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Mechanism of penicillin action: Penicillin and substrate bind covalently to the same active site serine in two bacterial D-alanine carboxypeptidases

(β-lactam/peptidoglycan/membrane enzyme/penicillinase/acyl enzyme)

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ABSTRACT **It has been hypothesized that penicillin acts as a structural analog of the acyl-D-alanyl-D-alanine terminus of nascent bacterial cell wall and that it consequently binds to and acylates the active site of the enzyme(s) that crosslinks the cell wall to form an inactive penicilloyl enzyme [Tipper, D. J. & Strominger, J. L. (1965) *Proc. Natl. Acad. Sci. USA* 64, 1133–1138]. This study directly proves that penicillin acylates the active site of two penicillin-sensitive enzymes, D-alanine carboxypeptidases from *Bacillus stearothermophilus* and *Bacillus subtilis*. Active site peptides were generated by chemical or enzymatic cleavage of these carboxypeptidases after covalently labeling with [^{14}C]penicillin G or after trapping an acyl-enzyme intermediate derived from the depsipeptide substrate, [^{14}C]diacetyl-L-lysyl-D-alanyl-D-lactate. The amino acid sequences of the penicillin- and substrate-labeled peptides were identical. Both penicillin and substrate were covalently bound via an ester linkage to the same active site residue, a serine at position 36 of the *B. stearothermophilus* carboxypeptidase and the corresponding serine in the *B. subtilis* carboxypeptidase. The two D-alanine carboxypeptidases showed significant homology around the active site. Moreover, homology between these two enzymes and four β-lactamases of known sequence suggests that these two groups of enzymes are evolutionally related.**

In 1965, Wise and Park (1) and Tipper and Strominger (2) demonstrated that penicillin kills susceptible bacteria by inhibiting the final step of cell wall biosynthesis, namely, the transpeptidation that crosslinks the peptide side chains of peptidoglycan strands. Tipper and Strominger (2) hypothesized that penicillin acts as a structural analog of the carboxy terminal D-alanyl-D-alanine of the pentapeptide side chain in nascent peptidoglycan. It was proposed that the highly reactive β-lactam of penicillin acylates the active site residue of the transpeptidase that catalyzes the crosslinking reaction, with formation of a stable, covalent penicilloyl enzyme. This penicilloyl enzyme would be analogous to an acyl enzyme, which might form as an intermediate during the catalytic reaction. Subsequently, a penicillin-sensitive transpeptidation was demonstrated *in vitro* by using a membrane fraction from *Escherichia coli* (3). A second, related, penicillin-sensitive enzyme, D-alanine carboxypeptidase (CPase), was also described (3).

Several predictions of the model proposed by Tipper and Strominger (2) have been verified experimentally. Proteins that covalently bind penicillin as the penicilloyl moiety (4, 5) were discovered in bacterial membranes. These include both transpeptidases and the related CPases (6, 7). It has been demonstrated that acyl-enzyme intermediates occur (8) and can be trapped (9–11) for several of these penicillin-binding proteins. The finding that the covalently bound penicilloyl moiety is susceptible to an enzymatically catalyzed release either in the presence or absence of added nucleophiles (12–15) clearly supports the hypothesis that penicillin is bound at a catalytically active site. One important prediction of the structural analog hypothesis is that penicillin and substrate both bind to the same active site amino acid residue of the penicillin-sensitive enzymes. An alternative proposal, suggested by Ghuysen and coworkers, predicts that penicillin binds at an allosteric site (16, 17).

Although penicillin-sensitive transpeptidation has now been demonstrated *in vitro* in several systems by using a crude membrane/wall fraction as a source of enzyme, all attempts to purify this transpeptidase have resulted in loss of activity. This could be because (*i*) detergents used to solubilize the enzyme from membranes inhibit enzymatic activity, (*ii*) an appropriate substrate (e.g., uncrosslinked linear peptidoglycan) was not employed, or (*iii*) close proximity of substrate and enzyme is required for transpeptidation and physical disruption of the system causes loss of activity.

Thus, studies of penicillin-sensitive enzymes have focused not on transpeptidases but on CPases, which specifically catalyze hydrolysis of the terminal D-alanine from cell wall-related substrates. Penicillin-sensitive CPases (M_r about 40,000–50,000) have been purified from various bacteria including *Bacillus subtilis, Staphylococcus aureus, Bacillus stearothermophilus, E. coli*, and species of *Streptomyces* (6, 9, 17–19). These enzymes, which often account for the major portion of the penicillin that can be covalently bound to bacterial membranes, can catalyze simple transpeptidation reactions when a nucleophilic amino acceptor (e.g., glycine or hydroxylamine) is supplied at sufficiently high concentration. Although the *in vivo* function of these CPases is unknown, they may function to limit cell wall crosslinking or they might be cell wall transpeptidases that have been "uncoupled" (6) during purification. In any case, they provide a useful model system for studying the inhibition of a cell wall enzyme by penicillin.

The depsipeptide substrate, diacetyl-L-Lys-D-Ala-D-lactate, can be used to trap acyl-enzyme intermediates with penicillin-sensitive CPases from various bacterial species (10). The acyl-enzyme intermediate derived from the CPase of *B. subtilis* was shown to have the same chemical stability as the corresponding penicilloyl enzyme (10, 14), consistent with that of a serine ester linkage (20, 21). This study provides direct evidence that penicillin and substrate acylate the identical amino acid residue, a serine at position 36 of the penicillin-sensitive CPases from two bacterial species, *B. subtilis* and *B. stearothermophilus*, as originally predicted by the Tipper–Strominger hypothesis.

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Abbreviations: CPase, D-alanine carboxypeptidase; CNBr, cyanogen bromide; Tryp, trypsin; Peps, pepsin; Staph, Staphylococcal protease; Pap, papain; -p*, peptide labeled with [^{14}C]penicillin G; -s*, peptide labeled with [^{14}C]diacetyl-L-lysyl-D-alanyl-D-lactate.

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MATERIALS AND METHODS

B. stearothermophilus ATCC 15952 and *B. subtilis* Porton were grown, membranes were isolated, and the CPases were purified by penicillin affinity chromatography, essentially as described (6, 22). [8-^{14}C]Penicillin G (54–56 Ci/mol; 1 Ci = 3.7 × 10^{10} becquerels) was from Amersham and di-[^{14}C]acetyl-L-Lys-D-Ala-D-lactate (2.6–119 Ci/mol) was synthesized as described (10). Manual and automated Edman sequence determination (23), digestion with carboxypeptidases A and B, and peptide composition and quantitation by amino acid analysis after 22–24 hr of acid hydrolysis (22) were as described.

Peptide notation is as follows: Peptides derived from digestion with cyanogen bromide (Eastman), pepsin (Sigma), trypsin (Worthington), Staphylococcal protease (Miles), and papain (Worthington) are designated by CNBr, Peps, Tryp, Staph, and Pap, respectively, followed by identification of the peptide's position in the NH_2-terminal sequence (Fig. 2) by numbers in parentheses. [^{14}C]Penicilloyl- and [^{14}C]diacetyl-L-lysyl-D-alanyl-labeled peptides are further identified by -p* and -s*, respectively.

Peptides Derived from *B. stearothermophilus* CPase. *B. stearothermophilus* CPase (480 nmol at 4 mg/ml) was labeled with a 2-fold molar excess of [^{14}C]penicillin G at pH 7.0 in 0.5 M Na acetate/0.05 M Na phosphate/1 mM EDTA/1 mM dithiothreitol/1% Triton X-100 for 5 min at 37°C. Cold acetone was added to 80%, and the precipitate of penicilloyl enzyme was pelleted at 2000 × *g* for 10 min. Substrate was trapped at the active site of *B. stearothermophilus* CPase (480 nmol at 4 mg/ml) in 5 mM [^{14}C]diacetyl-L-Lys-D-Ala-D-lactate (3 Ci/mol) in 0.5 M Na cacodylate, pH 6.0/1 mM EDTA/1 mM dithiothreitol/1% Triton X-100 at 0°C. Immediately after addition of substrate, cold trichloroacetic acid was added to 20%. The precipitated complex was centrifuged as above and washed once with 5% trichloroacetic acid and once with acetone, yielding 0.85 mol of covalently bound substrate per mol of enzyme.

B. stearothermophilus CPase, labeled with [^{14}C]penicillin G or [^{14}C]diacetyl-L-Lys-D-lactate, was dissolved in 70% formic acid to give 10 mg of protein per ml, and CNBr was added to give 100 mg/ml. The reaction vessel was flushed with N_2 and protected from light, and cleavage was allowed to proceed at 37°C for 3 hr, after which the volatiles were lyophilized.

CNBr fragments of CPase were fractionated on a 1.5 × 15 cm column of SP-Sephadex equilibrated in 8 M deionized urea and 20 mM hydroxylamine·HCl, pH 5.0. Elution was achieved by a 600-ml linear gradient of the same buffer to 50 mM ammonium acetate, pH 5.0.

Peptides Derived from *B. subtilis* CPase. The *B. subtilis* CPase (600 nmol at 3 mg/ml) was labeled by incubation with a 2-fold molar excess of [^{14}C]penicillin G in 0.05 M Tris·HCl, pH 7.5/0.5 M NaCl/1% Triton X-100 for 30 min at 25°C. Substrate was trapped at the active site by incubation of this CPase (300 nmol at 2.2 mg/ml) in 0.1 M Na acetate, pH 5.0/1% Triton X-100 with 7 mM [^{14}C]diacetyl-L-Lys-D-Ala-D-lactate (2.6 Ci/mol) at 0°C, followed by the immediate addition of 50% acetic acid to a final concentration of 10% (vol/vol). About 30% of the substrate was hydrolyzed prior to trapping. [^{14}C]Penicillin- and ^{14}C substrate-labeled enzymes were precipitated with 5 vol of cold acetone and centrifuged at 2000 × *g* for 10 min, and the pellets were dissolved in 2–3 ml of 6 M guanidine HCl/0.1 M HCl over 30 min at 37°C. Guanidine was removed by dialysis against 4 liters of 0.01 M HCl at 4°C, yielding a cloudy solution containing 0.8–0.9 mol of [^{14}C]penicillin or 0.6–0.7 mol of [^{14}C]diacetyl-L-Lys-D-Ala-D-lactate-derived label covalently bound per mol of CPase.

B. subtilis [^{14}C]CPase, 3–4.5 mg/ml in 0.01 M HCl, was digested with pepsin (Sigma) (60–90 μg/ml) for 5 hr at 37°C and lyophilized, and peptides soluble in 0.1 M acetic acid were fractionated by gel filtration on a 1.2 × 200 cm column of Sephadex G-50 (superfine) equilibrated with 0.1 M acetic acid. The [^{14}C]penicilloyl peptic peptide [Peps(24–37)-p*] was further purified by ion exchange chromatography on SP-Sephadex C25, followed by preparative thin layer chromatography on Cellulose (MN 300, Brinkmann) developed with pyridine/butanol/acetic acid/water (10:15:3:2) (R_f = 0.66). [^{14}C]Diacetyl-L-Lys-D-Alanyl peptic peptide [Peps(24–3)-s*] was further purified by SP-Sephadex ion exchange chromatography with a linear gradient of 0.1 M acetic acid to 0.3 M ammonium acetate, followed by ion exchange on a 0.28 × 15 cm column of Beckman AA20 resin at 55°C. The column was eluted with a four-chamber gradient (50 ml per chamber) containing (in order): 0.05 M pyridine acetate (pH 2.4), 0.3 M pyridine acetate (pH 3.75), 0.3 M pyridine acetate (pH 3.75), and 1.2 M pyridine acetate (pH 5.0).

Other Methods. Enzymatic digestions of peptides were carried out in the following buffers: trypsin, 0.2 M ammonium acetate (pH 8.2); Staphylococcal protease, 0.1 M ammonium acetate (pH 8.2); papain, 0.1 M ammonium acetate, pH 5.5/1 mM EDTA/1 mM dithiothreitol. Enzyme and substrate concentrations were 40–50 μg/ml and 400–800 μg/ml, respectively, with digestions proceeding for 30–60 min at 37°C. The resulting peptide mixtures were separated on a 0.9 × 170 cm column of Sephadex G-25 (superfine) equilibrated with 0.1 M acetic acid or by ion exchange on SP-Sephadex equilibrated in 0.1 M acetic acid and eluted with a linear gradient of this buffer to 0.2 M ammonium acetate, pH 5.3 (total volume of 200 ml). [^{14}C]Penicilloic acid was identified by thin-layer chromatography and [^{14}C]diacetyl-L-Lys-D-Ala, by high voltage paper electrophoresis at pH 3.5 (10).

RESULTS

Formation of [^{14}C]Acyl and [^{14}C]Penicilloyl Enzymes. The ester substrate, [^{14}C]diacetyl-L-Lys-D-Ala-D-lactate, has been used to trap acyl-enzyme intermediates with several penicillin-sensitive CPases (10). Incubation of *B. subtilis* CPase with this substrate at pH 5 and 0°C, followed by the immediate addition of acetic acid to 10%, resulted in the most efficient trapping of substrate at the enzyme's active site. Trapping as a function of concentration of [^{14}C]diacetyl-L-Lys-D-Ala-D-lactate (Fig. 1) suggested that a near stoichiometric acyl-enzyme complex could be trapped by using the ester substrate at a concentration equivalent to 10 times the K_m. Acyl enzyme was most efficiently trapped with *B. stearothermophilus* CPase at pH 6 by rapid denaturation with trichloroacetic acid.

Although data from an earlier study suggested that these CPases bind less than a stoichiometric amount of [^{14}C]penicillin (12), it has more recently been shown that both CPases form native complexes which can be isolated by gel filtration. They contain 0.8–1.0 mol of [^{14}C]penicillin covalently bound per mol of enzyme (ref. 22; unpublished results). It is likely that the lower values obtained previously reflect artifacts of the paper chromatographic procedure used to measure penicillin binding. Mild heat or detergent denaturation prevented binding of both [^{14}C]penicillin and [^{14}C]diacetyl-L-Lys-D-Ala-D-lactate, indicating that the binding is enzymatic.

Penicillin- and Substrate-Labeled Peptides of *B. stearothermophilus* CPase. [^{14}C]Penicillin-labeled *B. stearothermophilus* CPase was cleaved with CNBr and a ^{14}C-labeled 40-residue peptide [CNBr(1–40)-p*] was isolated on SP-Sephadex. This peptide was pure as determined by analytical isoelectric focusing (data not shown) and contained 0.71 mol of [^{14}C]penicilloyl label per mol of peptide. Treatment of this labeled peptide at pH 12 for 2 hr at 37°C caused the release of

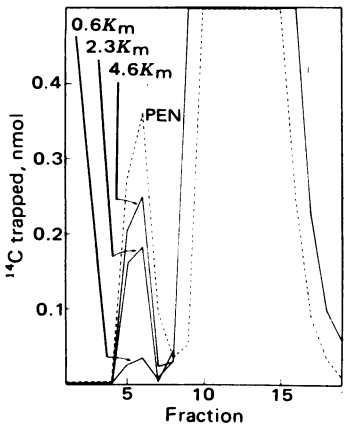

FIG. 1. Dependence of ^{14}C-labeled acyl-enzyme trapping on substrate concentration. *B. subtilis* CPase [0.8 nmol (40 μg) in 0.05 M Na acetate, pH 5.0/0.1% Triton X-100] was incubated with 36 μg of [^{14}C]penicillin per ml for 10 min at 25°C or with various concentrations of [^{14}C]diacetyl-L-Lys-D-Ala-D-lactate at 0°C, after which acetic acid was added to 10% (vol/vol) (see text). [^{14}C]Penicillin- or ^{14}C substrate-labeled CPase was separated from unbound label by gel filtration through a 0.6 × 10 cm column of Sephadex G-50 (superfine) equilibrated in 0.05 M Na acetate, pH 3.0/0.1% Triton X-100 at 4°C. [^{14}C]Penicillin (PEN) was at 54 Ci/mol; [^{14}C]diacetyl-L-Lys-D-Ala-D-lactate was at 109 Ci/mol (0.6 K_m), 42 Ci/mol (2.3 K_m), or 20.7 Ci/mol (4.6 K_m). K_m for substrate = 0.7 mM (10).

[^{14}C]penicilloic acid. The composition of CNBr(1–40)-p* is shown in Table 1 and the sequence is presented in Fig. 2. CNBr(1–40)-p* is the NH_2-terminal CNBr fragment, because the sequence obtained is identical to that of the first 15 amino acids of the intact *B. stearothermophilus* CPase (22). The [^{14}C]penicilloyl label was shown to be located between residues 26 and 40 of this peptide by isolation of a ^{14}C-labeled Staphylococcal protease subfragment, Staph(26–40)-p*, purified on Sephadex G-25 (Table 1 and Fig. 2). Papain cleavage of Staph(26–40)-p*, followed by fractionation of peptides on Sephadex G-25 yielded Pap(34–36)-p*, a tripeptide that contained 0.96 mol of [^{14}C]penicilloyl moiety per mol of peptide (Table 1 and Fig. 2), with an overall yield of 9%. Because serine is the only amino acid with a chemically functional side group in this tripeptide, the penicilloyl moiety must be covalently attached to this residue, serine 36.

B. stearothermophilus CPase was labeled with [^{14}C]diacetyl-L-Lys-D-Ala-D-lactate and cleaved with CNBr to give a peptide, CNBr(1–40)-s* (Fig. 2 and Table 1). This peptide contained 0.79 mol of substrate-derived label and had a composition similar to that of CNBr(1–40)-p* plus one lysine and one alanine contributed by substrate. Treatment of this peptide for 2 hr at 37°C and pH 12 released [^{14}C]diacetyl-L-Lys-D-Ala, as expected. CNBr(1–40)-s* was cleaved with Staphylococcal protease to give Staph(26–40)-s* which had a sequence identical to Staph(26–40)-p*. Staph(26–40)-s* was further cleaved with papain to give Pap(34–36)-s* in an overall yield of 11%. Again, serine was the only functional amino acid. Thus, it was concluded that the [^{14}C]diacetyl-L-Lys-D-Ala moiety was also covalently linked to serine 36 of the CPase.

Penicillin- and Substrate-Labeled Peptides of *B. subtilis* CPase. *B. subtilis* CPase was labeled with [^{14}C]penicillin and cleaved with pepsin, and the [^{14}C]penicilloyl-labeled peptide, Peps (24–37)-p*, was purified by a combination of gel filtration, ion exchange chromatography, and preparative thin-layer chromatography in an overall yield of 20%. The composition of this peptide is shown in Table 2 and its sequence is presented in Fig. 2. Mild base treatment (5% triethylamine for 1 hr at 37°C) quantitatively released the ^{14}C-label as [^{14}C]penicilloic acid. Trypsin cleavage of Peps(24–37)-p* yielded a mixture of peptides which were purified on SP-Sephadex. The ^{14}C-labeled tryptic subpeptide, Tryp(32–37)-p*, had a composition (Table 2) and sequence consistent with that of the COOH-terminal hexapeptide of Peps(24–37)-p* (Fig. 2). Enzymatic digestion of Peps(24–37)-p* with carboxypeptidases A and B removed the terminal methionine without loss of the [^{14}C]penicilloyl label. Because the only remaining functional amino acid of Tryp(32–37)-p* is a serine, it is concluded that the [^{14}C]penicilloyl moiety is covalently bound to the *B. subtilis* CPase via an ester linkage to this serine.

B. subtilis CPase was labeled with [^{14}C]diacetyl-L-Lys-D-

Table 1. Amino acid compositions of [^{14}C]penicilloyl and [^{14}C]diacetyl-L-Lys-D-Ala peptides from *B. stearothermophilus* CPase

	mol/mol peptide						
	CNBr(1–40)-p*	CNBr(1–40)-s*	Staph(26–40)-p*	Staph(26–40)-s*	Pap(34–36)-p*	Pap(34–36)-s*	Pap(34–36)†
Asx	5.12 (5)	4.78 (5)	2.15 (2)	2.00 (2)	0.20	0.13	0.17
Thr	2.88 (3)	2.97 (3)	1.97 (2)	1.77 (2)			0.10
Ser	1.76 (2)	1.70 (2)	0.99 (1)	0.91 (1)	0.95 (1)	0.97 (1)	0.95 (1)
Glx	3.40 (3)	3.23 (3)	0.29	0.32	0.15	0.13	0.11
Pro	0.93 (1)	0.85 (1)					
Gly	2.54 (2)	2.61 (2)	1.58 (1)	1.11 (1)	0.28	0.20	0.27
Ala	5.87 (6)	6.04 (6)	1.22 (1)	1.78 (1)‡	1.06 (1)	2.10 (1)‡	1.10 (1)
Val	2.50 (2)	2.62 (2)	1.16 (1)	1.03 (1)	0.17		
Met	2.29 (2)	2.37 (2)	2.18 (2)	2.34 (2)			
Ile	4.47 (5)	4.19 (5)	2.01 (2)	2.09 (2)	1.00 (1)	1.00 (1)	1.00 (1)
Leu	4.36 (4)	4.49 (4)	1.22 (1)	1.26 (1)	0.11		0.13
Tyr	1.37 (1)	1.35 (1)		0.12			
Phe	0.38 (0)	0.41 (0)					
His	0.29 (0)	0.31 (0)					
Lys	2.59 (3)	2.65 (3)	1.93 (2)	2.99 (2)‡		0.94‡	0.10
Arg	1.65 (1)	1.92 (1)	0.30	0.16		0.19	
Mol label per mol peptide	0.71	0.79	0.55	0.69	0.96	0.98	0.00

Data is shown only for those residues present in excess of 0.1 mol/mol of peptide. The parentheses denote the numbers of residues found in the sequence.

† The last column gives the composition of Pap(34–36)-s* after removal of substrate by treatment at pH 12 for 2 hr at 37°C followed by SP-Sephadex chromatography.

‡ One alanine and one lysine residue found in the composition but not found in the sequence are contributed by the covalently bound [^{14}C]diacetyl-L-Lys-D-Ala moiety. [^{14}C]Penicilloic acid gave no amino acids upon hydrolysis.

Table 2. Amino acid compositions of [^{14}C]penicilloyl and [^{14}C]diacetyl-L-Lys-D-Ala peptides from *B. subtilis* CPase

	mol/mol peptide†				
	Peps(24–37)-p*	Peps(24–37)-s*	Tryp(32–37)-p*	Tryp(32–37)-s*	Tryp(32–37)-s* + base‡
Asx	2.06 (2)	2.03 (2)	0.11	0.13	0.20
Ser	1.72 (2)	1.92 (2)	0.86 (1)	1.03 (1)	1.16 (1)
Glx				0.11	0.30
Pro	0.95 (1)	0.88 (1)	0.94 (1)	1.07 (1)	1.01 (1)
Gly§	0.20	0.18	0.34	0.52	1.51
Ala	2.21 (2)	3.19 (2)¶	0.99 (1)	2.01 (1)¶	1.17 (1)
Met	0.96 (1)	0.79 (1)	0.90 (1)	0.87 (1)	0.67 (1)
Ile	0.98 (1)	0.90 (1)	1.12 (1)	0.96 (1)	0.98 (1)
Leu	1.11 (1)	1.01 (1)	1.05 (1)	0.96 (1)	1.03 (1)
Tyr	1.13 (1)	0.93 (1)			
His	0.18	0.12			
Lys	1.91 (2)	3.09 (2)¶		0.94¶	
Arg	0.94 (1)	1.00 (1)			
Mol label per mol peptide	0.92	0.95	1.03	1.04	0

† Compositions after 22–24 hr of acid hydrolysis. Compositions by sequence analysis are shown in parentheses. Data is only shown for those amino acids present in excess of 0.1 mol/mol of peptide.
‡ Base treatment of Tryp(32–37)-s* (see text) yielded [^{14}C]diacetyl-L-Lys-D-Ala and an unlabeled peptide with the indicated composition.
§ Extent of glycine contamination was variable.
¶ Discrepancies between compositions obtained by acid hydrolysis and those obtained by sequence analysis reflect the Lys and Ala of the substrate-derived [^{14}C]diacetyl-L-Lys-D-Ala moiety. No ninhydrin-positive peaks were detected upon acid hydrolysis of [^{14}C]penicilloic acid.

Ala-D-lactate and digested with pepsin, and the [^{14}C]diacetyl-L-Lys-D-Alanyl-labeled peptide, Peps(24–37)-s*, was purified by gel filtration and ion exchange chromatography in an overall yield of 14%. The composition of Peps(24–37)-s* indicated an additional lysine and alanine when compared to that of Peps(24–37)-p* (Table 2). The sequence of Peps(24–37)-s* was, however, identical to that of Peps(24–37)-p*. Trypsin cleavage of Peps(24–37)-s* yielded the (unlabeled) peptides Tryp(24–26), Tryp(27–30), and Tryp(27–31), identical to those obtained from Peps(24–37)-p* (Fig. 2), and a labeled peptide, Tryp(32–37)-s*, having a composition (Table 2) equivalent to that of Tryp(32–37)-p* plus one alanine and one lysine. Base treatment of Tryp(32–37)-s* (5% triethylamine for 1 hr at 37°C) quantitatively removed the ^{14}C-labeled moiety as diacetyl-L-Lys-D-Ala. The unlabeled peptide was repurified on SP-Sephadex and had a composition identical to that of Tryp(32–37)-p* (Table 2, last column). As with Peps(24–37)-p*, the terminal methionine could be enzymatically removed from Peps(24–37)-s* with retention of the ^{14}C-label. Thus, both the *B. subtilis* penicilloyl-enzyme and acyl-enzyme intermediates derived from substrate contain an acyl (penicilloyl- or diacetyl-L-Lys-D-Ala) moiety covalently linked to a unique serine residue, corresponding to that of serine 36 of the *B. stearothermophilus* CPase. Because the NH_2-terminal 10 amino acids of the two CPases are 50% homologous and properly aligned (unpublished results), it is likely that this active site serine is also located at position 36 in the intact *B. subtilis* CPase.

DISCUSSION

The results presented here establish that one molecule of penicillin or substrate binds per molecule of penicillin-sensitive D-alanine carboxypeptidase. Both penicillin and substrate bind covalently to a single site on these CPases, serine 36. Partial sequences obtained for the CPases studied in this paper (Fig. 2 and unpublished data) indicate strong homology surrounding the active site serine.

Tipper and Strominger (2) predicted that penicillin acylates the catalytically active amino acid residue in susceptible enzymes involved in peptidoglycan crosslinking. This study shows this prediction to be correct for two model enzymes, CPases

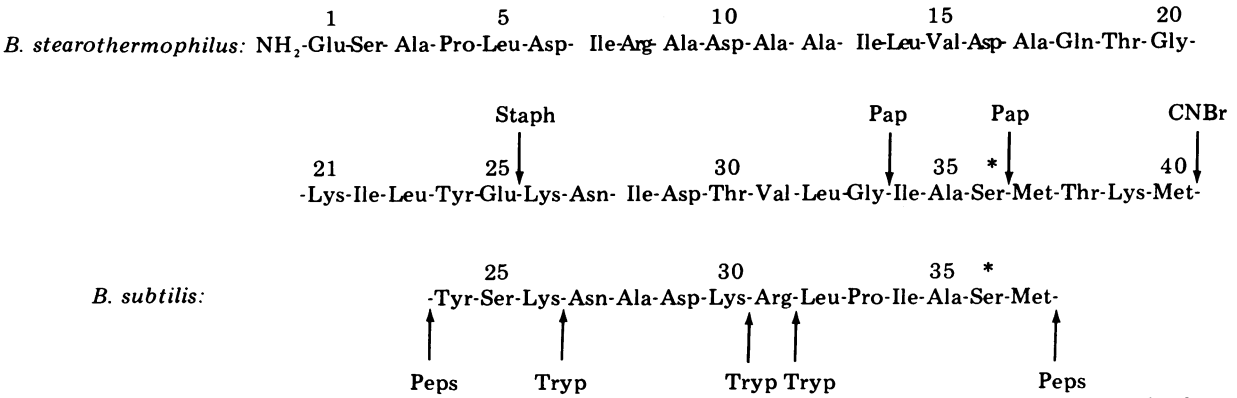

FIG. 2. Active site sequences for *B. stearothermophilus* and *B. subtilis* CPases. *B. stearothermophilus* CPase was labeled with [^{14}C]penicillin or ^{14}C-labeled substrate and cleaved with CNBr, and the ^{14}C-labeled peptide (the NH_2-terminal CNBr peptide, residues 1–40) was purified and the sequence was determined. This peptide was further cleaved with Staphylococcal protease (Staph) and papain (Pap), as shown. Both labels were located at serine 36 (*). *B. subtilis* CPase was similarly labeled with [^{14}C]penicillin or ^{14}C-labeled substrate, denatured, and cleaved with pepsin (Peps). The labeled peptides were purified, sequenced, and further cleaved with trypsin (Tryp) to yield a labeled hexapeptide, Tryp(32–37), (see text) allowing for identification of a serine (*) (equivalent to residue 36 of the *B. stearothermophilus* CPase) as the active site for both penicillin and substrate binding to the *B. subtilis* CPase.

from two bacilli. These data therefore suggest that penicillin, a structural or perhaps transition-state analog (2, 24, 25) of acyl-D-Ala-D-Ala, binds to the active site of susceptible enzymes. The β-lactam of penicillin is then attacked by a nucleophilic serine residue with formation of an ester of penicilloic acid. This ester is relatively stable in the native complex ($t_{1/2}$ = 2–3 hr at 37°C for these CPases) when compared to the ester intermediate which is analogously formed during cleavage of the terminal D-alanine during a transpeptidase or carboxypeptidase reaction. The extremely slow deacylation of the penicilloyl moiety results in continued occupation of the enzyme's active site. Thus, these vital cell wall enzymes are effectively inhibited and are unavailable for catalysis. Cell wall autolytic activity, essential for cell growth and expansion, does, however, continue with the resultant loss of an intact cell wall. The osmotically fragile bacterial membrane then ruptures, causing cell death.

Earlier work had shown that neutral hydroxylamine, H_2O_2, or thiols could reverse penicillin binding to a crude membrane fraction of the *B. subtilis* CPase (5). Because ester and amide bonds would not be susceptible to cleavage under these conditions, it was suggested that penicillin was bound to a cysteine residue of CPase via a thioester linkage. However, it has more recently been established that the hydroxylaminolysis of the bound penicilloyl moiety is an enzymatically catalyzed reaction (14). Thus, the penicilloyl-enzyme bond of the native complex *appeared* to be less stable than would be the analogous ester bond in a noncatalytic system. In fact, additional studies in this laboratory on the chemical stability of penicilloyl-enzyme complexes have shown that the bond has the stability of a serine ester (ref. 14; unpublished results). That the ester linkage was found for both penicillin and substrate using several methods of denaturation and cleavage (data not shown), suggests that this linkage is present in the native complex—i.e., it is unlikely that a base-catalyzed acyl migration occurred during peptide generation and purification.

Thus, CPase appears to be a "serine protease" with high specificity. However, because it is not inhibited by phenylmethylsulfonyl fluoride (18), it probably differs from classical serine proteases, such as chymotrypsin, in some essential features. Because these (ref. 13; unpublished results) and other (26) CPases catalytically (but very slowly) convert [^{14}C]penicillin to [^{14}C]phenacetylglycine rather than [^{14}C]penicilloic acid, the deacylation step for the penicilloyl moiety must be complicated (13, 15). Additional enzymological studies are needed to determine whether the deacylation steps for penicillin and substrate are as similar as the acylation steps.

Many bacteria have developed resistance to penicillins by production of β-lactamases, enzymes that hydrolyze the β-lactam of penicillin to form the bacteriocidally inactive penicilloic acid. Tipper and Strominger (2) also suggested that penicillinases may have evolved from enzymes of cell wall synthesis. If a penicilloyl-enzyme is attacked by water, penicilloic acid is released—i.e., the enzyme catalyzes the hydrolysis of penicillin. The findings that *E. coli* CPase IA has a weak β-lactamase activity (19) and that *S. aureus* PBP 4 catalyzes CPase and β-lactamase activities with equal maximal velocities (9) lend support to this hypothesis. Moreover, significant homology between the two CPases studied here and each of four β-lactamases of known sequence (27) can be seen by aligning serine 36 of the CPases with serine 44 of the *B. cereus* β-lactamase and equivalent serines of the other three β-lactamases (unpublished results). This homology suggests that the β-lactamase serine corresponding to the active site serine of the CPases (i.e., serine 44 of the *B. cereus* β-lactamase) may be involved in β-lactamase catalysis. In fact, during preparation of this manuscript, it was reported that serine 44 of the *B. cereus* β-lactamase is covalently labeled by β-bromo-penicillanic acid (28), an active site-directed β-lactamase inhibitor (29). The sequence homology and occurrence of the same active site serine lends strong support to the idea that penicillin-sensitive CPases and penicillin-inactivating β-lactamases are evolutionally related (2) and suggests that the catalytic mechanisms of these two groups of enzymes might be similar.

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