cell-free extract which was confirmed by microscopic examination. The following materials were added to 1 ml of cell-free extract in an 8.2-ml vial (covered with a single layer of a milk-filter disc (Kendall Co., Walpole, MA): 200-500 µg of penicillin N (Abbott Labs, approx. 5% purity) in 0.1 ml water, 5 µmoles ATP (Sigma), 10 µmoles of phosphoenolpyruvate (Sigma) in 0.1 ml 0.05M Tris buffer (pH 7.2) containing 0.01M NaCl and 0.01 $MgSO_4$ adjusted to pH 6.5, 100 µg of pyruvate kinase in 10 µl Tris buffer and water and co-factors in a volume of 100 µl (total volume = 1.32 ml).

The mixture was incubated for 3-5 hrs. at 25 C on a rotary shaker at 200 rpm. Samples were taken at intervals for assay of antibiotic activity using a β-lactam supersensitive strain of Pseudomonas aeruginosa (Pss) as the test organism (2). Bioassay was measured by placing paper discs (Schleicher & Schuell Co., Inc., Keene, NH, 6.35 mm diam.) on plates of 1/2 strength Nutrient Agar (Difco) supplemented with 0.1% dextrose with or without 100,000 units of penicillinase (Difco) per 10 ml agar seeded with the assay organism. When necessary, cephalosporinase, a crude preparation from sonicated cells of Enterobacter cloacae strain P99 (4), was also added in sufficient quantity to destroy cephalosporin C at a concentration of 10 µg/ml on equivalent paper discs to seeded agar in addition to the penicillinase. Cephalosporins were determined on plates containing penicillinase. Cephalosporin C, potassium salt, (Bristol Laboratories) was used as a standard at concentrations of 2.5, 5, and 10 µg/ml, to establish a calibration curve for each reaction measured. A typical calibration curve is presented in Fig. 1. Protein was determined by the Biuret reaction [Gornall et al (5)].

RESULTS

The ability of the cell-free extract to synthesize penicillinase-resistant material is shown in Table 1. The data also indicate that the synthesis is

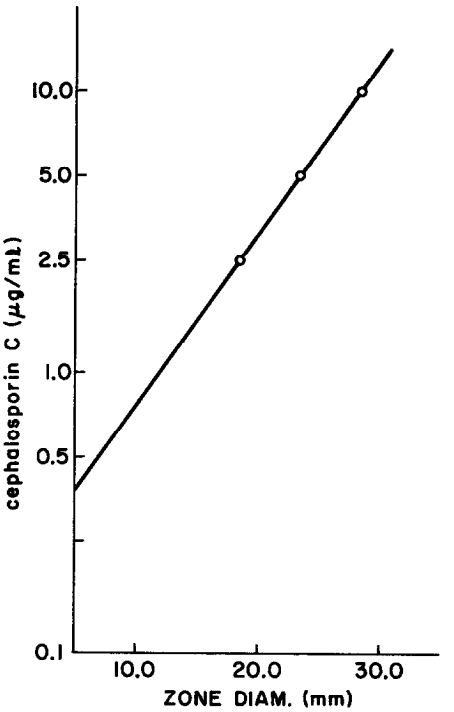

Fig. 1 Typical curve for calibration of cephalosporin C concentration vs diameter of inhibition zone

TABLE 1

Effect of co-factors on the conversion of penicillin N to a "cephalosporin" in the cell-free system

Condition	"Cephalosporin" (μg/ml cephalosporin C equivalent) 0 hr.	1.5 hr.	3.0 hr.	4.5 hr.
Control - no penicillin N	1.3	0.7	1.6	1.9
Complete system - no co-factors	2.3	2.3	2.5	3.8
Complete system + all co-factors*	2.5	5.6	7.4	6.6
Complete system + α-ketoglutarate ascorbate, $FeSO_4$ (100 nmoles each)	5.0	9.8	7.4	14.0

*Co-factors added so that the final reaction mixture contains 100 nmoles each of dithiothreitol, NADH, DL-6-methyl-5,6,7,8-tetrahydropterin, α-ketoglutarate, ascorbic acid and $FeSO_4$. The final reaction volume was 1.32 ml.

stimulated by α-ketoglutaric acid, ascorbic acid and ferrous ions but not by dithiothreitol, NADH, or DL-6-methyl-5,6,7,8 tetrahydropterin.

TABLE 2

Effect of various co-factors on the synthesis of a "cephalosporin" in the cell-free system

Condition	"Cephalosporin" (µg/ml cephalosporin C equiv.) 0 hr.	1.5 hr.	3.0 hr.
Control - no penicillin N	2.4	2.6	2.6
Complete system	3.2	3.1	3.6
Complete + 0.5 µmoles ascorbate and α-ketoglutarate	4.3	9.0	8.4
Complete + 0.5 µmoles α-ketoglutarate	3.4	4.3	4.4
Complete + 1.0 µmoles ascorbate and α-ketoglutarate	4.4	10.0	10.8
Complete + 1.0 µmoles ascorbate	7.3	10.8	10.4
Complete + 1.0 µmoles α-ketoglutarate	3.4	4.3	4.3
Complete + 1.0 µmoles ascorbate and α-ketoglutarate - $FeSO_4$	3.2	4.7	4.8

All reaction mixtures contain 100 nmoles $FeSO_4$ except where noted. Final reaction mixture was 1.32 ml.

TABLE 3

Effect of concentrations of co-factors on the conversion of penicillin N to a "cephalosporin"

Condition	"Cephalosporin" (µg/ml cephalosporin C equiv.) 0 hr.	1 hr.	2 hr.	3 hr.
Control - no penicillin N	0.0	0.0	0.0	0.0
Complete	0.0	0.0	0.0	0.0
Complete + 1.0 µmole α-KGA & ascorbate	2.7	4.6	3.9	4.3
Complete + 2.0 µmoles α-KGA & ascorbate	2.7	4.6	7.0	7.0
Complete + 3.0 µmoles α-KGA & ascorbate	2.7	6.6	7.0	7.4
Complete + 5.0 µmoles α-KGA & ascorbate	2.7	7.2	10.0	10.5
Complete + 1.0 µmole α-KGA & ascorbate (boiled CFE)	0.0	0.0	0.0	0.0

All reactions contain 100 nmoles $FeSO_4$. Final volume 1.32 ml. Final protein concentration was 8.3 mg/ml.

Table 2 indicates that ascorbate and ferrous ions stimulate the production of the penicillinase-resistant material, while the addition of α-ketoglutaric acid showed only slight stimulation. Both ascorbate and ferrous ions were required for maximal activity.

Table 3 shows that increasing the ascorbic acid level to 5 µmoles increased the amount of "cephalosporin" produced. Moreover, boiling of the cell-free

TABLE 4

Effect of storage of mycelia on ability to prepare active cell-free extract

	Condition	"Cephalosporin" (µg/ml cephalosporin C equiv.) 0 hr.	1.5 hr.
#1	Fresh 72-hr. mycelia		
	A. Control - no penicillin N	0.8	1.4
	B. Complete system	1.2	1.8
	C. Complete system + 5.0 µmoles α-KGA and ascorbate	2.4	4.8
#2	Frozen 72-hr. mycelia		
	A. Control - no penicillin N	0.0	0.0
	B. Complete system	0.0	0.7
	C. Complete + 5 µmoles α-KGA and ascorbate	0.9	4.0
#3	Frozen 96-hr. mycelia		
	A. Control - no penicillin N	0.7	0.5
	B. Complete system	0.9	1.0
	C. Complete + 5.0 µmoles α-KGA and ascorbate	1.0	2.8

All mixtures contain 100 nmoles $FeSO_4$ in a final reaction mixture of 1.32 ml. Final protein concentration: #1 CFE-13.6 µg/ml; #2 CFE-8.3 µg/ml; #3 CFE-7.1 µg/ml.

extract completely inhibited the reaction. Either fresh or frozen mycelia can be used as a source of active cell-free extracts (Table 4), although 66-hour old mycelia, whether fresh or refrigerated at -20 C, were more active than 96-hour mycelia.

When cephalosporinase sufficient to hydrolyze the equivalent of 10 µg/ml of cephalosporin C was added to the seeded assay medium in addition to the penicillinase, all of the detectable bioactivity was destroyed, indicating that "cephalosporin"(s) was formed as a product. (Table 5)

It appears from our results that the conversion of penicillin N to cephalosporin is rapid, especially when ascorbic acid and ferrous ions were used and this accounts for the large zero time values obtained in some cases. This zero time bioactivity was not due to ascorbate and ferrous ions inter-

TABLE 5

Effect of cephalosporinase plus penicillinase on reaction product.

Condition	"Cephalosporin" (µg/ml cephalosporin C equiv.) Penicillinase only 0 hr.	2 hr.	3 hr.	Penicillinase + Cephalosporinase 0 hr.	2 hr.	3 hr.
Control - no penicillin N	1.2	1.8	1.6	0.0	0.0	0.0
Complete	1.8	2.7	2.5	0.0	0.0	0.0
Complete + 1.0 µmole αKGA and ascorbate	3.3	5.2	4.6	0.0	0.0	0.0
Complete + 5.0 µmoles αKGA and ascorbate	3.7	9.2	9.3	0.0	0.0	tr.
Complete + 5.0 µmoles αKGA	1.7	2.7	2.1	0.0	0.0	0.0
Complete + 5.0 µmoles ascorbate	3.0	10.0	10.0	0.0	0.0	tr.

All reactions contain 100 nmoles-$FeSO_4$. Final reaction volume was 1.32 ml. Final protein concentration was 7.1 µg/ml.

tr = trace

TABLE 6

Effect of penicillin N on the production of "cephalosporin" in *C. acremonium* CW19 and mutant M-0198

Condition	"Cephalosporin" (µg/ml cephalosporin C equiv.) 0 hr.	3 hr.
A. CW 19 Strain (72-hr. mycelia)		
Control - no penicillin N	0.0	0.9
Control - no penicillin N + 5.0 µmoles ascorbate + 100 µmoles $FeSO_4$	1.0	1.0
Complete + 5.0 µmoles ascorbate + 100 µmoles $FeSO_4$	2.5	8.8
B. Mutant M-0198 (72-hr. mycelia)		
Control - no penicillin N + 5.0 µmoles ascorbate + 100 µmoles $FeSO_4$	0.0	0.0
Complete + 5.0 µmoles ascorbate + 100 µmoles $FeSO_4$	1.9	9.2

Final reaction volume was 1.37 ml.

acting with the cell-free extract since a control reaction containing these components without penicillin N had only a small background amount of penicillinase-resistant material (Table 6).

DISCUSSION

These results appear to confirm and extend those reported by Kohsaka and Demain (1) and by Yoshida et al (2), concerning the biosynthesis of cephalosporinase-sensitive material when a crude source of penicillin N was added to cell-free extracts of strains of C. acremonium.

We have found that the reaction is stimulated by 3-4 fold upon the addition of ascorbic acid and ferrous ions. The reaction was only slightly stimulated by the addition of α-ketoglutarate. This suggests the enzyme involved in the conversion of penicillin N to cephalosporin could be a monooxygenase, similar to dopamine-β-monooxygenase (5) or a dioxygenase similar to thymine-7-hydroxylase (6), proline hydroxylase (7) or the enzyme involved in the conversion of deacetoxycephalosporin C to deacetylcephalosporin C [Turner et al (8)].

The activity of the cell-free extract is enzymic in nature because boiling the extract abolishes activity even in the presence of ascorbic acid, α-ketoglutarate and ferrous ions. Moreover, these co-factors themselves are not responsible for the bioactivity even in the presence of unboiled cell-free extracts.

The transformation of penicillin N to deacetoxycephalosporin C is an oxidative process in which the present work implicates the involvement of ascorbic acid and ferrous ions in cell-free extracts.

Our results not only confirm that the cell-free extract from the parent culture of Cephalosporium acremonium CW19 will convert penicillin N to a "cephalosporin" but that a cell-free extract from Demain's mutant M-0198 which is blocked in penicillin N production also catalyses the reaction (Table 6).

Since the conversion of the δ-(α-aminoadipyl)-cysteinylvaline tripeptide (ACV) to isopenicillin N is also an oxidative process, ascorbic acid and ferrous ion might also be co-factors in this biosynthetic transformation, a postulate which we are presently investigating.

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