Purification to Homogeneity and Properties of Two D-Alanine Carboxypeptidases I from *Escherichia coli*®

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Three homogeneous preparations of D-alanine carboxypeptidases I have been obtained from *Escherichia coli* strain H2143, termed enzymes IA, IB, and IC. Enzyme IA purified from the membrane after extraction with Triton X-100 appeared on sodium dodecyl sulfate gel electrophoresis to be a polypeptide doublet whose monomer molecular weights were about 32,000 and 34,000. In addition to D-alanine carboxypeptidase activity, it catalyzed a transpeptidase reaction with several substrates, bound [14C]penicillin G, had a weak penicillinase activity, but was devoid of endopeptidase activity. Enzyme IB obtained from the membrane after LiCl extraction and enzyme IC obtained from the supernatant solution were either identical or extremely similar. They were composed of a single polypeptide whose monomer molecular weight was about 41,000. In addition to carboxypeptidase activity, they catalyzed an endopeptidase reaction, had weak penicillinase activity, and had very poor transpeptidase activity, but did not bind [14C]penicillin G. Some data relating to the mechanism of catalysis by these enzymes are described. Their possible physiological role is discussed.

A number of penicillin-sensitive enzymatic activities have been identified in various bacterial species. These include transpeptidases, D-alanine carboxypeptidases, and endopeptidases (for a recent review, see Ref. 1). The relationship of the various activities to one another (i.e. whether they are catalyzed by one or more than one protein) and the physiological functions of some of these activities require much further clarification, as does the precise mechanism(s) by which they are inhibited by ß-lactam antibiotics. D-Alanine carboxypeptidases I and II which remove, respectively, the terminal and penultimate D-alanine residues of the uridine nucleotide substrate, UDP-acetyl-muramyl-D-alanine were first described in *Escherichia coli* and partially purified (2). D-Alanine carboxypeptidase I is competitively inhibited by penicillins, and D-alanine carboxypeptidase II was insensitive to ß lactam antibiotics. Subsequently, partially purified preparations of D-alanine carboxypeptidase I were shown to possess both endopeptidase (3) and transpeptidase (4) activity (the latter assayed using both natural and synthetic substrates). However, since none of the preparations studied had been purified to homogeneity, it was difficult to state whether or not the various activities observed were functions of the same enzyme protein. Penicillin-sensitive endopeptidase activities in *E. coli* had also been studied independently of D-alanine carboxypeptidase and transpeptidase (5).

The purpose of the present paper is to report the purification to homogeneity of two distinct proteins from *Escherichia coli* which catalyze D-alanine carboxypeptidase I activity. Some properties of these proteins will also be described and compared to earlier work.

MATERIALS AND METHODS

Substrates

UDP-MurNAc-LAla-DGlu-mesoDap-[14C]nAla-[14C]nAla was prepared as previously described (2, 6) (specific activity, 37.4 × 10⁶ cpm/μmol). UDP-MurNAc-LAla-DGlu-mesoDap-[14C]nAla was prepared by hydrolysis of the above compound with nAla carboxypeptidase I (2). D-[14C]acetyl-L-lysyl-D-alanyl-D-alanine (specific activity, 152 × 10⁶ cpm/μmol) and diacetyl-L-lysyl-D-alanyl-[14C]D-alanine (specific activity, 74.3 × 10⁶ cpm/μmol) were prepared chemically (7). Bis(disaccharide-tetrapeptide), the dimer C-3 (8), used as a substrate for endopeptidase, was prepared after hydrolysis of cell walls of *Escherichia coli* H2143/T1 (see below) grown in the presence of [14C]diaminomitic acid and lysozyme. Its specific activity could not be measured, but the specific activity of the [14C]diaminomitic acid used in its preparation was 10 μCi/μmol. [14C]Penicillin G (38 μCi/μmol) was obtained from Amersham/Searle.

Assays for Enzymatic Activity

**D-Alanine Carboxypeptidase I**—The routine assay was carried out in a reaction mixture containing in a total volume of 25 μl, 1 μl of 1.25 μM Tris-HCl buffer, pH 8.6, 1 μl of 0.5 mM MgCl₂, 1 μl of 25% Triton X-100, 2 μl of UDP-MurNAc-L-Ala-D-Glu-mesoDap-[14C]nAla-[14C]nAla (22,000 cpm, 2.4 μM final concentration) and enzyme. After incubation at 37° for 15 min, the reaction mixture was heated for 5 min in a boiling water bath and then subjected to paper electrophoresis on Whatman 3MM filter paper at pH 3.5 (acetic acid/pyridine/water, 10/1/1000) at 5000 volts for 30 min. Markers of unlabeled alanine were located with ninhydrin, and corresponding areas of the reaction mixtures were cut out and counted. Usually under these conditions alanine migrated to the anode 5 cm and the substrate to the cathode 20 cm.

**D-Alanine Carboxypeptidase II**—The reaction mixture contained in
a total volume of 25 μl, 1 μl of 1.25 M Tris-HCl buffer, pH 8.6, 1 μl of 0.5 M MgCl₂, 2 μl of UDP-MurNAc-L-Ala-γ-glutaryl-meso-DAP-[¹⁴C]p-Ala (1260 cpm) and enzyme. After incubation at 37°C for 1 hour the reaction was stopped by heating in a boiling water bath and analyzed as described above.

**Endopeptidase**—The reaction mixture contained in a final volume of 30 μl, 2 μl of 2 M Tris-HCl buffer, pH 7.5, 3 μl of the E. coli dimer (C-3, 8000 cpm), and enzyme. After incubation at 37°C for 1 hour, 20 μl of isotonic acid/1 n NH₄OH (5/3) were added, and the reaction mixture was subjected to paper chromatography overnight on Whatman 3MM filter paper and the solvent absorbance. The chromatogram was subjected to radioautography, and the area corresponding to the E. coli monomer, C-6 (Rₒ = 0.4, Rₚ of the substrate C-3 = 0.2) was cut out and counted. Alternatively the reaction mixture could be subjected to paper electrophoresis at pH 1.8 (7% formic acid) at 1700 volts for 5 hours, which also separates C-3 and C-6.

**Transpeptidase Activity**—The reaction mixture contained in a final volume of 25 μl, 1 μl of 1.25 M Tris-HCl buffer, pH 8.6, 1 μl of 0.5 M MgCl₂, 1 μl of 25% Triton X-100, 2 μl of unlabeled 15 mM UDP-MurNAc-L-Ala-γ-glutaryl-meso-DAP-p-Ala-p-Ala, 3 μl of 3 mM glucose, and 10 μl of [¹⁴C]glycine (8.9 x 10⁶ cpm, specific activity, 98 μC/mmol). After a 30-minute incubation at 37°C, 400 μl of the reaction mixture was heated in a boiling water bath for 5 min and then subjected to paper electrophoresis on Whatman 3MM filter paper at pH 1.8 (7% formic acid) at 1700 volts for 1.5 hours. Under these conditions [¹⁴C]glycine migrated 30 cm to the anode, while the transpeptidation product migrated 7 cm to the cathode in approximately the same position as the dye, xylene cyanol. Alternatively, when the substrate was di[¹⁴C]acetyl-L-lysyl-o-alanyl-o-alanine, the reaction mixture was the same except that 3 μl of 80 mM acetic acid was employed, and incubation was for 1 hour. Paper electrophoresis was carried out at pH 3.5 at 5000 volts for 3 hours. Under these conditions the relative migrations of various substances to the anode were: diacetyl-L-lysyl-o-alanyl-o-alanine 30.5 cm, diacetyl-L-lysyl-o-alanyl-o-alanyl-cysteine 27.0 cm, and diacetyl-L-lysyl-o-alanyl-o-alanyl-cysteine 21.5 cm. In both cases the paper electrophoreograms were subjected to radioautography for approximately 2 days to locate the appropriate compounds for counting.

In transpeptidase assays with hydroxylamine as acceptor, the reaction mixture contained in a final volume of 25 μl, 1 μl of 1.25 M Tris-HCl buffer, pH 8.6, 1 μl of 0.5 M MgCl₂, 5 μl of UDP-MurNAc-L-Ala-γ-glutaryl-meso-DAP-[¹⁴C]p-Ala-[¹⁴C]p-Ala (55,000 cpm) and 6 μl of 0.5 M hydroxylamine hydrochloride (neutralized with NaOH). After incubation for 8 hours at 37°C the reaction was stopped by heating for 5 min in a boiling water bath and then subjected to paper electrophoresis on 85-cm sheets of Whatman 3MM filter paper at pH 3.5 and 5000 volts for 1 hour. Under these conditions the uridine nucleotide substrate migrated 52 cm, its hydrolysis product 49 cm, and the transpeptidation product 44 cm. A small amount of an additional unidentified compound migrated 57 cm from the anode and the transpeptidation product 44 cm from the anode. The chromatogram was subjected to radioautography on Whatman 3MM filter paper in isobutyric acid/l-NH₄OH (5/3). The protein-bound [¹⁴C]glycine was eluted from the filter paper with 3 ml of 1 N NH₄OH and counted.

**Other Procedures**

Protein was measured by the method of Lowry et al. (10). The cells employed were E. coli strain H2143/T1. It is a previously employed Dap⁻, Lys⁻ auxotroph (11) from which a variant resistant to phage T₁ was isolated. It was grown in Difco antibiotic medium 3 supplemented with 1% yeast extract and 36 μM dl-aminobipicolinic acid. Normally a 150 liter batch culture was grown from which the yield was 250 to 300 grams of cells at mid-log phase.

**RESULTS**

**Purification of D-Alanine Carboxypeptidases I from Escherichia coli**

Cells of Escherichia coli strain H2143/T1 were disrupted in the Ribi cell fractionator. This procedure was employed because it was applicable to large scale preparations under constant conditions. Under these conditions (10,000 p.s.i.) approximately 70 to 75% of the activity was soluble and 25 to 30% was insoluble (defined by centrifugation at 40,000 rpm for 1 hour in the IEC model B 60 ultracentrifuge, using an A1/6 rotor). The latter was presumably located in the membrane fraction. Various procedures were employed in an attempt to solubilize the material in the membrane fraction. Initially, total solubilization could be obtained only by treatment with 2% Triton X-100 and 0.5 M LiCl in 0.02 M Tris-maleate buffer, pH 6.8. Two fractions of enzyme activity could be separated from this solubilized material by chromatography on a column of DEAE-cellulose, one fraction coming through the column (enzyme A) and the other fraction being absorbed onto the column and eluted from it with salt (enzyme B). However, subsequently it was found that enzyme B was only solubilized with LiCl and that the residual material which was then solubilized with Triton X-100 contained mainly enzyme A. This procedure was therefore employed as an initial step in purification, and both enzymes A (soluble in Triton X-100) and B (soluble in LiCl) have been purified to homogeneity. In addition, enzyme C, the o-alanine carboxypeptidase I in the supernatant solution, has been purified to homogeneity but as will be shown, enzyme B and enzyme C appear to be identical.

The details of the purification procedures together with Tables I to III and Figs. 1 to 9 appear in miniprint following the body of this paper.

**Sodium dodecyl sulfate Gel Electrophoresis—o-Alanine carboxypeptidase IA gave a single band on sodium dodecyl sulfate gel electrophoresis on cylindrical gels run in 7.5% acrylamide. However, on sodium dodecyl sulfate slab gels, using a gradient of acrylamide from 5 to 10%, the major band was resolved into two bands at apparent molecular weights around 32,000 and 34,000 (Fig. 10). The significance of this is not clear. It could indicate that the enzyme has two subunits. However, the high resolution of the system is compatible with separation of two apparent polypeptide species with only minor differences, e.g., charge differences due to different extents of amidation. Catalase is also separated into two species in this system.
Purification of E. coli D-Alanine Carboxypeptidases I

Properties of the Purified Enzymes

D-Alanine Carboxypeptidase Activity—Each of these enzymes released only the terminal d-alanine residue from the uridine substrate, UDP-MurNAc-L-Ala-d-Glu-mesoDap-d-Ala-dAla (Fig. 11). D-Alanine carboxypeptidase II had been removed during the purification; no activity on the substrate UDP-MurNAc-L-Ala-d-Glu-mesoDap-[^14C]dAla was detected even after prolonged incubation. In addition, d-alanine carboxypeptidase IA catalyzed the release of the terminal d-alanine residue from UDP-MurNAc-L-Ala-d-Glu-Lys-dAla-dAla (Fig. 12A) and from the synthetic substrate diacetyl-L-lysyl-d-alanine (Fig. 12B). Neither of the latter compounds was a very good substrate, and under the conditions of the experiment in Fig. 12, release of the terminal d-alanine residue was incomplete. Data on the K_m and V_max of these enzymes are reported below.

Endopeptidase Activity—The ability of the three enzymes to catalyze the hydrolysis of the dimer isolated from the cell wall of Escherichia coli (C-3, bis(disaccharide-tetrapeptide)) was examined. This hydrolysis was catalyzed by d-alanine carboxypeptidases IB and IC, but d-alanine carboxypeptidase IA was totally inactive on this substrate (Fig. 13). It should be noted that the hydrolysis did not go to completion. This could be due to impurities in the substrate, inactivation of the enzyme during the reaction, or an equilibrium phenomenon. It has not been examined further.

Transpeptidase Activities—Several reactions which can be termed transpeptidation have been examined. The substrates employed were either UDP-MurNAc-L-Ala-d-Glu-mesoDap-dAla-dAla or diacetyl-L-lys-dAla-dAla, and the acceptors examined in each case were glycine and hydroxylamine.

D-Alanine carboxypeptidases IA, IB, and IC all catalyzed transpeptidation reactions under some conditions. A comparison of the time course of transpeptidation with UDP-MurNAc-L-Ala-d-Glu-mesoDap-dAla-dAla as substrate and glycine as transpeptidation acceptor at a substrate/acceptor ratio of 1/100 is shown in Fig. 14. The amount of enzyme added in each case was such as to catalyze equivalent carboxypeptidase activity on the substrate. It is evident that the extent of transpeptidation under these conditions was greater with d-alanine carboxypeptidase IA than with either d-alanine carboxypeptidase IB or IC.

A detailed examination of transpeptidation reactions catalyzed by d-alanine carboxypeptidase IA was carried out with UDP-MurNAc-L-Ala-d-Glu-mesoDap-dAla-dAla as substrate and glycine as acceptor (Fig. 15A). Under these conditions total "hydrolysis" of the substrate (release of d-alanine) was constant up to a concentration of about 120 mM glycine, and the products were partitioned between the hydrolysis product, UDP-MurNAc-L-Ala-d-Glu-mesoDap-dAla, and the transpepti-
transpeptidation product, UDP-MurNAc-L-Ala-pGlu-mesoDap-dAla-Gly. At concentrations of glycine above 120 mM, there was rate acceleration; at 1.2 M glycine, where the hydrolysis product was totally eliminated, the extent of utilization of substrate for formation of transpeptidation product was 140% of its utilization in the absence of glycine.

A similar result was obtained with diacetyl-L-Lys-d-Ala-d-Ala as substrate. However, in this case the rate acceleration with 1.2 M glycine amounted to 220% of the rate of substrate utilization in the absence of glycine (Fig. 15B).

The data obtained with hydroxylamine as transpeptidation acceptor were less satisfactory (Fig. 15, C and D) because hydroxylamine (whether containing salt or salt-free) was a marked inhibitor of the total reaction. The salt (NaCl, equimolar to NH₂OH) itself produced some inhibition but not nearly as marked as that found with hydroxylamine. From these experiments it was possible to demonstrate only that in the presence of the appropriate substrate small amounts of either UDP-MurNAc-L-Ala-pGlu-mesoDap-dAla-NH₂OH or diacetyl-L-Lys-d-Ala-NH₂OH were formed (Fig. 15, C and D).

A similar result was obtained with d-alanine carboxypeptidase IB using UDP-MurNAc-L-Ala-pGlu-mesoDap-dAla-dAla as substrate and glycine as acceptor, except that much higher concentrations of acceptor were required to form transpeptidation products (Fig. 16). For example, the concentration of glycine required to effect formation of equivalent amounts of hydrolysis and transpeptidation products was 600 to 700 mM with enzyme IB and about 90 mM with enzyme IA. Enzyme IB did not catalyze any hydrolysis of diacetyl-L-lysyl-d-alanyl-d-alanine, and similarly no transpeptidation reaction occurred. The reactions of enzyme IB with hydroxylamine as transpeptidation acceptor, if it occurred at all, would require extremely high concentrations of the acceptor. Although enzyme IB can catalyze transpeptidation reactions, it does so less efficiently than enzyme IA.
Sensitivity of Enzymatic Activities to β-Lactam Antibiotics

Sensitivity—All three enzymes were sensitive to inhibition by various β-lactam antibiotics. The concentrations of several of these antibiotics required to produce 50% inhibition of hydrolysis of UDP-MurNAc-Ala-γ-Glu-mesoDAP-D-Ala-D-Ala by d-alanine carboxypeptidases IA and IB are summarized in Table IV. It should be noted that enzyme IB is markedly more sensitive than enzyme IA to inhibition by these substances.

Binding of [14C]Penicillin G—As shown in the purification procedure, particularly in the elution profiles of the various columns, d-alanine carboxypeptidase IA activity coincided with a peak of [14C]penicillin G binding activity (Fig. 2). On the other hand a second penicillin-binding protein(s) was seen in the initial purification steps in the same region as d-alanine carboxypeptidases IB and IC (Fig. 4); this penicillin-binding profile did not coincide with enzyme activity (Figs. 5 and 7), and during the preparation of enzyme IB and IC it was removed.

The amount of [14C]penicillin G bound by purified enzyme IA was in the range of 50 to 100 cpm/μg of protein, and for enzymes IB and IC it was less than 7 cpm/μg. Sodium dodecyl sulfate gel electrophoresis of enzyme IA was carried out after prebinding of [14C]penicillin G. The gel profile of bound [14C]penicillin G coincided exactly with the Coomassie blue staining material (Fig. 17).

Release of Bound [14C]Penicillin G—Binding of penicillin to E. coli carboxypeptidases had not been observed previously. Moreover, the inhibition of this enzyme activity by penicillins was reported to be reversible on addition of penicillinase (2). A similar situation had been observed earlier in the study of the Bacillus stearothermophilus carboxypeptidases which released an unidentified compound (12).1 Examination of the purification procedure for d-alanine carboxypeptidase IA suggests that this stepwise release of bound radioactive penicillin but released it with a half-time which was short relative to the incubation time ordinarily used in the assay. Because of this prior experience with Bacillus stearothermophilus, the rate of release of bound [14C]penicillin G from E. coli carboxypeptidase IA was examined. It was found to be remarkably rapid at 37° with a half-time of about 5 min. At 4° the half-time was about 1 hour (Fig. 18).

It has recently been reported (12) that both the Bacillus subtilis and Bacillus stearothermophilus carboxypeptidases (the latter at a very slow rate) released bound [14C]penicillin G as a compound which is neither penicillin G nor penicilloic acid. The nature of the material released by the E. coli d-alanine carboxypeptidase IA was examined by incubating enzymes with the [14C]penicillin G overnight, and then subjecting the mixture to thin layer chromatography, using the same technique as had been employed earlier in a study of the product released by the bacillus enzymes. In the present case it was found that [14C]penicilloic acid was apparently the product (Fig. 19), in contrast to the B. subtilis and Bacillus stearothermophilus carboxypeptidases which released an unidentified compound (12). Examination of the purification procedure for d-alanine carboxypeptidase IA suggests that this compound has recently been identified as phenylacetylglycine (20).

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1. The value with UDP-MurNAc-Ala-γ-Glu-mesoDAP-D-Ala-D-Ala by d-alanine carboxypeptidases IA and IB are summarized in Table IV. It should be noted that enzyme IB is markedly more sensitive than enzyme IA to inhibition by these substances.

2. This compound has recently been identified as phenylacetylglycine (20).
pCMB inhibition of carboxypeptidase activities

| Enzyme | Carboxypeptidase | Transpeptidase | Endopeptidase | PcmG (bound) |
|--------|------------------|----------------|--------------|-------------|
|        | Control | pCMB | Control | pCMB | Control | pCMB | Control | pCMB | pCMB | pCMB |
| IA     | 4850    | 240  | 3290    | 27     | 3560    | 3100  | 997     | 2647  |
| IB     | 4490    | 5700 | 1440    | 1140   | 3670    | 3310  |         |       |
| IC     | 4570    | 5560 | 1830    | 1820   | 3670    | 3310  |         |       |

Under these conditions the activity due to p-alanine carboxypeptidase IA was entirely inhibited, and the residual activity was exactly equivalent to the activity of the amount of p-alanine carboxypeptidase IB added in the absence of pCMB.

In addition, pCMB inhibited the transpeptidase activity of enzyme IA. It had no effect on either the transpeptidase or the endopeptidase activity of enzymes IB and IC (Table V). Penicillinase activity of enzyme IA, like its other activities was also inhibited by pCMB (Table VI). Penicillinase activity of enzymes IB and IC was, however, resistant to pCMB. The E. coli penicillinase (free of all carboxypeptidase activity, obtained from the hydroxylapatite column flow through in Fig. 2)
was sensitive to pCMB. Therefore, the penicillinase activity of enzymes IB and IC cannot be due to contamination by the major E. coli penicillinases. The penicillinase-like activity of the B. steatorrhophilus d-alanine carboxypeptidase and the commercial B. cereus penicillinase were both inhibited by pCMB.

pCMB did not inhibit the binding of [14C]penicillin G to d-alanine carboxypeptidase IA, in contrast to its effects on the enzymatic activities of this preparation. In fact the amount of penicillin G apparently bound was 3 times that bound in the absence of pCMB (Table V). These data could suggest that pCMB inhibited the release of [14C]penicillin G from the enzyme, but not its binding. More detailed experiments are being undertaken.

**Effects of Phenylmethanesulfonylfluoride—**Phenylmethanesulfonylfluoride (an inhibitor of serine proteases) at concentrations up to 1 mM had no effect on the activity of either d-alanine carboxypeptidase IA or IB or on [14C]penicillin G binding to d-alanine carboxypeptidase IA. At higher concentrations the inhibition by phenylmethanesulfonylfluoride was equivalent to the inhibition produced by ethanol (the solvent in which phenylmethanesulfonylfluoride is dissolved).

**Other Properties of the Enzymes**

Values for the \( K_m \), \( V_{max} \), pH optimum, and metal ion requirements of the three enzymes are summarized in Tables VII and VIII and Fig. 21. Enzyme IA differs markedly from IB and IC in that it is not activated by metal ions nor inhibited by addition of EDTA. Rather both EDTA and metal ions inhibit it.

**DISCUSSION**

The properties of the three d-alanine carboxypeptidases purified from a strain of *E. coli* are summarized in Table IX. The similarity of d-alanine carboxypeptidases IB and IC and their differentiation from d-alanine carboxypeptidase IA are clearly evident. The physiological role of these d-alanine carboxypeptidases is not clear (for a full discussion, see Ref. 1). Several additional facts need to be considered in relation to a discussion of this problem. It has been shown that the d-alanine carboxypeptidases of *B. subtilis* and *B. steatorrhophilus* are not essential for growth and division of these organisms (13, 14). Moreover, an *E. coli* mutant has been obtained with normal growth physiology in which the d-alanine carboxypeptidase activity is reduced to 25% of the control level (15). This latter mutant has normal transpeptidase activity as measured both in *vivo* and in *vitro* using uridine nucleotide substrates. *E. coli* has been reported to contain six to 10 penicillin binding components (16), and improved techniques have demonstrated that there are in fact six penicillin binding components in *E. coli* (17). One of these binding components may be d-alanine carboxypeptidase IA, but the reactions and role of the others remain to be elucidated. In view of these facts...
Table IX

Summary of properties of Escherichia coli carboxypeptidases

| Carboxypeptidase activity | IA | IB | IC |
|---------------------------|----|----|----|
| Substrate: UDP-MurNAc-Ala-Glu-Dap-Ala-Ala | + | + | + |
| UDP-MurNAc-Ala-Glu-Lys-Ala-Ala | + | - | - |
| Diacetyl-Lys-Ala-Ala | + | - | - |
| Endopeptidase activity | - | + | + |
| Transpeptidase activity | - | + | + |
| Substrate: UDP-MurNAc-Ala-Glu-Dap-Ala-Aly-Gly or NH₂OH | + | - | - |
| DiAcetyl-Lys-Ala-Ala | + | - | - |
| Gly or NH₂OH | - | + | + |
| Penicillinase activity | - | + | + |
| pCMB sensitivity of enzymatic activities | + | + | + |
| [¹⁴C]Penicillin G binding | - | + | + |
| Absorption to DEAE- and CM-cellulose | - | + | + |
| Absorption to hydroxylapatite | - | - | - |

It seems difficult to equate the physiological transpeptidase activity with the *in vitro* "transpeptidation" reactions catalyzed by the *E. coli* carboxypeptidases which have been described in this paper and by other workers (4). Another interesting phenomenon was observed first in 1940 (18). At low penicillin concentrations, cells of *E. coli* are not killed but form long filaments, *i.e.* presumably septation is specifically inhibited. The marked difference in penicillin sensitivities of *E. coli* D-alanine carboxypeptidase IA on the one hand and IB and IC on the other could suggest some relation to the process of filamentation. But again, the multiplicity of other proteins with which penicillin interacts in *E. coli* suggests that the data which are presently available should be interpreted with great caution.

An especially interesting feature of the *E. coli* carboxypeptidases described here is their weak penicillinase activity. Evidence has been presented that this penicillinase activity is an intrinsic property of these enzymes and not due to contamination with the major *E. coli* penicillinase. If further investigation confirms this finding, then these data suggest that the cleavage of the β-lactam ring of penicillin is analogous to the cleavage of the terminal D-alanyl-D-alanine bond of the various substrates for the carboxypeptidases, *i.e.* the data support the idea that penicillins are substrate analogs, and also incidentally the idea that D-alanine carboxypeptidases and penicillinases had a common evolutionary origin (19).

The data obtained may be compared to other published data on this enzyme (4). Early studies from this laboratory on D-alanine carboxypeptidase IA (2) were carried out with another strain, *E. coli* B, and thus some of the differences (*e.g.* precise levels of penicillin sensitivity) may be ascribed to strain differences. The two D-alanine carboxypeptidases reported here (IA and IB; IC) correspond to the two major D-alanine carboxypeptidases reported in a preliminary communication from this laboratory (16). If there are additional minor D-alanine carboxypeptidases and/or endopeptidases (see Fig. 6 in Ref. 16), they will have been discarded in the present purification. Some of the properties of these enzymes correspond to properties reported in a study of the same enzymes from another laboratory (4), for example, their separation by DEAE-cellulose, the differences in their sensitivities to β-lactam antibiotics, and the transpeptidation reaction. However, enzyme IA was devoid of endopeptidase activity in the present study although the analogous Fraction A (4) had considerable activity. It is conceivable that this difference is due to the higher degree of purification obtained in the present study.

Finally, the present paper contains some preliminary information concerning the mechanism of catalysis by these carboxypeptidases. The partitioning of products between hydrolysis and transpeptidation products in the presence of glycine and the marked rate acceleration seen with high levels of glycine strongly suggest the occurrence of an acyl enzyme intermediate in the reaction catalyzed by carboxypeptidase IA. The hydrolysis of this intermediate would be rate limiting and its decomposition would be accelerated by the nucleophile, glycine. It should be compared to more extensive data obtained with the *B. subtilis* and *B. stearothermophilus* carboxypeptidases. Since pCMB inactivates the hydrolysis of both UDP-acetylaminomethyl-pentapeptide and diacetyl-L-lysyl-D-alanyl-D-alanine by carboxypeptidase IA (as well as the transpeptidase and penicillinase activities of carboxypeptidase IA), it seems likely that an —SH group participates in some manner in the reactions catalyzed by this enzyme. pCMB is more effective as an inhibitor with the synthetic substrate, diacetyl-L-lysyl-D-alanyl-D-alanine than with the uridine nucleotide substrate, *i.e.* the natural substrate is more effective in protecting the —SH group from reaction. On the other hand penicillin G binding to D-alanine carboxypeptidase IA is not inhibited at all by pCMB, and in fact the level of binding in the presence of pCMB is 3 times that in its absence. These data could suggest that the presumed acyl enzyme intermediate (of which penicillloyl enzyme may be an analog) is not located on a sulfhydryl group but that a sulfhydryl group is involved in the deacylation reaction. Thus, the penicillloyl enzyme would be stabilized by a substitution of this —SH group. D-Alanine carboxypeptidases IB and IC obviously have a very different catalytic mechanism since they do not bind [¹⁴C]penicillin G and moreover are not inhibited by pCMB. Extension of these preliminary findings to obtain further information about the catalytic mechanism of these enzymes is in progress.

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Purification of E. coli D-Alanine Carboxypeptidases I

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Figure 1 - Purification of D-alanine carboxypeptidases I by chromatography on DEAE-cellulose. Step 30, for details see text. Portions of 4 ml were collected.

Figure 2 - Purification of D-alanine carboxypeptidases I by chromatography on DEAE-cellulose. Step 30, for details see text. Portions of 4 ml were collected.

Table 2. Purification of D-alanine carboxypeptidases I

| Step | Volume (ml) | Total Protein (mg) | Total Activity (nmol HCl per hr) | Yield | Specific Activity (nmol HCl per hr per mg protein) | Fold Purification |
|------|-------------|-------------------|---------------------------------|-------|---------------------------------------------------|------------------|
| 30   | 200         | 18.4 ± 0.5        | 39.1 ± 0.0                      | 20.0±1.0 | 2.0 ± 0.1                                         | 1.0 ± 0.1       |
| 31   | 190         | 17.8 ± 0.5        | 37.5 ± 0.0                      | 20.0±1.0 | 2.0 ± 0.1                                         | 1.0 ± 0.1       |
| 32   | 180         | 16.5 ± 0.5        | 36.0 ± 0.0                      | 20.0±1.0 | 2.0 ± 0.1                                         | 1.0 ± 0.1       |
| 33   | 170         | 15.3 ± 0.5        | 34.5 ± 0.0                      | 20.0±1.0 | 2.0 ± 0.1                                         | 1.0 ± 0.1       |
| 34   | 160         | 14.0 ± 0.5        | 33.0 ± 0.0                      | 20.0±1.0 | 2.0 ± 0.1                                         | 1.0 ± 0.1       |
| 35   | 150         | 12.7 ± 0.5        | 31.5 ± 0.0                      | 20.0±1.0 | 2.0 ± 0.1                                         | 1.0 ± 0.1       |
| 36   | 140         | 11.4 ± 0.5        | 29.0 ± 0.0                      | 20.0±1.0 | 2.0 ± 0.1                                         | 1.0 ± 0.1       |
| 37   | 130         | 10.1 ± 0.5        | 26.5 ± 0.0                      | 20.0±1.0 | 2.0 ± 0.1                                         | 1.0 ± 0.1       |
| 38   | 120         | 8.8 ± 0.5         | 24.0 ± 0.0                      | 20.0±1.0 | 2.0 ± 0.1                                         | 1.0 ± 0.1       |
| 39   | 110         | 7.5 ± 0.5         | 21.5 ± 0.0                      | 20.0±1.0 | 2.0 ± 0.1                                         | 1.0 ± 0.1       |
| 40   | 100         | 6.2 ± 0.5         | 19.0 ± 0.0                      | 20.0±1.0 | 2.0 ± 0.1                                         | 1.0 ± 0.1       |

* About 70% of the total enzyme activity is eluted at Step 30.
Purification to homogeneity and properties of two D-alanine carboxypeptidases I
From Escherichia coli.
T Tamura, Y Imae and J L Strominger

J. Biol. Chem. 1976, 251:414-423.

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