FURTHER STUDIES ON THE CYCLIZATION OF THE UNNATURAL TRIPePTIDE L-(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 L-(d-α-aminoadipyl)-L-cysteinyl-D-valine (LDL-ACV) to deacetoxycephalosporin C when incubated with Fe²⁺, ascorbate, dithiothreitol, α-ketoglutarate and ATP. This conversion appears to result from cyclization of the unnatural substrate LDL-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 LDL-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 LDL-ACV in reaction mixtures containing a-ketoglutarate and ATP. This inability to observe formation of penicillin N in the absence of a-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 LDL-ACV as substrate and following the reaction by bioassay with Micrococcus luteus ATCC 381. Under these conditions we saw no inhibition, 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 LDL-ACV to deacetoxycephalosporin C was strengthened by the findings of BALDWIN et al.4), that the conversion of LDL-ACV→penicillin N does take place, although at an extremely slow rate. This slow conversion of LDL-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, FeSO₄ 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 LDL-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 LDL-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 LDL-ACV cyclization) and isopenicillin N (product of LDL-ACV cyclization). 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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but also supplemented with α-ketoglutarate 1 mM, KCl 7.5 mM and MgSO₄ 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 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 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>
<thead>
<tr>
<th>Substrate</th>
<th>Zone of inhibition (mm)</th>
<th>Amount of antibiotic (µg/ml)</th>
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<tbody>
<tr>
<td>LLD-ACV</td>
<td>25.5 (0)</td>
<td>97.5 (0)</td>
</tr>
<tr>
<td>DLD-ACV</td>
<td>8.5 (0)</td>
<td>6.6 (0)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Substrate</th>
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</tr>
</thead>
<tbody>
<tr>
<td>LLD-ACV</td>
<td>22.5 (22.0)</td>
<td>8.25 (7.5)</td>
</tr>
<tr>
<td>DLD-ACV</td>
<td>16.5 (16.0)</td>
<td>2.75 (2.5)</td>
</tr>
</tbody>
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Table 2. Activity of salt precipitated cell-free extract from S. clavuligerus on LLD-ACV and DLD-ACV.
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 1tBondapak column (3.9 x 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 Omniscribe Strip Chart Recorder (Austin, TX, U.S.A.).

Reactions were conducted and terminated essentially as described previously1) 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.

Acknowledgments

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References


