FURTHER STUDIES ON THE CYCLIZATION OF THE UNNATURAL TRIPEPTIDE δ-(D-α-AMINOADIPYL)-L-CYSTEINYL-D-VALINE TO PENICILLIN N

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We reported recently that a partially purified extract of *Cephalosporium acremonium* CW-19 (*Acremonium chrysogenum* ATCC 36225) could convert the tripeptide δ-(D-α-aminoadipyl)-L-cysteinyl-D-valine (DLD-ACV) to deacetoxycephalosporin C when incubated with Fe^{2+}, ascorbate, dithiothreitol, α-ketoglutarate and ATP[1]. This conversion appears to result from cyclization of the unnatural substrate DLD-ACV to penicillin N by isopenicillin N synthetase (cyclase) followed by ring-expansion of the penicillin N to deacetoxycephalosporin C by deacetoxycephalosporin C synthetase (expandase). The existence of penicillin N as an intermediate in the DLD-ACV to deacetoxycephalosporin C conversion was supported by the finding that penicillinase (which does not attack cephalosporins) totally inhibited the formation of deacetoxycephalosporin C from DLD-ACV in reaction mixtures containing α-ketoglutarate. However, FAWCETT *et al.*[2] reported no production of penicillin N from DLD-ACV, and we also were unable to demonstrate penicillin N formation when we omitted α-ketoglutarate, a co-substrate in the expandase reaction[1]. Similarly, early studies using cell-free extracts prepared from *Streptomyces clavuligerus* failed to show antibiotic production in the absence of α-ketoglutarate[3], but cephalosporin production was observed in complete reaction mixtures containing α-ketoglutarate (unpublished data).

This inability to observe formation of penicillin N in the absence of α-ketoglutarate suggested that penicillin N might be an inhibitor of the cyclase. Antibiotic formation then might be observable only under conditions which remove the penicillin N (*e.g.* by subsequent conversion to deacetoxycephalosporin C). This possibility was investigated by adding penicillin N to a normal cyclase assay mixture with LLD-ACV as substrate and following the reaction by bioassay with *Micrococcus luteus* ATCC 381. Under these conditions we saw no inhibition[1], but the inherent sensitivity of *M. luteus* to penicillin N makes this result uncertain. Large zones of inhibition resulted from the exogenous penicillin N and could have obscured any effect due to cyclase inhibition.

Despite this inconclusive result, evidence for the role of penicillin N as an intermediate in the conversion of DLD-ACV to deacetoxycephalosporin C was strengthened by the findings of BALDWIN *et al.*[4], that the conversion of DLD-ACV→penicillin N does take place, although at an extremely slow rate. This slow conversion of DLD-ACV into penicillin N in the absence of expandase activity was confirmed using highly purified cyclase from *S. clavuligerus* (Table 1). Cyclase assays containing; dithiothreitol (DTT) 4 mM, ascorbate 2.8 mM, $FeSO_4$ 45 μM, Tris-HCl (pH 7.0) 50 mM and highly purified cyclase (0.06 mg protein) in a final volume of 0.4 ml gave a zone of inhibition of 25.5 mm when incubated with 100 μg of LLD-ACV as substrate for 30 minutes. Under identical reaction conditions, cyclase assays gave a zone of 9.0 mm when incubated with 100 μg of DLD-ACV for 1 hour. Antibiotic formed from both tripeptide substrates was completely destroyed by penicillinase. *M. luteus* was used as the indicator organism in these studies since it is about equally sensitive to both penicillin N (product of DLD-ACV cyclization) and isopenicillin N (product of LLD-ACV cyclization)[5]. When salt precipitated cell-free extract (2.4 mg protein containing cyclase, epimerase and expandase activities) from *S. clavuligerus* was used as the enzyme source in reaction mixtures containing the cyclase assay components as listed above

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Table 1. Activity of highly purified cyclase[a] from *S. clavuligerus* on LLD-ACV and DLD-ACV.

Substrate	Zone of inhibition[b] (mm)	Amount of antibiotic[b] (μg/ml Isopenicillin N equivalents)
LLD-ACV		
30 minutes incubation	25.5 (0)	97.5 (0)
1 hour incubation	25.0 (0)	84.0 (0)
DLD-ACV		
30 minutes incubation	8.5 (0)	6.6 (0)
1 hour incubation	9.0 (0)	7.2 (0)

[a] Highly purified cyclase was prepared from *S. clavuligerus* cell-free extracts by a combination of salt precipitation, ion exchange and gel filtration chromatography (manuscript in preparation).

[b] Reaction mixtures were inactivated by the addition of 0.4 ml of methanol after 30 minutes and 1 hour of incubation, and 20 μl samples were bioassayed against *M. luteus* ATCC 9341 as the indicator organism. Numbers in parentheses represent the values obtained when penicillinase (Difco Laboratories, Detroit, MI, U.S.A.) was included in the bioassay medium at 1,000 U/ml.

but also supplemented with α-ketoglutarate 1 mM, KCl 7.5 mM and $MgSO_4$ 7.5 mM, the preferential conversion of LLD-ACV over DLD-ACV was much reduced (Table 2). Reaction mixtures containing LLD-ACV gave a zone of 22.0 mm due to penicillinase resistant antibiotic (deacetoxycephalosporin C) after a 30 minutes incubation period while reaction mixtures containing DLD-ACV gave a zone of 16.0 mm after 30 minutes incubation under identical reaction conditions. *Escherichia coli* ESS was used as the indicator organism in these studies since *M. luteus* is insensitive to cephalosporins.

These results indicate that LLD-ACV is cyclized about 15× more rapidly by purified cyclase than is DLD-ACV, but that enzyme mixtures which allow removal of penicillin N by subsequent expandase activity show only a three-fold preference for LLD-ACV over DLD-ACV. Similar results, demonstrating the slow conversion of DLD-ACV to penicillin N in the absence of α-ketoglutarate and the more rapid conversion of DLD-ACV to deacetoxycephalosporin C in the presence of α-ketoglutarate were obtained using purified cyclase and cyclase/expandase enzyme mixtures respectively from

Table 2. Activity of salt precipitated cell-free extract[a] from *S. clavuligerus* on LLD-ACV and DLD-ACV.

Substrate	Zone of inhibition[b] (mm)	Amount of antibiotic[b] (μg/ml Cephalosporin C equivalents)
LLD-ACV	22.5 (22.0)	8.25 (7.5)
DLD-ACV	16.5 (16.0)	2.75 (2.5)

[a] Crude enzyme mixture was prepared from *S. clavuligerus* cell-free extract as described previously[7].

[b] Reaction mixtures were inactivated by the addition of 0.4 ml of methanol after 30 minutes of incubation, and 20 μl samples were bioassayed against *E. coli* ESS as the indicator organism. Numbers in parentheses represent the values obtained when penicillinase was included in the bioassay medium at 1,000 U/ml.

C. acremonium (data not shown).

Upon confirmation of this slow production of penicillin N from DLD-ACV in reaction mixtures lacking α-ketoglutarate, we returned to the consideration of penicillin N as an inhibitor of the cyclase reaction. We reasoned that the rapid conversion of DLD-ACV to deacetoxycephalosporin C must necessarily involve a rapid production of the penicillin N intermediate. The slow accumulation of penicillin N in reaction mixtures lacking α-ketoglutarate strongly indicated that penicillin N inhibited the reaction. Although our earlier experiments did not demonstrate inhibition by penicillin N, the shortcomings of the method used (bioassay with *M. luteus*) necessitated the development of a more reliable assay. Thus, the inhibition of cyclase activity by penicillin N was examined using HPLC to assay for disappearance of the tripeptide. A highly purified preparation of cyclase from *C. acremonium*[8] was used in these studies to give a high enzyme concentration without problems of α-ketoglutarate carry-over.

The HPLC method used to assay LLD-ACV and DLD-ACV was as follows: To 0.5 ml of the reaction mixture was added 6 μl of 0.2 M DTT at pH 8.0 and the mixture incubated for 10~15 minutes at 21°C. To this mixture was added 25 μl of 0.2 M 5,5′-dithiobis-2-nitrobenzoate (DTNB), (the DTNB solution was prepared by dissolving 793 mg DTNB in 10 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing

Table 3. Inhibition of DLD-ACV disappearance by penicillin N.

Initial DLD-ACV concentration (μg/ml)	Initial penicillin N concentration (μg/ml)	Rate of disappearance of DLD-ACV (μg/ml/minute)
50	0	0.050
50	200	0.017
50	300	0.008
100	0	0.058
100	200	0.033
100	300	0.025
200	0	0.13
200	200	0.050
200	300	0.025

5 mM EDTA and adjusting the pH to 8.0 with 0.5 M 3-[*N*-morpholino]propane sulfonic acid (MOPS) in 6 M KOH). The derivatized solution was analyzed on a C-18 μBondapak column (3.9×300 mm) using models 6000A and M45 pumps and a model 660 solvent programmer (all from Waters Scientific Ltd., Milford, MA, U.S.A.). Elution was accomplished using a 30 minutes linear gradient starting with 95% 50 mM ammonium formate (pH 7.5) - 5% methanol and ending with 100% methanol at a flow rate of 2 ml/minute. Detection was at 280 nm (Waters model 440 absorbance detector) and recording was done using a Houston Instruments Onmiscribe Strip Chart Recorder (Austin, TX, U.S.A.).

Reactions were conducted and terminated essentially as described previously[1] except that 30 μl enzyme was used (0.19 mg protein). The substrate concentration was varied between 50 and 200 μg/ml. As can be seen in Table 3, DLD-ACV slowly disappeared and this disappearance was inhibited by penicillin N. When LLD-ACV was used as substrate, the reaction proceeded at a faster rate, but penicillin N showed roughly the same degree of inhibition (Table 4).

In conclusion, cyclization of both LLD-ACV and DLD-ACV is inhibited by penicillin N to a similar degree. Using a purified cyclase with no epimerase activity, the conversion of LLD-ACV is much faster than that of DLD-ACV. Presumably, isopenicillin N, the normal product of the cyclization of LLD-ACV, is less inhibitory to cyclase than is its epimer, penicillin N. Since the immediate product of the DLD conversion is penicillin N, the reaction is much slower due to product inhibition. Only in the presence of α-ketoglutarate does the DLD reaction proceed rapidly due to the rapid removal of penicillin N by expandase action.

Table 4. Inhibition of LLD-ACV disappearance by penicillin N.

Initial LLD-ACV concentration (μg/ml)	Initial penicillin N concentration (μg/ml)	Rate of disappearance of LLD-ACV (μg/ml/minute)
25	0	0.92
25	100	0.38
25	200	0.29
25	400	0.26
50	0	2.2
50	100	1.0
50	200	0.69
50	400	0.69
100	0	3.5
100	100	1.9
100	200	1.2
100	400	0.90

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References

1) SHEN, Y.-Q.; S. WOLFE & A. L. DEMAIN: Enzymatic conversion of the unnatural tripeptide δ-(D-α-aminoadipyl)-L-cysteinyl-D valine to β-lactam antibiotics. J. Antibiotics 37: 1044~1048, 1984

2) FAWCETT, P. A.; J. J. USHER, J. A. HUDDLESTON, R. C. BLEANEY, J. J. NISBET & E. P. ABRAHAM: Synthesis of δ-(α-aminoadipyl)cysteinylvaline and its role in penicillin biosynthesis. Biochem. J. 157: 651~660, 1976

3) JENSEN, S.E.; D.W.S. WESTLAKE, R.J. BOWERS, C. F. INGOLD, M. JOUANY, L. LYUBECHANSKY & S. WOLFE: Penicillin formation by cell-free extracts of *Streptomyces clavuligerus*. Behavior of aminoadipyl modified analogs of the natural peptide precursor δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV). Can. J. Chem. 62: 2712~2720, 1984

4) BALDWIN, J. E.; E. P. ABRAHAM, R. M. ADLINGTON, G. A. BAHADUR, B. CHAKRAVARTI, B. P. DOMAYNE-HAYMAN, L. D. FIELD, S. L. FLITSCH, G. S. JAYATILAKE, A. SPAKOVSKIS, H.-H. TING, N. J. TURNER, R. L. WHITE & J. J. USHER: Penicillin biosynthesis: Active site mapping with aminoadipyl-cysteinylvaline variants. J. Chem. Soc. Chem. Commun. 1984: 1225~1227, 1984

5) WOLFE, S.; C. LÜBBE, S. E. JENSEN, H. HERNANDEZ & A. L. DEMAIN: Effect of side-chain substitution of a CH_2 group by sulfur on the antimicrobial activity of natural penicillins and cephalosporins. J. Antibiotics 38: 1550~1554, 1985

6) HOLLANDER, I. J.; Y.-Q. SHEN, J. HEIM, A. L. DEMAIN & S. WOLFE: A pure enzyme catalyzing penicillin biosynthesis. Science 224: 610~612, 1984

7) JENSEN, S. E.; D. W. S. WESTLAKE & S. WOLFE: Production of penicillins and cephalosporins in an immobilized enzyme reactor. Appl. Microbiol. Biotechnol. 20: 155~160, 1984