Proc. Natl. Acad. Sci. USA
Vol. 75, No. 1, pp. 84–88, January 1978
Biochemistry

Utilization of a depsipeptide substrate for trapping acyl–enzyme intermediates of penicillin-sensitive D-alanine carboxypeptidases

(esterase activity/rate acceleration/penicillin binding site)

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Contributed by Jack L. Strominger, October 11, 1977

ABSTRACT **The penicillin-sensitive D-alanine carboxypeptidases of *Bacillus subtilis, Escherichia coli,* and *Staphylococcus aureus* catalyzed the hydrolysis of the D-lactic acid residue from the depsipeptide diacetyl-L-lysyl-D-alanyl-D-lactic acid. The ester substrate was hydrolyzed faster than the peptide analogue, diacetyl-L-lysyl-D-alanyl-D-alanine, by the *B. subtilis* (15-fold) and *E. coli* (4-fold) carboxypeptidases, presumably because acylation (k_2), which is the rate-limiting step of the peptidase reaction, occurred more rapidly during cleavage of the ester bond than during cleavage of the amide bond. No rate acceleration was observed with the *S. aureus* carboxypeptidase for which deacylation (k_3) is already the rate-determining step with the peptide substrate. The efficiency of utilization of the depsipeptide (V_{max}/K_m) was greatly enhanced (19- to 147-fold) for all three enzymes. After incubation of the *B. subtilis* carboxypeptidase and [^{14}C]diacetyl-L-lysyl-D-alanyl-D-lactic acid at pH 5.0 and lowering of the pH to 3.0, a radioactive acyl–enzyme intermediate containing 0.43 mol of substrate per mol of enzyme was isolated by Sephadex G-50 chromatography. After acetone precipitation, the acyl group of the denatured acyl–enzyme complex appeared to be bound to the protein by an ester bond. Acyl enzymes were also detected for the *S. aureus* and *E. coli* carboxypeptidases after sodium dodecyl sulfate/polyacrylamide gel electrophoresis and fluorography of enzyme incubated with [^{14}C]depsipeptide and precipitated with acetone.**

Bacterial D-alanine carboxypeptidases (CPases) catalyze the hydrolysis of the terminal D-alanine from the cell wall precursor UDP-MurNAc-pentapeptide and from synthetic substrates that resemble the uridine nucleotide substrate. CPases from several bacterial species have been purified and found to possess common features. These enzymes are membrane-bound proteins [Gram-negative bacteria often possess a second, soluble CPase activity (1, 2)] with a molecular weight of 40,000–50,000 (1–4). They frequently account for 1–2% of the total membrane protein and constitute the major penicillin-binding component of the bacteria (3, 5). Although β-lactam antibiotics inhibit CPase activity, the enzymes do not appear to be the penicillin killing site (6–8). The CPase of *Gaffkya homari* is a possible exception because its activity appears to be essential for generating a tetrapeptide substrate for transpeptidation (9). In addition to CPase activity, many of the enzymes catalyze transpeptidase and penicillinase reactions. The *in vivo* function of this class of enzyme is uncertain.

The development of methods to prepare large amounts of pure CPase has led to their use as models for the study of the interaction of penicillin with enzymes. In the two cases examined to date, the CPase of *Bacillus subtilis* (10) and of *Streptomyces* (11), penicillin was covalently bound to the enzyme via an ester linkage formed between the acyl group of the hydrolyzed β-lactam ring and a serine residue of the enzyme. Kinetic data (12, *) have suggested that substrate and penicillin bind at the same catalytic site rather than at different sites. A more conclusive answer to this question, however, could be obtained by trapping an acyl–enzyme intermediate from the reaction between a CPase and a suitable substrate, digesting this acyl–enzyme with trypsin, and comparing the acyl–peptide with a similarly obtained penicilloyl–peptide (10).

An acyl–enzyme intermediate has previously been detected in the reaction between the CPase of *Staphylococcus aureus* and [^{14}C]diacetyl-L-lysyl-D-alanyl-D-alanine (diAc-L-Lys-D-Ala-D-Ala) by acetone precipitation of the acyl–enzyme, sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis, and fluorography (12). The relative difficulty in obtaining suitable quantities of the *S. aureus* CPase and the high concentration of substrate ($K_m \approx 100$ mM) that would be required for preparative isolation of acyl–enzyme prompted a search for a CPase that was more amenable to large-scale operations. Unfortunately, trapping experiments with [^{14}C]-diAc-L-Lys-D-Ala-D-Ala and the *Escherichia coli* CPase gave only small amounts of acyl–enzyme* and none was obtained from the *B. subtilis* CPase (unpublished data). These results and other kinetic data (13) suggested that, although the acylation constant, k_2, was larger than the deacylation constant, k_3, for the *S. aureus* CPase and thereby allowed acyl–enzyme to accumulate, k_2 was equal to or smaller than k_3 for the *E. coli* and *B. subtilis* CPases with which little or no acyl–enzyme was observed.

$$E + S \underset{}{\overset{k_s}{\rightleftarrows}} E \cdot S \xrightarrow{k_2} E\text{–}P \xrightarrow{k_3} E + P$$

In many cases in which no intermediate is observed in the reaction between a protease and a peptide substrate, it has been possible to isolate an acyl–enzyme complex by using a more reactive ester substrate (14–16). In anticipation of a similar acceleration of k_2 and accumulation of acyl–enzyme in the reactions between the CPases and ester substrates, the depsipeptide diacetyl-L-lysyl-D-alanyl-D-lactic acid (diAc-L-Lys-D-Ala-D-Lac) has been synthesized. This report describes the kinetics of its interaction with the *B. subtilis, E. coli,* and *S. aureus* CPases and the results of acyl–enzyme trapping experiments using the depsipeptide and these enzymes.

MATERIALS AND METHODS

[8-^{14}C]Penicillin G potassium salt (specific activity, 54 μCi/μmol) and [1-^{14}C]acetic anhydride (specific activity, 122.8 μCi/μmol) were obtained from Amersham/Searle. Cephradine was a gift from E. R. Squibb and Sons. The synthesis of [^{14}C]-

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Abbreviations: CPase, carboxypeptidase; $NaDodSO_4$, sodium dodecyl sulfate.

* S. J. Curtis and J. L. Strominger, unpublished data.

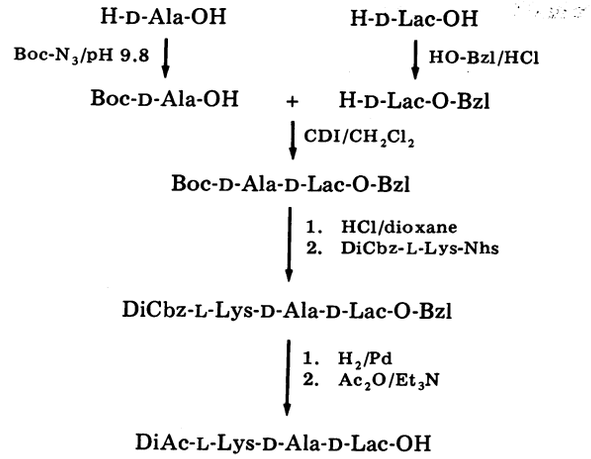

FIG. 1. Synthesis of DiAc-L-Lys-D-Ala-D-Lac. Abbreviations: Boc, *t*-butyloxycarbonyl; Bzl, benzyl; CDI, carbonyldiimidazole; Nhs, *N*-hydroxysuccinimide; Cbz, benzyloxycarbonyl.

diAc-L-Lys-D-Ala-D-Ala has been described (17). Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. Amino acid analysis was conducted on a Beckman model 121M amino acid analyzer. Discontinuous NaDodSO$_4$/polyacrylamide slab gel electrophoresis (18, 19) and gel fluorography (20) were performed according to published procedures (12). High-voltage electrophoresis was with Whatman 3MM paper and water/acetic acid/pyridine, 1000:10:1 (vol/vol) buffer, pH 3.5. Protein concentration was measured by the procedure of Lowry *et al.* (21).

Synthesis of DiAc-L-Lys-D-Ala-D-Lac. The synthesis is outlined in Fig. 1 and will be described in detail elsewhere. Unlabeled depsipeptide was purified by high-voltage paper electrophoresis at pH 3.5 and isolated as a glassy solid, mp 62°–65°. Amino acid analysis gave a molar ratio for alanine/lysine of 1.0/0.9. Calcd for $C_{16}H_{29}N_3O_8$ (monohydrate): C, 49.11; H, 7.46; N, 10.74. Found: C, 47.92; H, 7.29; N, 10.73. [^{14}C]DiAc-L-Lys-D-Ala-D-Lac (specific activity, 122.8 μCi/μmol) was prepared by using [*1*-^{14}C]acetic anhydride (specific activity, 122.8 μCi/μmol). The purified product gave a single radioactive spot after high-voltage electrophoresis (pH 3.5) under conditions that would detect >0.2% radioactive impurity (relative mobilities: diAc-L-Lys-D-Ala-D-Lac, +15 cm; diAc-L-Lys-D-Ala, +7 cm; mono-*N*-α-Ac-L-Lys-D-Ala-D-Lac, −3.5 cm; mono-*N*-ϵ-Ac-L-Lys-D-Ala-D-Lac, −2 cm; and for reference, diAc-L-Lys-D-Ala-D-Ala, +5 cm). *B. subtilis* CPase, which is specific for the D,D configuration, converted >98% of the depsipeptide to a single product that comigrated with an authentic sample of [^{14}C]diAc-L-Lys-D-Ala.

Enzyme Purification. *B. subtilis* CPase (5) and *E. coli* CPase IA (1) were purified to homogeneity by published procedures. *S. aureus* CPase (12) was isolated by affinity chromatography after incubation of solubilized membranes with cephradine (50.0 μg/ml) for 10 min at room temperature (D. J. Waxman, personal communication).

Enzyme Assays and Kinetic Measurements. Enzyme assays of *S. aureus* CPase were performed at 37° in mixtures containing 0.1 M Tris·HCl (pH 7.5), 1% Triton X-100, 0.25 M NaCl, 5 mM $MgCl_2$, and [^{14}C]diAc-L-Lys-D-Ala-D-Lac (300,000 cpm) diluted with unlabeled depsipeptide to the desired concentration and 4 μg of enzyme in a total volume of 50 μl. Assays of *B. subtilis* CPase were performed at 25°in mixtures containing 0.2 M cacodylate (pH 6.0), 1% Triton X-100, [^{14}C]diAc-L-Lys-D-Ala-D-Lac (300,000 cpm) diluted with unlabeled depsipeptide and 1.5 μg of CPase in a volume of 50 μl. The *E. coli* CPase IA assays were conducted at 37° and the assay solution was similar to that used for the *S. aureus* enzyme except that Mg^{2+} was omitted. Reactions were stopped by adding 15-μl aliquots of the assay solution to 4 μl of 0.5 M HCl. The extent of conversion of diAc-L-Lys-D-Ala-D-Lac to diAc-L-Lys-D-Ala was determined by separating the compounds by high-voltage electrophoresis at pH 3.5, locating each by autoradiography, and assaying the radioactivity. K_m and V_{max} values were determined from initial velocity rates, V_0, which were calculated from three points at which less than 15% of the substrate was converted to product. V_0 measurements for each substrate were performed in duplicate.

Table 1. Kinetic constants for CPase reactions

CPase	Substrate*	K_m, mM	V_{max}, μmol/mg/min	Efficiency (V_{max}/K_m)
B. subtilis	R-Lac	0.68	3.0	4.4
	R-Ala	6.7	0.2	0.03
S. aureus	R-Lac	0.94	0.16	0.17
S. aureus†	R-Ala	100	0.24	0.0024
E. coli	R-Lac	4.2	1.6	0.38
	R-Ala	19.2	0.42	0.022

* R = DiAc-L-Lys-D-Ala-D-.
† Taken from ref. 12.

Acyl-Enzyme Detection. Incubation mixtures for acyl-enzyme trapping experiments contained 0.2 M buffer, 1% Triton X-100, 0.9 mM [^{14}C]diAc-L-Lys-D-Ala-D-Lac (specific activity 122.8 μCi/μmol), and 20 μg of enzyme in a total volume of 20 μl. The protein was precipitated with 100 μl of acetone and isolated by centrifugation. The formation of a covalent acyl-enzyme intermediate was demonstrated by the presence of radioactivity associated with the protein band after NaDodSO$_4$/polyacrylamide gel electrophoresis and fluorography.

RESULTS

Kinetic Studies of the Reaction of DiAc-L-Lys-D-Ala-D-Lac with D-Alanine CPases. The *B. subtilis*, *E. coli*, and *S. aureus* CPases catalyzed the hydrolysis of the terminal D-lactic acid from the synthetic substrate diAc-L-Lys-D-Ala-D-Lac. In the presence of an appropriate nucleophile such as glycine or alanine, all three enzymes efficiently transferred the diAc-L-Lys-D-Ala moiety to the amino group of the acceptor. In addition, the *S. aureus* CPase exhibited endopeptidase activity,

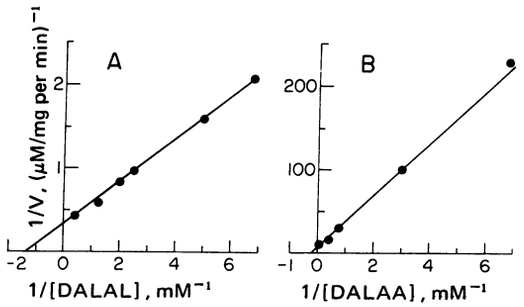

FIG. 2. Lineweaver–Burk plots used to calculate K_m and V_{max} for the reactions between *B. subtilis* CPase and (*A*) DiAc-L-Lys-D-Ala-D-Lac (DALAL) and (*B*) DiAc-L-Lys-D-Ala-D-Ala (DALAA). The intercepts and slopes of the lines were determined by linear regression analysis, and the correlation coefficients were 0.996 (*A*) and 0.994 (*B*).

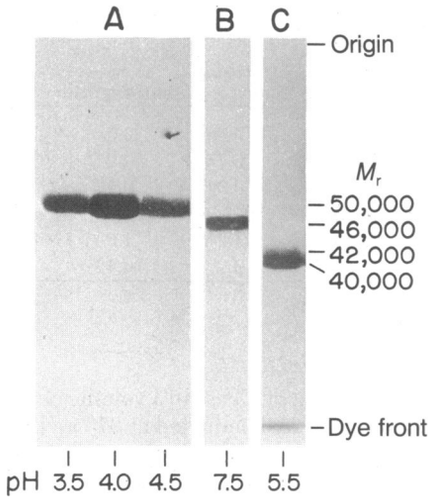

FIG. 3. Fluorograms of $NaDodSO_4$/polyacrylamide slab gels (*A*) *B. subtilis* CPase; (*B*) *S. aureus* CPase; (*C*) *E. coli* CPase IA. Enzymes were incubated with 0.9 mM [^{14}C]diAc-L-Lys-D-Ala-D-Lac. The reaction times and temperatures of the incubation mixtures prior to acetone precipitation, and the exposure times of the films were: (*A*) 0.5 min, 25°, and 15 hr; (*B*) 10 min, 37°, and 42 hr; and (*C*) 2.0 min, 25°, and 20 days. The pH of each incubation is indicated. M_r, molecular weight.

cleaving the ε-acetyl group from the lysine of the depsipeptide. The CPases utilized diAc-L-Lys-D-Ala-D-Lac more efficiently than the alanine analogue (V_{max}/K_m, Table 1). The Lineweaver–Burk plots from which the K_m and V_{max} values for the reactions between the *B. subtilis* CPase and diAc-L-Lys-D-Ala-D-Lac and diAc-L-Lys-D-Ala-D-Ala were calculated are shown in Fig. 2. The depsipeptide had a significantly lower K_m with each enzyme than did the peptide substrate, and it was turned over faster than diAc-L-Lys-D-Ala-D-Ala by the *B. subtilis* (15-fold) and *E. coli* (4-fold) CPases. No rate acceleration was observed with the *S. aureus* CPase but, because deacylation (k_3) is already the rate-limiting step with the alanine substrate (12), none was expected.

Isolation of Acyl–Enzyme Intermediates. Incubation of [^{14}C]diAc-L-Lys-D-Ala-D-Lac with *B. subtilis*, *S. aureus*, or *E. coli* CPase produced a significant accumulation of acyl–enzyme over a wide pH range. Acetone precipitation of the protein yielded a denatured acyl–enzyme complex that was stable on $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 3). After correction for factors of substrate concentration and differences in specific activity, the intensities of the radioactive bands from acyl–enzyme complex were comparable to those obtained from the penicilloyl–enzyme complex, indicating a good yield of acyl–enzyme. It was necessary to expose the *E. coli* CPase product film for a longer time because the concentration of substrate (0.9 mM) was considerably below the K_m for this enzyme (4.2 mM). The efficiency for trapping the *B. subtilis* CPase intermediate was pH dependent with the optimum at pH 4.0.

The stoichiometry of binding for the *B. subtilis* CPase and diAc-L-Lys-D-Ala-D-Lac was determined by isolating the acyl–enzyme complex by gel filtration (Fig. 4). After chromatography on Sephadex G-50 (0.9 cm × 40 cm column), a small peak of radioactivity (50,600 cpm; 9.4 nmol) remained associated with the protein fraction. Assuming a molecular weight of 50,000 for the *B. subtilis* CPase (4), 0.43 mol of substrate was bound per mol of CPase. A ratio of 0.45–0.50 is typically observed for pencillin–CPase binding (10). The reason that a precisely stoichiometric (1:1) complex was not isolated

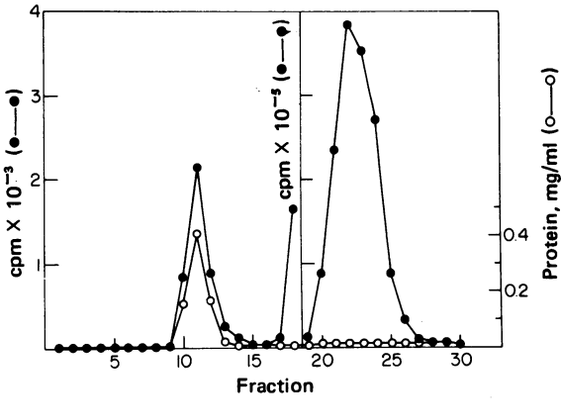

FIG. 4. Gel filtration chromatography of the [^{14}C]diAc-L-Lys-D-Ala- *B. subtilis* CPase intermediate. The enzyme (1.1 mg) was incubated at pH 5.0 for 30 sec with a saturating concentration of diAc-L-Lys-D-Ala-D-Lac (3.0 mM; specific activity 2.45 μCi/μmol) and then the pH was lowered rapidly to 3.0 by addition of 30% acetic acid. Chromatography was performed on Sephadex G-50 at pH 3.0, and 1.25-ml fractions were collected. The fractions were assayed for radioactivity (0.1-ml aliquots) and for protein concentration by the method of Lowry *et al.* (21).

is unclear. Because CPase activity is completely inhibited by penicillin under the incubation conditions, it may be that the enzyme was partially inactivated during its purification (for example, by 0.8 M hydroxylamine during elution from the affinity chromatography column). Alternatively, acetone precipitation may be a relatively inefficient denaturation method, allowing the release of a portion of the label. When enzyme incubation and gel filtration were performed at pH 4.0 without prior acetone precipitation [the optimum pH for acyl–enzyme trapping by acetone precipitation (Fig. 3*A*)], no radioactivity was present in the protein fractions. Because the CPase is enzymatically active at pH 4.0, it is not surprising that deacylation occurred during chromatography.

Chemical Stability of the Acyl–Enzyme Bond. Initial experiments suggested that the acyl group of the denatured *B. subtilis* CPase acylated intermediate may be bound to the enzyme by an ester linkage. The complex was relatively stable at low (pH 3.0) and neutral pH, and largely survived during 5 min of boiling in the $NaDodSO_4$ sample buffer for polyacrylamide gel electrophoresis. Treatment of the denatured acyl–enzyme complex with 0.05 *M* sodium carbonate (pH 11.6) at 37° for 2 hr released >95% of the bound radioactivity as [^{14}C]diAc-L-Lys-D-Ala (identified by mobility after high-voltage paper electrophoresis). Similarly, 1.0 M hydroxylamine, pH 7.5, at 37° effected complete release of the acyl group in 2 hr. The lability of the intermediate under mildly alkaline conditions and its relative stability at low pH are characteristic of a serine ester (12).

Inhibition of Penicilloyl–Enzyme Formation by DiAc-L-Lys-D-Ala-D-Lac. Saturating concentrations of diAc-L-Lys-D-Ala-D-Lac protected the *B. subtilis* CPase from inhibition by penicillin G (Fig. 5). Because penicillin binding is essentially irreversible (k_3 very small) but substrate is constantly being turned over, the conditions of the experiment are important in obtaining the result shown in Fig. 5. Prolonged incubation of the reaction mixture or incubation at high concentrations of penicillin produced equivalent amounts of penicillin binding at all depsipeptide concentrations.

DISCUSSION

Over a decade ago it was proposed that penicillin, acting as a structural analogue of the acyl-D-alanyl-D-alanine moiety found

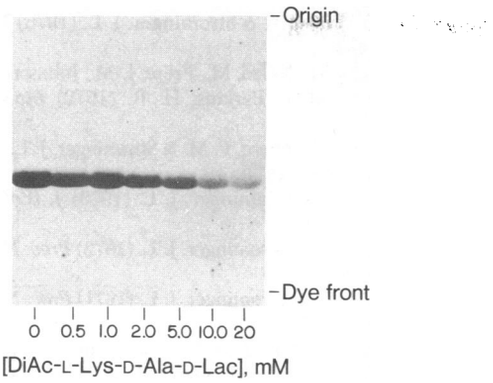

FIG. 5. Fluorogram of a $NaDodSO_4$/polyacrylamide slab gel of *B. subtilis* CPase incubated with [^{14}C]penicillin G in the presence of varying concentrations of unlabeled diAc-L-Lys-D-Ala-D-Lac. CPase (15 μg) was preincubated for 20 sec with diAc-L-Lys-D-Ala-D-Lac in 0.3 M cacodylate buffer, pH 6.0 (total volume = 25 μl). [^{14}C]Penicillin G (5 μl; 0.063 μg/μl; 54 μCi/μmol) was added. After 30 sec, the reaction was diluted with a 250-fold excess of unlabeled penicillin G and the protein was precipitated with 4 volumes of acetone.

in the bacterial peptidoglycan, binds irreversibly at the active site of the transpeptidase that crosslinks the peptide chains of bacterial cell walls (22). According to this hypothesis, the transpeptidase cleaves the peptide bond between the terminal D-alanine residues to form a reactive acyl–enzyme intermediate. The acyl group is then transferred from the enzyme to a free amino residue on an adjacent peptide chain to complete the crosslink. In a similar manner, the reactive β-lactam bond of penicillin, acting as the structural equivalent of the D-alanyl-D-alanine peptide bond, acylates the active site of the enzyme; however, the enzyme is now unable to catalyze the deacylation of the penicillin group, and an inactive penicilloyl–enzyme is produced.

Strong support for this theory has come from studies with bacterial CPases. Like the transpeptidases, this class of enzyme cleaves the D-alanyl-D-alanine peptide bond but transfers the acyl group to water rather than to an amino group. Under appropriate conditions, CPases also catalyze transpeptidase reactions but the possible *in vivo* significance of these transpeptidase reactions is unresolved. As predicted by the model of penicillin action, the inactivation of the CPases of *B. subtilis* (10) and *Streptomyces* (11) occurred with concomitant formation of a covalent penicilloyl–enzyme complex. The postulate that cleavage of the terminal alanine residue from substrate occurs via formation of an acyl–enzyme complex has been substantiated previously in the reactions between the synthetic substrate diAc-L-Lys-D-Ala-D-Ala and the CPases of *S. aureus* (12) and *E. coli*.* In the cases of the *S. aureus* and *B. subtilis* CPases, it has been established that the substrate binds to the enzyme as a diAc-L-Lys-D-Ala moiety, indicating that a nucleophilic residue (probably a serine) has hydrolyzed the D-Ala-D-Ala bond of the substrate. It remains to be demonstrated conclusively that acylation by substrate and by penicillin occurs at the same active site residue. Kinetic evidence from this laboratory suggests that this is indeed the case for the *E. coli* CPase IA.* Incubation of the CPase with sulfhydryl reagents apparently blocks a thiol group of the enzyme near the active site. This enzyme modification effects the rates of acylation and deacylation by substrate and penicillin in an identical manner. A definitive demonstration that both substrate and penicillin bind at the active site would be to isolate and compare the acyl- and penicilloyl–peptide from one of these enzymes.

Isolation of acyl–enzyme on a preparative scale requires a satisfactory method of obtaining the intermediate. In earlier experiments, only a small amount of acyl–enzyme was isolated from the reaction between diAc-L-Lys-D-Ala-D-Ala and *E. coli* CPase IA and none with *B. subtilis* CPase. Good yields of acyl–enzyme were obtained in trapping experiments (12) with the *S. aureus* CPase (and the other penicillin-binding components of this organism), but it is relatively difficult to obtain suitable quantities of this protein and, in addition, the substrate K_m is inconveniently high (100 mM) for large-scale experiments at saturating concentrations of substrate.

Kinetic analysis of the reaction of *B. subtilis* CPase and diAc-L-lys-D-Ala-D-Ala showed that the failure to observe an acyl–enzyme intermediate was a consequence of $k_3 \gg k_2$ (13). Addition of a nucleophile to the reaction mixture increased the overall rate of substrate conversion because the rate of deacylation was enhanced. In many cases this unfavorable k_2/k_3 ratio can be remedied by choosing a substrate that acylates the enzyme more rapidly so that $k_2 > k_3$. Typically a *p*-nitrophenyl ester derivative has been used to "titrate" the active site (14–16). In these cases, "rapid burst" kinetics indicate that k_3 has become rate-limiting (22). Less reactive ester substrates also show large increases in reaction rates over the analogous amide substrates. For example, trypsin hydrolyzes specific ester substrates about 300 times faster than the amide substrates (23). Likewise, the rate of hydrolysis of a compound such as acetyl-L-tyrosine ethyl ester by chymotrypsin is about 850 times faster than its hydrolysis of acetyl-L-tyrosinamide (24).

Anticipating an increase in k_2, the lactyl analogue of diAc-L-Lys-D-Ala-D-Ala was synthesized. The resulting depsipeptide, diAc-L-Lys-D-Ala-D-Lac, was hydrolyzed by *B. subtilis* CPase with a 15-fold rate acceleration over the peptide analogue. Because the hydrolysis of the two substrates results in the same product (diAc-L-Lys-D-Ala), identical acyl–enzyme intermediates are presumably formed and the acceleration is therefore the result of an increased rate of acylation (k_2). The isolation of a nearly stoichiometric acyl–enzyme complex from the reaction of *B. subtilis* CPase and diAc-L-Lys-D-Ala-D-Lac indicates that k_2 is indeed larger than k_3. A 4-fold rate acceleration and a good yield of acyl–enzyme was also observed in the reaction of the *E. coli* CPase IA and the depsipeptide substrate. When corrections are made for the differences in K_m, the amounts of acyl–enzyme from *E. coli* and *B. subtilis* CPases are comparable. No rate acceleration was observed with *S. aureus* CPase with which deacylation was already rate-limiting with the peptide substrate. With all three enzymes, it appears that k_3 is the rate-limiting step in the hydrolysis of the depsipeptide.

The lactyl substrate had a significantly lower K_m with all three enzymes than did the alanyl substrate. Ester analogues in general have lower K_ms than amide substrates because the equation $K_m = K_s(k_3/k_2 + k_3)$ reduces to $K_m = K_s$ for amides ($k_3 > k_2$) and to $K_m = K_s(k_3/k_2)$ for esters ($k_2 > k_3$). For example, in the reaction of CPase A with hippuryl-L-phenylalanine and hippuryl-L-phenyllactic acid, the ester substrate was hydrolyzed 4 times faster and had a K_m value that was smaller by a factor of 40 (25).

The efficiency of acyl–enzyme trapping by acetone precipitation in the reaction between the *B. subtilis* CPase and depsipeptide was pH dependent (Fig. 3A). This was not simply a kinetic effect because similar observations were made when different incubation times were used. Apparently, the rate of deacylation is influenced by an amino acid residue with a $pK_a \approx 4.0$. Deacylation does not stop altogether, however, because the CPase is enzymatically active at pH 4.0 and no acyl–enzyme intermediate can be isolated by gel filtration at pH 4.0. Similar

pH effects have been observed for a number of serine proteases. For example, acyl–enzyme intermediates of chymotrypsin (14), trypsin (15), and subtilisin (16) have been isolated either by incubation at low pH or by lowering the pH after the initial incubation.

An earlier observation that Zn^{2+} enhanced the catalytic activity of the *B. subtilis* CPase toward the substrate UDP-MurNAc-pentapeptide (4) suggested that its mechanism of action might resemble that of CPase A in which transient reaction intermediates have only been detected under optimal conditions (26). Because Zn^{2+} was required in substrate quantities by the CPase, it was also possible that the only function of the metal was to neutralize the negative charges of the UDP phosphate groups. This latter explanation appears to be the case because, in the reactions of the CPase with the substrates diAc-L-Lys-D-Ala-D-Ala and diAc-L-Lys-D-Ala-D-Lac, there was no Zn^{2+} requirement. Because penicillin binds to a serine residue of the enzyme (10), and it appears that the depsipeptide also reacts to form an ester linkage with the enzyme, the *B. subtilis* CPase is probably a serine protease. It is unlikely that its active site will contain the classical serine-histidine-aspartic acid cleavage relay system (27), however, because of its lower pH optimum and its insensitivity to diisopropylphosphofluoridate (10).

In summary, all the results to date support the original hypothesis of the mechanism of penicillin action (22). Previous studies established that penicillin inhibits the *B. subtilis* CPase by binding to a serine residue of the enzyme (10). In the present experiments with *B. subtilis*, *S. aureus*, and *E. coli* CPases we isolated acyl–enzyme intermediates in the reactions between the enzymes and a depsipeptide substrate and presented evidence that suggests that the acyl group of diAc-L-Lys-D-Ala is attached to the *B. subtilis* CPase via an ester linkage. In addition, because the *B. subtilis* CPase activity is inhibited by penicillins, and penicillin binding to the enzyme is retarded by diAc-L-Lys-D-Ala-D-Lac, it appears likely that penicillin and the depsipeptide bind at the same site. Isolation and sequencing of the acyl- and penicilloyl-peptides from the *B. subtilis* CPase may provide direct proof that penicillin acylates the active site.

We thank R. Nochimow for growing the bacterial cells. This work was supported by Research Grants PCM75-16405 from the National Science Foundation and AI 10952 from the National Institutes of Health.

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