Comparison of Periplasmic and Membrane Associated β-Lactamase

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β-Lactamase encoded by a plasmid pBR 322 was produced during the active growth phases of Escherichia coli IA 199. The maximal specific activity was about 15 times higher in shock fluid than in the cells disrupted by sonic disintegration. β-Lactamase activity found in the membrane preparations increased gradually parallel to the cell growth. The amount of β-lactamase in the membrane fraction, however, was only 0.2 – 0.4% of that found in shock fluid. β-Lactamase was purified to homogeneity from shock fluid by a one-step procedure in a DEAE-Sepharose column. Most of the β-lactamase activity present in the membrane fraction was released by salt extraction. β-Lactamase solubilized from the membrane fraction by Triton X-100 treatment after salt extractions had the same molecular weight and immunological properties as β-lactamase purified from the periplasmic space. Membrane associated β-lactamase did not contain any covalently linked phospholipid.

Secreted proteins like periplasmic and outer membrane proteins in Escherichia coli undergo a proteolytic cleavage during secretion.1-3 Processed β-lactamase is a monomer with a molecular weight of 28900.4 The protein is secreted into the periplasmic space of gram-negative species of bacteria. The results of Kosland and Botstein5 suggest that the synthesis of β-lactamase is completed before secretion and processing. The processing of another periplasmic protein precursor, maltose-binding protein, have been reported to occur co-translationally as well as post-translationally.3 Evidence for conformational differences between precursor and processed forms has been obtained.6 It is possible that the conformational changes associated with the conversion of the precursors to the processed forms play a role in the secretion of proteins.3

In this paper we compare the secreted and membrane associated β-lactamase to each other. We conclude that β-lactamase is trapped and kept in the membrane through hydrophobic association during secretion. Most β-lactamase activity in the membrane fraction, however, is loosely bound and could be removed by salt extraction.

MATERIALS AND METHODS

Materials. Na125I and [32P]P0.1 (1.5 GBq/ml) were obtained from the Radiochemical Centre, Amersham, England. Lactoperoxidase was from Sigma, St. Louis, Mo., U.S.A.

Growth of Escherichia coli IA 199. E. coli IA 199 carrying plasmid pBR 322 was cultured in ACH-medium containing the salts,7 0.2% glucose, 0.2% casamino acids, 1 mg/ml thiamine hydrochloride and 0.1 mg/ml ampicillin. For labeling with [32P]P0, cells were grown in media containing 0.2 mM inorganic phosphate and 75 MBq of [32P]P0/liter. Samples of 10 ml were withdrawn as indicated and the cells disrupted by sonication. The culture supernatants and the cell extracts were assayed for β-lactamase.

Preparation of spheroplasts and membrane vesicles. Spheroplasts were prepared from the cells by lysozyme-EDTA treatment.8 The formation of spheroplasts was ensured under a phase contrast microscope. The spheroplasts were incubated at 30°C in ACH-medium in the presence of 0.25 M sucrose. The samples were taken as indicated and after centrifugation (10,000 × g, 15 min) the supernatants were assayed for β-lactamase. Membrane preparations were made from the spheroplasts by disrupting in a French press. The membranes were pelleted (30,000 × g, 1 h) and washed with 0.05 M Tris/HCl, 0.01 to 1 M KCl, pH 7.0. The membranes were solubilized with 0.5%
Triton X-100 and after centrifugation (30 000 × g, 30 min) the supernatants were assayed for β-lactamase.

Osmotic shock treatment. The cells were washed twice with 25 volumes of 10 mM Tris/HCl, 30 mM NaCl, pH 7.3. The washed cells were broken by suspending them rapidly in 40 volumes of 0.5 mM ice cold MgCl₂ as described by Heppel.⁹

Immunological experiments. β-Lactamase was iodinated by using the lactoperoxidase system.¹⁰ Rabbit antiserum to purified β-lactamase was prepared as described.¹¹ Immunoprecipitation was carried out as described.¹²

β-Lactamase assay. β-Lactamase was assayed using a chromogenic cephalosporin compound 87/312 (a generous gift from Glaxo Research Ltd., Greenford, Middx., England) as a substrate. β-Lactamase was incubated in 1 ml at room temperature with 0.1 M potassium phosphate, pH 7.0 and 25 μg of the cephalosporin compound. Increase in absorbance was measured at 486 nm. One unit was defined as the amount of enzyme that causes an increase in absorbance at 486 nm of 1.0 per min.

SDS-Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis in the presence of SDS was performed as described.¹³ A gradient gel (5 to 20% polyacrylamide) was used.

RESULTS

Formation of β-lactamase during the growth of Escherichia coli IA 199. Fig. 1 shows the formation of β-lactamase during the growth of E. coli IA 199/pBR 322. β-Lactamase is produced during the active growth phases; maximal concentration being reached at the beginning of the stationary phase of growth. When the cells were disrupted by osmotic shock the specific activity was about 15 times higher than in the cells disrupted by ultrasonic disintegration. Because total activities were almost equal after the treatments osmotic shock was used as the starting step in the purification of β-lactamase.

Salt extraction of β-lactamase from E. coli membranes. Amounts of β-lactamase in the membrane preparations were measured after successive extractions with 50 mM Tris/HCl (pH 7.0), 50 mM Tris/HCl—0.1 M to 1 M KCl and 50 mM Tris/HCl—0.5% Triton X-100. As seen in Table 1 most β-lactamase activity was released from the membranes by Tris—KCl treatments. From 0.2 to 0.4% of total β-lactamase was so tightly associated

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Fig. 1. Formation of β-lactamase during the growth of E. coli IA 199/pBR 322. The washed cells were broken osmotically or by ultrasonic disintegration and the supernatants were assayed for β-lactamase. (○) Turbidity. A. Total activity, (▲) osmotic shock treatment; (△) ultrasonic disintegration. B. Specific activity. The symbols are the same as above.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Released activity/U ml⁻¹</th>
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</thead>
<tbody>
<tr>
<td>50 mM Tris</td>
<td>0.68</td>
</tr>
<tr>
<td>50 mM Tris-0.1 M KCl</td>
<td>0.43</td>
</tr>
<tr>
<td>50 mM Tris-0.5 M KCl</td>
<td>0.81</td>
</tr>
<tr>
<td>50 mM Tris-1.0 M KCl</td>
<td>0.18</td>
</tr>
<tr>
<td>50 mM Tris-0.5% Triton X-100</td>
<td>0.24</td>
</tr>
<tr>
<td>Shock fluid</td>
<td>64.8</td>
</tr>
</tbody>
</table>

Table 1. Release of β-lactamase from the E. coli IA 199 membranes by consecutive extractions. The extraction mixtures were shaken for 10 min at 30 °C, centrifuged (30 000 × g, 1 h) and the supernatants were assayed for β-lactamase.
Fig. 2. Correlation between membrane-associated \( \beta \)-lactamase activity and growth of \( E. \text{coli} \) IA 199. \( E. \text{coli} \) was shaken in ACH-medium. At various times, aliquots were removed, centrifuged and the cells disrupted and treated as described under "Materials and Methods". After solubilization with Triton X-100 the extracts were assayed for \( \beta \)-lactamase.

with the membrane that it was released only by detergent treatments.

Association of \( \beta \)-lactamase in membranes during different growth phase of \( E. \text{coli} \). Concentrations of \( \beta \)-lactamase associated with membranes were studied. The cells were taken as indicated, washed with 50 mM Tris/HCl–0.5 M KCl and disrupted in a French press (2 \( \times \), 70 MPa). The formation of membrane vesicles was ensured under a phase contrast microscope. The membrane vesicles were washed three times with the same Tris–KCl buffer and solubilized by shaking for 30 min at 37°C. The supernatants (30,000 \( \times g \), 1 h) were assayed for \( \beta \)-lactamase. As seen in Fig. 2 concentrations of \( \beta \)-lactamase in the Triton X-100 supernatants increased parallel to cell growth suggesting that secreted \( \beta \)-lactamase may correlate with activities associated with membranes. The ratio of secreted and membrane-associated \( \beta \)-lactamase was from 250 to 500.

Purification of extracellular and membrane-associated \( \beta \)-lactamase. \( \beta \)-Lactamase was purified from the shock fluid and the membrane fraction of \( E. \text{coli} \) IA 199. The cells (19.8 g) were washed twice with 500 ml of 10 mM Tris/HCl–30 mM NaCl, pH 7.3. The shock fluid (940 ml) was prepared as described. After concentration (Amicon PM 10 membrane) and dialysis against 15 mM Tris/HCl, pH 7.0, \( \beta \)-lactamase was purified in a DEAE-Sepharose column (2.4 \( \times \) 30 cm). The column was washed with a linear Tris/HCl gradient (15 to 100 mM, 200 ml and 200 ml). \( \beta \)-Lactamase was eluted between 80 and 90 mM Tris/HCl (Fig. 3). The remaining cell fraction after shock treatment was treated in a French press (70 MPa) and washed three times with 15 mM Tris/HCl–0.5 M KCl, pH 7.0. The membranes were solubilized by shaking (30 min at 37°C) with 0.5\% Triton X-100. After centrifugation (30,000 \( \times g \), 1 h) and dialysis \( \beta \)-lactamase was purified by DEAE-
Table 2. Purification of β-lactamase from shock fluid. The enzyme was purified from 2 l of culture as described under “Materials and Methods”.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Activity/ U</th>
<th>Protein/ mg</th>
<th>Specific activity/ U mg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shock fluid</td>
<td>54 400</td>
<td>39.3</td>
<td>1380</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>50 320</td>
<td>9.2</td>
<td>5460</td>
</tr>
</tbody>
</table>

chromatography as described above. The membrane associated enzyme was eluted at the same Tris/HCl concentration as the periplasmic enzyme (Fig. 3). The purifications are summarized in Tables 2 and 3. When ¹²⁵I-labeled β-lactamases were precipitated with anti-β-lactamase to the periplasmic enzyme and subjected to polyacrylamide gel electrophoresis in the presence of SDS they migrated at the same speed (Fig. 4). Only one band was detected on the gel when purified periplasmic β-lactamase was subjected to gel electrophoresis without immunoprecipitation, as well (results not shown).

**Immunological comparison of secreted and membrane associated β-lactamase.** Fig. 5 shows an immunological comparison of secreted β-lactamase and β-lactamase purified from the membrane preparation. As seen, equal amounts of β-lactamase units were inhibited and precipitated with anti-β-lactamase to periplasmic β-lactamase.

Is there any phospholipid associated with β-lactamase purified from membranes? In order to reveal any attachment of phospholipid to β-lactamase obtained from the membranes of E. coli 1A 199 the cells were grown in the presence of [³²P]P₁ (75 MBq/liter). After washing and disrupting the cells the membrane preparation was solubilized with 0.5% Triton X-100 as described above. β-Lactamase was precipitated with anti-β-lactamase as above and counted for radioactivity. β-Lactamase isolated either from membranes or from extracellular supernatant did not contain any radioactivity. Before immunoprecipitation the Triton X-100 extract contained 463 000 cpmp of ³²P/reaction mixture. After three successive washings with the buffer, the pellets contained about 0.005% of radioactivity which was the same amount of radioactivity as in the Staphylococcus aureus cells without the antibody. These results suggest that no covalently linked phospholipid is attached to β-lactamase during secretion.

**DISCUSSION**

The following pieces of evidence support the idea that there is no intermediate form of β-lactamase covalently linked to phospholipid or a form tightly attached to E. coli membrane: (i) No covalently labeled [³²P]P₁ was detected in β-lactamase after immunoprecipitation or SDS-polyacrylamide gel electrophoresis. (ii) Secreted and membrane associated β-lactamase were eluted at the same Tris/HCl concentration from the DEAE-Sepharose column. (iii) Secreted β-lactamase and membrane associated β-lactamase co-migrated in SDS-polyacrylamide gel electrophoresis. (iv) Anti-β-lactamase to the periplasmic enzyme precipitated

phosphopeptides are attached to staphylococcal penicillinase and to the enzyme purified from *B. licheniformis*. These phosphopeptides may contain phosphatidylserine which might explain the hydrophobic properties of membrane-derived penicillinase from *B. licheniformis*. Membrane-derived $\beta$-lactamase from *E. coli* has the same molecular weight as the periplasmic enzyme suggesting that both forms have the same N-terminal end. The N-terminal end of periplasmic $\beta$-lactamase is rather more hydrophilic than hydrophobic. Because the membranes were extracted consecutively with increasing salt concentrations, and then with Triton X-100 the results suggest that the remaining $\beta$-lactamase after the salt treatments has hydrophobic interactions with the membranes. These interactions, however, may result from the presence of phosphatidylserine attached to $\beta$-lactamase. The significance of this hydrophobicity remains obscure but it might play some role during secretion. The presence of periplasmic $\beta$-lactamase as a contaminant in the membranes was excluded by incubating purified $\beta$-lactamase from the shock fluid with the isolated membranes and then extracting the membranes with 1 M KCl. After this treatment $\beta$-lactamase was entirely removed from the membranes.

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**REFERENCES**


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