Isolation of the penicillin-binding peptide from D-alanine carboxypeptidase of Bacillus subtilis

(penicilloyI-carboxypeptidase/peptide fragments/ester bond)

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ABSTRACT The D-alanine carboxypeptidase of B. subtilis is a membrane-bound enzyme which is inhibited by penicillins and binds them covalently. The enzyme has been labeled with [14C]- or [35S]penicillin. After tryptic or Pronase digestion of the labeled, denatured, reduced, and carboxymethylated enzyme, a radioactive peptide was isolated in each case. The amino acid compositions of these two peptides are reported. The Pronase peptide was a subset of the tryptic peptide. Neither contained a cysteine residue and the only amino acid in the Pronase peptide to which the penicillin could be bound was a serine residue.

The D-alanine carboxypeptidase (CPase) of Bacillus subtilis is a membrane-bound enzyme which catalyzes the hydrolysis of the terminal D-alanine residue of UDP-MurNAc-pentapeptide, the uridine nucleotide intermediate in cell wall peptidoglycan synthesis. The reaction is similar to the transpeptidase reaction involved in cell wall biosynthesis, except that water rather than an amino group is the acceptor. Both reactions, it was suggested, proceed through an acyl enzyme intermediate (1), and both are inhibited by penicillins. CPase is a moderate-sized protein (50,000 molecular weight) (2), is the major penicillin binding component in B. subtilis (3), and has been purified to homogeneity by affinity chromatography (4). Its function is not known, but it might be involved in the regulation of crosslinking during cell wall biosynthesis.

It was suggested earlier that the penicilloyI–enzyme complex might be a penicilloyI thioester involving a cysteine residue in the susceptible enzyme. This suggestion was based on the inhibition of penicillin binding by thiol reagents and the release of a penicilloyI derivative by hydroxylamine and ethanethiol (5). However, the concentrations of thiol inhibitors which were effective may have been too high to be specific (6). Furthermore, an alternative interpretation of the release of penicilloyI by hydroxylamine has been obtained, namely, the release is an enzymatically catalyzed transfer of penicilloyI to hydroxylamine, and denatured penicilloyI enzyme is stable under conditions in which acyl thioesters do not migrate and are cleaved by hydroxylamine (refs. 6–10; S. J. Curtis and J. L. Strominger, manuscript submitted for publication). The chemical reactivity of penicilloyI-enzyme, denatured under several conditions, was compatible with the presence of a serine ester (8). However, at least in the case of Escherichia coli, D-alanine carboxypeptidase IA, a sulhydryl group, presumably in the active site, is involved in the decylation of the penicilloyI- and acyl-enzyme. Its substitution by thiol reagents results in stabilization of the penicilloyI-enzyme and in accumulation of an acyl-enzyme intermediate derived from a normal substrate (ref. 8; S. J. Curtis and J. L. Strominger, manuscript submitted for publication). These findings prompted a close examination of the penicilloyI-enzyme linkage by isolating and characterizing peptide fragments containing bound penicillin. In this communication, we report two such fragments resulting from a tryptic and Pronase digestion, respectively.

MATERIALS AND METHODS

Materials. Deoxyribonuclease, lysozyme, and dithiothreitol were purchased from Sigma; 1-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-trypsin from Worthington; Pronase from Calbiochem; Sephadex G-50 (fine) from Pharmacia; guanidine hydrochloride from Heico; dapsyl chloride, Chen-Ching polyamide sheets, standard amino acid mixture, 6 M HCl, ninhydrin, fluorescamine, and triethyamine from Pierce; [8-14C]penicillin G, potassium salt (specific activity: 54 mCi/mmol) from Amersham/Searle. [35S]Penicillin G, potassium salt (specific activity: 1.6 mCi/mmol) was kindly synthesized by Richard Thoma of E. R. Squibb and Sons. Pyridine was purified by distillation after 2 hr of refluxing with ninhydrin (5 g of ninhydrin per liter of pyridine). Iodoacetic acid was purchased from Eastman and purified by recrystallization from petroleum ether. All other reagents were of analytical grade.

Preparation of Labeled CPase. CPase was isolated by affinity chromatography (4) from B. subtilis (strain Porton) membranes obtained by lysozyme DNase treatment (11). In a typical experiment, 10 mg of enzyme was obtained from 300 g of cells. The enzyme in 5 ml of 0.05 M Tris-HCl buffer (pH 7.5) containing 1% (vol/vol) Triton X-100, 0.5 M NaCl and 1 mM 2-mercaptoethanol [14] was incubated with labeled penicillin G ([8-14C]penicillin G, 25 μCi, or [35S]penicillin G, 0.8 μCi) for 10 min at 20°C. The incubation was stopped by adding 4 volumes of cold acetone containing a 100-fold molar excess of unlabeled penicillin G. The resulting suspension was centrifuged at 11,000 × g for 20 min, and the pellet was dried under a stream of nitrogen.

Denaturation, Reduction, Carboxymethylation, and Trypsin or Pronase Digestion. The precipitated carboxypeptidase, labeled with penicillin G (160 nmol, 1.1 × 107 dpm) was dissolved in 2 ml of 6 M guanidine-HCl containing 2 mM EDTA, and 0.2 M Tris-HCl (pH 8.2). After 6 mg of diithiothreitol had been added, the solution was incubated under nitrogen at 37°C for 1 hr. Then 20 mg of iodoacetate was added and the solution was incubated under nitrogen and in the dark at room temperature for 1 hr. The solution was dialyzed against distilled water at 4°C during which the carboxypeptidase precipitated. The resulting suspension was lyophilized. After lyophilization, the carboxymethylated carboxypeptidase was dissolved in 3 ml of 0.1 M NH2HCO3 and TPCK-trypsin was added to a final concentration of 10% (wt/wt) relative to the carboxypeptidase. The hydrolysis was carried out at 37°C for 1 hr, and the reaction was stopped by freezing with dry ice-acetone followed by lyophilization. Pronase digestion was performed under the same conditions as trypsin digestion. If longer incubation times were used, much of the bound penicillin was released.
Isolation of Labeled Peptides. Fractionation was first performed on a Sephadex G-50 (fine) column (1.2 × 100 cm) with 0.1 M NH₄HCO₃ as eluent at a flow rate of 10 ml/hr at 4°. Fractions of 2 ml were collected and monitored for both ¹⁴C content (by counting 50 μl aliquots in Aquasol) and peptide content (by measuring A₃₂₀ nm). The fractions comprising the major radioactive peak were pooled and lyophilized. Further purification was achieved by high voltage electrophoresis at pH 6.5 and 3.5 and descending chromatography on Whatman 3MM paper (46 × 57 cm) preswashed with 1% acetic acid. The electrophoresis buffers (all vol/vol) were pyridine/acetic acid/water (50/2/950; pH 6.5) and pyridine/acetic acid/water (1/10/89; pH 3.5) (12), and the electrophoreses were carried out on a flat plate and in a tank, respectively. Chromatography of the peptides was carried out in 1-butanol/acetic acid/}

RESULT

Properties of the penicilloyi-enzyme linkage

Labeled penicillin was bound to the B. subtilis M-alanine carboxypeptidase under conditions that led to complete inhibition of the enzyme (18). After carboxymethylation, there was 0.5 mol of labeled penicillin per mol of enzyme. The penicilloyi-enzyme complex was stable near neutral pH (electrophoresis at pH 6.5 and descending chromatography) but only partially so in basic (Sephadex G-50 chromatography, pH 8–9) or acidic (electrophoresis at pH 3.5) conditions. This might account for the low overall yield of radioactive peptide (typically, 2%, Fig. 1). At pH 8.5 and 37°, the half-life of the penicilloyl-enzyme linkage is 4 hr (S. Hammarström, unpublished). Thus, after preliminary experiments, the trypsin or Pronase digestions were carried out for 1 hr with a high trypsin or Pronase to carboxypeptidase ratio. Finally, during the isolation of the Pronase peptide, the electrophoresis step at pH 3.5 was used last, this
being the harshest condition of the isolation procedure (Fig. 3). At pH 3.5, the penicilloyl moiety itself might decompose; in a companion experiment using \(^{35}\)S penicillin, the radioactivity did not migrate with the peptide at this step (Fig. 2). These properties of the penicilloyl–enzyme linkage are consistent with either an ester or thioester type of bond. However, as will be discussed later, amino acid analysis of the tryptic peptide after performic acid oxidation revealed no cysteic acid (Fig. 2), thus eliminating the possibility of a thioester bond.

Purification and characterization of labeled tryptic peptide

The radioactive peptide present in the tryptic digest of labeled and carboxymethylated CPase was first subjected to gel filtration as described in Materials and Methods. The major radioactive fractions eluted from the Sephadex G-50 column (Fig. 2A) contained a single labeled peptide with an anode mobility of \(-2.5\) cm/hr on electrophoresis at pH 6.5 (30 min; 60 V/cm). Three more radioactive bands with no peptide content were observed at \(-4, +5\), and \(+11\) cm (Fig. 2B) when the \(^{14}\)C label was in the side chain of penicillin, but were absent when the \(^{35}\)S label was in the nucleus. This suggests that these products were degradation products of the penicilloyl moiety (compare refs. 19 and 20). The radioactive peptide was further purified by electrophoresis at pH 3.5 (Fig. 2C) (2 hr; 40 V/cm, mobility of \(-11\) cm/hr) and descending chromatography (\(R_F = 0.56\) with solvent front at 35 cm) (Fig. 2D). The distribution of radioactivity and fluorescamine-positive material is shown in Fig. 2. The peptide obtained was homogeneous as judged by analytical chromatography and had glycine as its sole NH\(_2\)-terminal amino acid. The amino acid composition of the purified tryptic peptide is shown in Table 1.

Purification and characterization of labeled Pronase peptide

The radioactive peptide obtained from Pronase digestion was smaller than the tryptic peptide as judged by its elution profile in Sephadex G-50 chromatography (Fig. 3A). The penicillic acid peak, a discrete peak in the case of the tryptic peptide, was buried under the major radioactive peak in the case of the Pronase peptide. On electrophoresis at pH 6.5 (30 cm; 60 V/cm) (Fig. 3B), the labeled peptide had a mobility of \(-3\) cm/hr and on descending chromatography (Fig. 3C) an \(R_F\) value of 0.82

Table 1. Amino acid composition of labeled tryptic and Pronase peptides

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Tryptic</th>
<th>Pronase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asn*</td>
<td>1.2 (1)</td>
<td>1.0 (1)</td>
</tr>
<tr>
<td>Thr</td>
<td>0.9 (1)</td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>1.4 (1)</td>
<td>1.3 (1)</td>
</tr>
<tr>
<td>Glu</td>
<td>1.2 (1)</td>
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</tr>
<tr>
<td>Pro</td>
<td>0.8 (1)</td>
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<tr>
<td>Gly</td>
<td>2.5 (2)</td>
<td>2.5 (2)</td>
</tr>
<tr>
<td>Ala</td>
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<td>1.2 (1)</td>
</tr>
<tr>
<td>Ile</td>
<td>0.7 (1)</td>
<td>1.4 (1)</td>
</tr>
<tr>
<td>Leu</td>
<td>1.0 (1)</td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>1.0 (1)</td>
<td></td>
</tr>
<tr>
<td>Total residues</td>
<td>11</td>
<td>6</td>
</tr>
</tbody>
</table>

* Based on the electrophoretic mobility of the Pronase peptide (see Discussion).

† The assumed integral values are given in parentheses.

DISCUSSION

Previous studies in this laboratory have indicated that penicillin acts on the susceptible enzymes by binding covalently to them. In most of these studies, D-alanine carboxypeptidase from B. subtilis has been chosen as a model system because of its accessibility. Nevertheless, even in this case, the mode of attachment of penicillin and its spatial relationship to the binding of the substrate is poorly understood. It is likely that the \(\beta\)-lactam bond of penicillin is opened by a nucleophilic attack on the carboxyl group because this is the most reactive part of the penicillin molecule. In a previous report, it was suggested that the nucleophile is an SH group because penicillin bound to D-alanine carboxypeptidase could be released with hydroxylamine or ethanethiol (5). More recently, it was shown that penicillin release by hydroxylamine or ethanethiol was prevented by denaturation of penicilloyl-carboxypeptidase under a variety of conditions (7–9). These reactions are enzymatically catalyzed. Thus, the present evidence does not support a thioester bond between penicillins and D-alanine carboxypeptidase (reviewed in refs. 6 and 9).

The possibility of an ester bond, involving a serine in the enzyme, has also been examined. Surprisingly, the enzyme was insensitive to di-isopropylfluorophosphate [up to 10 mM (N. H. Georgopapadakou, unpublished)], an organophosphorus reagent that generally inhibits serine hydrolases [alkaline phosphatase being a notable exception (21, 22)]. It is also insensitive to phenylmethylsulfonyl fluoride, another, although less potent, inhibitor of several serine hydrolases (23).

The present study indicates the binding of penicillin to D-alanine carboxypeptidase from B. subtilis involves a serine residue (Table 1). Cysteine was excluded on the basis of the
absence of cysteic acid after performic acid oxidation. The oxidation was necessary because unmodified cysteines do not survive acid hydrolysis of peptide bonds whereas the penicilloyl group could be hydrolyzed off rapidly under these conditions. Serine is the only group in the Pronase peptide to which the penicilloyl moiety could be attached. The stability of an acid anhydride of glutamic acid (present in the tryptoic peptide but not in the Pronase peptide) or aspartic acid would preclude its isolation (24), and the electrophoretic mobilities of the tryptoic and Pronase peptides can be accounted for only if the aspartic acid residue is an asparagine one.

It should be emphasized that the fact that penicillin binds to a serine residue does not necessarily imply a serine protease (i.e., a protease with a "superactive" serine). The high reactivity of the β-lactam ring, which early workers have compared to that of an acid chloride (25), might compensate for the lack of reactivity of serine. In this case, the question arises whether the same serine residue is involved in the acyl–enzyme complex formed from substrate (ref. 9; J. W. Kozarich and J. L. Strominger, manuscript submitted for publication), i.e., whether, as has been suggested in the past (1), penicillin acts as a substrate analogue and binds at the same site. This can only be resolved by isolating tryptoic acyl–peptide and comparing it to the penicilloyl–peptide.

During the preparation of this manuscript, the isolation of a penicilloyl–peptide from a soluble D-alanine carboxypeptidase from Streptomyces was reported (26). In that case too, the penicillin was bound to a serine residue. The isolation of penicilloyl–proteins (6) and of these penicilloyl–peptides from both B. subtilis and Streptomyces strongly supports the mechanism of inactivation of penicillin-sensitive proteins originally proposed by Tipper and Strominger (1).

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