Polymyxin Acylase: An Enzyme Causing Intramolecular $N^2 \leftrightarrow N^6$ Acyl Transfer in $N$-Monooctanoyl-L-Lysine

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Polymyxin acylase from Pseudomonas sp. M-6-3 can deacylate not only polymyxin antibiotics, but also $N$-fatty acyl-peptides and $N$-fatty acyl-amino acids. We found that this enzyme causes intramolecular $N^2 \leftrightarrow N^6$ acyl transfer in monooctanoyl-L-lysine; when $N^2$-octanoyl-L-lysine is the substrate, $N^6$-octanoyl-L-lysine is produced at pH 10.5, but when $N^6$-octanoyl-L-lysine is the substrate, $N^2$-octanoyl-L-lysine is produced at pH 8.0. In these reactions, the deacylation proceeded gradually at the final stage and eventually, both $N^2$-octanoyl-L-lysine and $N^6$-octanoyl-L-lysine were hydrolyzed to L-lysine and octanoic acid. Furthermore, this enzyme showed intermolecular acyltransferase activity, transferring several $N$-octanoyl-DL-amino acids to $N$-octanoyl-hydroxylamine. This acyltransfer ability of polymyxin acylase offers a new method of enzymic $N$-acylation of compounds containing amino components.

Polymyxin acylase from Pseudomonas sp. M-6-3 is an enzyme useful for preparing semi-synthetic polymyxins.1) It can deacylate not only polymyxin antibiotics, but also $N$-fatty acyl-peptides and $N$-fatty acyl-amino acids. The cell-free enzyme solubilized from acetone-dried cell powder has been purified to a homogeneous state and its properties have been clarified with special reference to its broad substrate specificity.2) In the last stage of the enzymatic deacylation for colistin, HPLC reveals new small peaks with retention times slightly longer than those of colistins A and B. Analysis of these new peaks by the DNP method suggested that an acyl group linked with an $\alpha$-amino group migrates to a $\gamma$-amino group in colistin.3)

In this study, we examined the acyltransferase activity of polymyxin acylase on $N^2$-octanoyl-L-lysine as a mimic substrate. This transferase activity has not been found in many known amino acylases. We report here that polymyxin acylase catalyzes not only $N^2 \leftrightarrow N^6$ acyl migration on $N$-monoacyl-L-lysine, but also intermolecular acyl migration from $N$-acyl-DL-amino acids to $N$-acyl-hydroxylamine.

Materials and Methods

Chemicals. $N^2$-Octanoyl-L-lysine was prepared by removing the benzyloxycarbonyl group from $N^2$-octanoyl-$N^6$-benzyloxycarbonyl-L-lysine, which was prepared by the Shotten-Baumann reaction of $N^6$-benzyloxycarbonyl-L-lysine and octanoyl chloride. $N^6$-Octanoyl-L-lysine was prepared in the same manner from L-lysine copper chelate and octanoyl chloride. All other chemicals were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Preparation of polymyxin acylase. Polymyxin acylase was purified from the acetone-dried cell powder of Pseudomonas sp. M-6-3 by the procedure described in our preceding paper.2) The purified enzyme (Type I) was homogeneous by SDS-polyacrylamide gel electrophoresis and the activity for colistin B was 1750 units/mg; one unit of enzyme activity was defined as the amount of enzyme that produced 1 nmol of deacyl colistin B per min.

Enzyme assay. Intramolecular acyltransferase activity

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(\(\beta\)-aminoethyl ether)-\(N, N\), \(N', N\)'-tetraacetic acid; DFP, diisopropylphosphorofluoridate; PMSF, phenylmethylsulfonylfluoride.
was assayed from the ninhydrin color spot of N<sup>2</sup>- or N<sup>6</sup>-monooctanoyl-L-lysine on the TLC plate (Method 1). Intermolecular acyltransferase activity was measured using hydroxylamine as the acyl acceptor and N-acyl-DL-amino acids as the acyl donor and the rate was assayed by the FeCl<sub>3</sub>-color reaction of hydroxamic acid (Method 2).

**Assay method 1.** The reaction mixture, 200 µl, containing 2.5 µmol of N<sup>2</sup>-octanoyl-L-lysine and the enzyme solution (10 units) in 100 mM carbonate buffer (pH 10.5), was incubated at 37°C for several hours. For the reverse reaction with N<sup>6</sup>-octanoyl-L-lysine, the phosphate buffer (pH 8.0) was used.

A 1 µl portion of the reaction mixture was put on a thin layer plate of silica gel and developed with a solvent system of phenol-water (4:1). To detect the product and residual substrate, ninhydrin reagent (80 mg ninhydrin, 1 ml pyridine, and 20 ml ethanol) was sprayed on the plate, which was kept at 100°C for 5 min. The amount of the product was monitored by the ninhydrin color with Chromoscan 200/201 (Joyce-Loebl, England).

**Assay method 2.** A reaction mixture, 200 µl, containing 25 µmol of N-octanoyl-DL-amino acid, 75 µmol hydroxylamine sulfate, and the enzyme solution (30 units) in 100 mM Tris-HCl buffer (pH 8.0) was incubated at 37°C for several hours. To this mixture (50 µl), 5.7 mM FeCl<sub>3</sub> solution in 160 mM HCl, 450 µl, was added, and the absorbance at 525 nm was measured.

### Results

**Production of a ninhydrin-positive compound in the reaction of N<sup>2</sup>-octanoyl-L-lysine with polymyxin acylase**

The reaction of N<sup>2</sup>-octanoyl-L-lysine and polymyxin acylase yielded a ninhydrin-positive compound which appeared at a position on the thin-layer plate different from that of lysine. With time, the lysine gradually appeared. This compound was found to be N<sup>6</sup>-octanoyl-L-lysine by comparison of its thin-layer chromatographic and high-voltage electrophoretic data with those of an authentic sample.

**Effects of pH on intramolecular acyltransferase activity**

The migration of the octanoyl group from N<sup>2</sup> to N<sup>6</sup> on L-lysine was examined. In the reaction mixture with N<sup>2</sup>-octanoyl-L-lysine and polymyxin acylase, the optimal pH for N<sup>6</sup>-octanoyl-L-lysine formation (N-acyltransferase activity) was 10.5, and that for L-lysine formation (deacylase activity) was 8.5 (Fig. 1).

![Fig. 1. Effects of pH on Intramolecular A cyltransferase Activity in N-Mono octanoyl-L-lysine.](image)

- ○—, N<sup>2</sup>-<br>—N<sup>6</sup> acyltransferase activity; —■—, N<sup>6</sup>-<br>—N<sup>2</sup> acyltransferase activity.

The reaction mixture, 200 µl, containing 2.5 µmol of N<sup>2</sup>-octanoyl-L-lysine (or N<sup>6</sup>-octanoyl-L-lysine) and 10 units of enzyme in the following buffers (final concentration of 100 mM): citrate-phosphate (pH 6.0), sodium phosphate (pH 6.5~8.5), Tris-HCl (pH 7.5~9.0), and sodium carbonate (pH 9.5~11.0), was incubated for 1 hr at 37°C. The relative activity is expressed as the percentage of the maximum activity (2700 nmol/min/mg protein) attained under these experimental conditions.

![Fig. 2. Course of N<sup>6</sup>-Octanoyl-L-lysine Formation from N<sup>2</sup>-Octanoyl-L-lysine.](image)

- ○—, N<sup>6</sup>-octanoyl-L-lysine; —■—, L-lysine.

The reaction mixture, 500 µl, containing 10 µmol of N<sup>2</sup>-octanoyl-L-lysine and 30 units of enzyme in 100 mM carbonate buffer (pH 10.5), was incubated at 37°C.

At pH 10.5, the deacylase activity was much weaker than the N-acyltransferase activity. The courses of the N-acyltransfer and deacylation reactions at pH 10.5 showed a predom-
Acyltransferase Activity of Polymyxin Acylase

The acyltransferase activity was measured from the migration of the octanoyl group from \( N^2 \) to \( N^6 \) on L-lysine. The enzyme activity was markedly inhibited by \( \text{Hg}^{2+} \) and \( \text{Ag}^+ \), inhibited (50%) by \( \text{Cu}^{2+} \), but slightly enhanced by \( \text{Co}^{2+} \) and \( \text{Mg}^{2+} \) at the concentration of 1 mM. Inhibition by other agents at 1 mM concentration was as follows: metal ion chelating agents (EDTA, EGTA, \( \alpha \)-phenanthroline and 8-hydroxyquinoline) 5 – 10% inhibition; thiol-blocking reagents, 20 – 30% inhibition, and an oxidizing agent, \( \text{N-bromosuccinimide} \), complete inhibition.

**Tolerance for organic solvents on the intramolecular acyltransferase activity**

The enzyme showed tolerance for several organic solvents, acetone, ethanol, ethylene glycol, and acetonitrile. Among them, ethylene glycol was best tolerated. Interestingly, addition of 20 – 30% ethylene glycol enhanced, and did not inhibit, the enzyme activity, and even in 50% ethylene glycol, half of the activity remained. All organic solvents examined increased the enzyme activity 10 – 30% when added as 10% solutions (Fig. 3).

**Intermolecular acyltransferase activity of polymyxin acylase**

The migration of the octanoyl group on \( N \)-octanoyl-DL-amino acids from DL-amino acid to hydroxylamine was examined. The reaction mixture with \( N \)-octanoyl-DL-glutamic acid, hydroxylamine, and polymyxin acylase gave \( N \)-octanoyl-hydroxylamine and the optimal pH was 8.5. The apparent \( V_{\text{max}} \) and \( K_m \) values were 800 nmol/min/mg and 36 mM for hy-

**Table 1. Substrate Specificity of Acyltransfer of \( N \)-Octanoyl-DL-amino Acids to \( N \)-Octanoyl-hydroxylamine by Polymyxin Acylase (Type I)**

<table>
<thead>
<tr>
<th>Acyl donors</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( N )-Octanoyl-DL-glutamic acid</td>
<td>100</td>
</tr>
<tr>
<td>( N )-Octanoyl-glycine</td>
<td>100</td>
</tr>
<tr>
<td>( N )-Octanoyl-DL-valine</td>
<td>90</td>
</tr>
<tr>
<td>( N )-Octanoyl-DL-aspartic acid</td>
<td>90</td>
</tr>
<tr>
<td>( N )-Octanoyl-DL-serine</td>
<td>75</td>
</tr>
<tr>
<td>( N )-Octanoyl-DL-threonine</td>
<td>75</td>
</tr>
<tr>
<td>( N )-Octanoyl-DL-alanine</td>
<td>75</td>
</tr>
<tr>
<td>( N )-Octanoyl-DL-methionine</td>
<td>60</td>
</tr>
<tr>
<td>( N )-Octanoyl-DL-leucine</td>
<td>40</td>
</tr>
<tr>
<td>( N )-Octanoyl-DL-phenylalanine</td>
<td>10</td>
</tr>
</tbody>
</table>

The reaction was done at 37°C for 1 hr by assay method 2 (pH 8.0) using 30 units of enzyme. The formation of \( N \)-octanoyl-hydroxylamine, corresponding to 800 nmol/min/mg protein, was taken as 100%.
Droxyamine, respectively. The other N-octanoyl-DL-amino acids were also effective as acyl donors, and the substrate specificity for amino acid residues on intermolecular acyltransferase activity was slightly different from that of the amino acid acylase activity of the same enzyme. As shown in Table 1, N-octanoyl derivatives of some amino acids (Gly, Val, Asp, and Glu) were also effective as acyl donors. Neither metal ion chelating agents nor thiol-blocking reagents, except for p-chloromercuribenzoate, affected the intermolecular acyltransferase activity at 1 mM. N-Bromosuccinimide inhibited the activity in a similar manner to its inhibition of intramolecular acyltransferase activity.

Discussion

Previously, we found that polymyxin acylase from Pseudomonas sp. M-6-3 displays N<sub>2</sub>-<del>→</del>A<sub>4</sub> acyl migration activity on 2,4-diaminobutyrate in the polymyxin molecule. To clarify the mechanism of this activity, N<sub>2</sub>-octanoyl-L-lysine was used as a mimic substrate, because polymyxin acylase has a high affinity for long chain fatty acyl groups and lysine is a typical basic amino acid. The optimal pH for the acyl migration from N<sub>2</sub>-octanoyl-L-lysine to N<sub>6</sub>-octanoyl-L-lysine was 10.5, which is equal to the pK value of the ε-amino group of lysine. This means that the un-protonated form of the ε-amino group is required for the acceptance of the acyl group and a much higher concentration of hydroxy ion may disturb this activity by a conformational change of the enzyme. In the reverse reaction (from N<sub>6</sub>-octanoyl-L-lysine to N<sub>2</sub>-octanoyl-L-lysine), the optimal pH was 8.0, which was also approximately equal to the pK value of the ε-amino group of lysine. These results suggest that the optimal pH of N,N-acyltransferase is approximately equal to the pK value of the amino group that will accept an acyl group. The courses of N<sub>2</sub>→N<sub>6</sub> and N<sub>6</sub>→N<sub>2</sub> acyl migration on lysine by polymyxin acylase indicated that the acyltransfer reaction occurs first and is followed by deacylation (Fig. 4). A small amount of direct liberation of the acyl group from the N<sub>2</sub>-octanoyl-L-lysine or N<sub>6</sub>-octanoyl-L-lysine was also recorded. Our results showed that the migration by polymyxin acylase from N<sub>2</sub>-octanoyl-L-lysine to N<sub>6</sub>-octanoyl-L-lysine occurred much more easily than that from N<sub>6</sub>-octanoyl-L-lysine to N<sub>2</sub>-octanoyl-L-lysine, which offers evidence in support of the acyltransfer occurring before the deacylation in the polymyxin molecule.

The acyltransferase activity showed tolerance to several organic solvents as seen for the deacylase activity on polymyxin. The tolerance observed for the acyltransferase activity was a little higher than that for the deacylase activity. For example, addition of 20% ethylene glycol led to 80% activation rather than inhibition of the acyltransferase activity. This
may have been due to repression of protonation on the amino group, which serves as the acceptor, by the addition of organic solvents. The optical specificity of the enzyme for lysine was not exact; it acted on both N-octanoyl-L-lysine and N-octanoyl-D-lysine, but the activity for the L-form was 10 times higher than that for the D-form. The substrate specificity for acyl groups other than the octanoyl group has not yet been studied, but the deacylase activity for many fatty acyl groups in N-acyl-methionine has been reported.

This enzyme showed not only intramolecular acyltransferase activity, but also intermolecular acyltransferase activity from N-octanoyl-DL-amino acids to N-octanoyl-hydroxylamine. However, these $V_{\text{max}}$ values were lower than that in the intramolecular acyltransferase activity for the acyltransfer of $N^2$-octanoyl-L-lysine to $N^6$-octanoyl-L-lysine. The $K_m$ value for hydroxylamine was much higher than the usual values. Generally, the acyl donor of acyltransferase is acyl-S-CoA, but we found here the presence of $N,N$-acyltransferase as the donor for $N$-acyl-amino acids. However, there is little possibility of this enzyme displaying this activity in living cells because of its high $K_m$ value.

Intramolecular acyltransferase activity displayed by polymyxin acylase was inhibited by Hg$^{2+}$ and Ag$^+$, but was affected little by metal chelators and various thiol-blocking reagents, other than $p$-chloromercuribenzoate. These properties are very similar to those of its deacylation activity. Both reactions, deacylation and transacylation, may occur at the same active site of the enzyme. None of the known aminoacylases that we examined showed this acyltransferase activity. This means that the enzymic reaction mechanism of polymyxin acylase is different from those of usual aminoacylases. Similar enzymes, penicillin acylases, produced from *E. coli*, *Bacillus megaterium*, or *Xanthomonas citri* are also distinguishable from polymyxin acylase, because they are serine enzymes, inhibited by DFP or PMSF.

In sum, polymyxin acylase shows both intramolecular and intermolecular acyltransferase activities. It can cause $N,N$-acylmigration of the $N$-monoacyl derivatives of basic peptides, which shows promise for use in preparing the acyl isomers of these peptides. It can also be a very useful deacylating reagent for many $N$-acyl-peptides. This intermolecular acyl transfer ability of polymyxin acylase shows promise for the development of a new method of enzymic $N$-acylation of compounds containing amino components.

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References