Penicillin Is an Active-Site Inhibitor for Four Genera of Bacteria

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The hypothesis that penicillin acts as an active-site inhibitor of cell wall biosynthesis was tested by a method of partial proteolytic mapping of penicillin-binding sites versus substrate-binding sites in cell wall D-alanine carboxypeptidases. This enzyme was obtained from four genera of bacteria, purified, and tested.

Penicillin kills susceptible bacteria by specifically inhibiting the transpeptidase(s) and D-alanine carboxypeptidase(s) that catalyze the final cross-linking step in cell wall peptidoglycan biosynthesis (15). However, there has been disagreement on the chemical details of this inhibition. Although it is known that penicillin and related β-lactam antibiotics bind covalently to a defined set of membrane-bound and exocellular bacterial proteins, including the cell wall carboxypeptidases and transpeptidases, it has been suggested that penicillins bind at an active site (i.e., the substrate-binding site) or, alternatively, at an allosteric site (4, 11). In the case of D-alanine carboxypeptidases from Bacillus stearothermophilus (14) and Bacillus subtilis (12), it was recently shown that penicillin acylates the active-site serine. However, evidence in support of the allosteric model was accumulated from studies of D-alanine carboxypeptidases from several Streptomyces strains (4), raising the possibility that the mechanism of penicillin inhibition is different in different bacteria or with other enzymes. Rigorous proof that penicillin binds covalently to the active site of sensitive enzymes from Bacillus spp. required a large amount of purified protein for the isolation of active-site peptides and the determination of their amino acid sequences. Thus, it was desirable to develop a method that required only small amounts of the enzymes in question and that would facilitate a relatively rapid analysis to determine whether penicillin is bound at the substrate-binding site. This paper describes such a method and its application to penicillin-binding proteins (PBP) (all D-alanine carboxypeptidases) from Streptomyces R61, Escherichia coli, and Staphylococcus aureus, as well as the application of the method to the D-alanine carboxypeptidases from Bacillus spp. studied previously (12, 14).

Most of the PBP that have been isolated as active enzymes have D-alanine carboxypeptidase activity; i.e., they catalyze the removal of the terminal D-alanine from natural and cell wall-related substrates such as UDP-N-acetylmuramyl-L-alanyl-γ-D-glutamyl-meso-2,6-diaminopimelyl-D-alanyl-D-alanine (2). All of these enzymes also catalyze hydrolysis of the terminal D-alanine of the synthetic substrate diacetyl-L-lysyl-D-alanyl-D-alanine (2, 6), and some of them remove D-lactate from the ester analog diacetyl-L-lysyl-D-alanyl-D-lactate (9). Both of these synthetic substrates, labeled with [14C] in the diacetyl portion of the molecule, have been used to trap substrate-derived covalent acyl-enzyme intermediates (6, 9, 14). This was possible because of a rate of acceleration of the acylation step relative to the deacylation step, giving an increase in the steady-state concentration of the covalent intermediate. Thus, these [14C]-labeled substrates can be used to specifically label the active site of D-alanine carboxypeptidases from a variety of bacteria.

In this study, an enzyme labeled with [14C] diacetyl-L-lysyl-D-alanyl-D-lactate ([14C] Ac2-lys-ala-lac), which will be referred to hereafter as the substrate, or [14C] penicillin G, was denatured and digested with CNBr cyanogen bromide staphylococcal protease, or chymotrypsin under conditions that give partial proteolytic cleavage. The partial cleavage products were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the radioactively labeled peptides were detected by fluorography. If the [14C] penicillin G and [14C] Ac2-lys-ala-lac are bound covalently to the same amino acid residue of an enzyme and if the two labels do not alter the mobility of peptides differentially, then an identical pattern of labeled peptides should result. The resolution of this method is limited to the cleavage sites nearest the labeled amino acid residue. With the combination of CNBr, staphylococcal protease, and chymotrypsin (which cleave preferentially after
methionine; aspartic acid and glutamic acid; and phenylalanine, tyrosine, tryptophan, and leucine residues, respectively), typical proteins are cleaved every three to four residues, on the average. Moreover, only serine, threonine, tyrosine, lysine, and cysteine contain functional groups likely to form a covalent penicillin-enzyme or substrate-enzyme complex of the requisite chemical stability. Thus, it is reasonable to expect that distinct binding sites for penicillin and the substrate would be revealed by this method.

This approach was first applied to *B. stearothermophilus*-d-alanine carboxypeptidase, for which it was already known from sequencing studies that penicillin and substrate acylate the same active-site serine. As expected, the partial proteolytic maps for this enzyme labeled with either \([1^{14}C]\)penicillin G or \([1^{14}C]A_{2C}\)-lys-ala-lac were indistinguishable (Fig. 1). A similar proteolytic map for this *B. subtilis* d-alanine carboxypeptidase (data not shown). Thus, the method appears to be valid for comparing penicillin-binding and substrate-binding peptides in two independent cases.

The method was then applied to purified PBP from other bacteria. Of special interest was the *Streptomyces* R61 exocellular d-alanine carboxypeptidase, whose kinetic and spectral properties had been interpreted as suggesting that the penicillin- and substrate-binding sites were different. Labeling of the *Streptomyces* R61 enzyme with \([1^{14}C]\)penicillin G and \([1^{14}C]A_{2C}\)-lys-ala-lac, followed by partial proteolysis and gel electrophoresis, showed indistinguishable patterns of labeled peptides (Fig. 2). Thus, penicillin is an active-site inhibitor for the streptomyceses R61 enzyme, in contradiction with the conclusion of Ghuysen et al. (4).

Membranes of *E. coli* contain at least seven PBP, several of which have been purified. Two, designated PBP 5 and 6, have recently been shown to have d-alanine carboxypeptidase activity (1). PBP 6 was favorable for trapping the \(1^{14}C\)-substrate-derived covalent complex, but it was available only in microgram amounts. Thus, it was well suited for the partial proteolysis mapping technique. Again, \([1^{14}C]\)penicillin and \([1^{14}C]A_{2C}\)-lys-ala-lac gave identical maps when three different methods of cleavage were used (Fig. 3). Incidentally, an adaptation of this technique has been used to demonstrate that PBP 5 and 6 contain primary structures distinct from each other, making it unlikely that PBP 6 is a proteolytic derivative of PBP 5 (1).

Although most recent studies on the mode of action of penicillin have focused on *Streptomyces, Bacillus* spp., and *E. coli*, many of the early studies on the modes of action of \(\beta\)-lactam antibiotics used *Staphylococcus aureus* as a model organism (11, 13). PBP from *Staphylococcus aureus* have been particularly difficult to isolate in large amounts, so structural studies have been scant. Recently, microgram amounts of *Staphylococcus aureus* PBP 4 have been purified. In addition to d-alanine carboxypeptidase activity, this enzyme possesses model
non has PBP active-site residue. at cleavage that ly at and substrate be labeled enzyme catalyze to that bands tional the with tides; i.e., be to enzyme G-labeled case, peptides to generated from the CNBr for G', at trypsin and D', uncleaved; and A', uncleaved; B and B', staphylococcal protease at 20 μg/ml; C and C', staphylococcal protease at 100 μg/ml; D and D', chymotrypsin at 0.08 μg/ml; E and E', chymotrypsin at 0.4 μg/ml; F and F', CNBr for 5 min; G and G', CNBr for 15 min.

transpeptidase and weak penicillinase activity (6). It can be covalently labeled with [14C]penicillin G or [14C]Ac2-lys-ala-lac, so it too was subjected to active site mapping (Fig. 4). In this case, peptides generated from the [14C]penicillin G-labeled enzyme gave a pattern similar but not identical to that of peptides derived from the [14C]Ac2-lys-ala-lac-labeled enzyme in the case of cleavages with CNBr (Fig. 4, lanes 2, 3, 9, and 10) and chymotrypsin (Fig. 4, lanes 4, 5, 11, and 12). The [14C]penicillin G peptides appeared to be a subset of the 14C-substrate–labeled peptides; i.e., several additional labeled bands were present in the latter case. In the case of cleavage with staphylococcal protease (Fig. 4, lanes 6, 7, 13, and 14), some of the bands obtained from the [14C]penicillin G-labeled enzyme were found in the 14C-substrate–labeled enzyme, but the additional bands were particularly prominent. It appears that staphylococcal protease was less able to catalyze hydrolysis of the 14C-substrate–labeled enzyme to completion. These data suggest that penicillin and substrate are bound covalently at the same site but that the covalently bound substrate and penicillin differentially influence cleavage at one or more sites adjacent to the active-site residue. Precedence for this phenomenon has been previously documented; B. subtilis PBP 5 binds [14C]penicillin G, [14C]cefoxitin (a 7α-methoxycephalosporin), and the 14C-substrate in ester linkage to the identical serine residue, but cefoxitin suppresses a pepsin cleavage site which is accessible in the substrate- or penicillin-labeled enzyme (12). Whether this is the correct interpretation will require further study.

Thus, at least four of the five penicillin-sensitive cell wall enzymes that have been tested so far bind penicillin covalently at a site which is indistinguishable from the substrate-binding site. Since enzymes isolated from the genera of

Fig. 2. Partial proteolysis of [14C]penicillin G and [14C]Ac2-lys-ala-lac-labeled Streptomyces R61 d-alanine carboxypeptidase. R61 carboxypeptidase was purchased from UCB Bioproducts, Brussels, Belgium. Labeling and cleavage procedures were as described in the legend to Fig. 1, except that Triton X-100 was omitted from the labeling buffer and [14C]Ac2-lys-ala-lac was used at 10 mM. Lanes A, B, C, D, E, F, and G, [14C]penicillin G labeled; lanes A', B', C', D', E', F', and G', [14C]Ac2-lys-ala-lac labeled. A and A', uncleaved; B and B', staphylococcal protease at 20 μg/ml; C and C', staphylococcal protease at 100 μg/ml; D and D', chymotrypsin at 0.08 μg/ml; E and E', chymotrypsin at 0.4 μg/ml; F and F', CNBr for 5 min; G and G', CNBr for 15 min.

Fig. 3. Partial proteolysis of [14C]penicillin G and [14C]Ac2-lys-ala-lac-labeled E. coli PBP 6. E. coli PBP 6 was purified as described previously (11). Binding of [14C]penicillin G at 30 μg/ml was performed in 80 mM potassium phosphate (pH 7.2)–1% Triton X-100 for 20 min at 25°C. Trapping of [14C]Ac2-lys-ala-lac at 2.1 mM was performed in 0.1 M Tris-hydrochloride (pH 8.5)–1% Triton X-100. Otherwise, procedures were as described in the legend to Fig. 1. A, Gel stained with Coomassie blue; B, fluorograph. Odd-numbered lanes, [14C]penicillin G labeled; even-numbered lanes, [14C]Ac2-lys-ala-lac labeled. Lanes 1 and 2, uncleaved; lanes 3 and 4, CNBr for 3 min; lanes 5 and 6, CNBr for 8 min; lanes 7 and 8, staphylococcal protease at 10 μg/ml; lanes 9 and 10, staphylococcal protease at 50 μg/ml; lanes 11 and 12, chymotrypsin at 1 μg/ml; lanes 13 and 14, chymotrypsin at 2 μg/ml.
bacteria studied in the present experiment (including both gram-negative and gram-positive organisms) appear to be identical in this respect, it is probable that this mechanism of action of penicillin will be true for many enzymes that bind penicillin covalently. The studies presented here and elsewhere (1, 4, 12, 14) have focused on the lower-molecular-weight cell wall carboxypeptidases because they can be purified in active form. Until very recently, the higher-molecular-weight transpeptidases have been refractory to similar studies because they appear to be present in minute amounts in bacterial membranes and because they lose activity upon purification. However, the putative E. coli transpeptidases, PBP 1A and 1Bs have recently been cloned, overproduced, and purified in partially active form (5, 8, 10). The method described in this paper should be useful in examining the general-

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