Purification and Characterization of a Carboxypeptidase-
Transpeptidase of *Bacillus megaterium* Acting on the Tetrapeptide
Moiety of the Peptidoglycan*

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The enzyme carboxypeptidase-IIW of *Bacillus megaterium* incorporates free diaminopimelate into purified bacterial walls. This enzyme can be solubilized from toluene-treated cells by LiCl extraction and has now been purified 106-fold to one major band on polycrylamide gel electrophoresis. The enzyme has an apparent molecular weight of approximately 60,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by Sephadex G-100 gel filtration. Carboxypeptidase-IIW requires divalent cations and thiol group(s) for optimal activity. Product analysis indicates that the enzyme can hydrolyze the terminal d-alanine from the tetrapeptide of the peptidoglycan or replace it with a variety of amino acids with D-asymmetric centers for transpeptidation. Substrate specificity studies reveal that the enzymatic activity depends on the presence of N-acetyl-D-glucosamine of the GlcNAc-MurNAc-tetrapeptide. This specificity of carboxypeptidase-IIW for the N-acetyl-D-glucosamine explains in part the affinity of the enzyme for the cell wall of *B. megaterium*. The enzyme is compared to the carboxypeptidases-transpeptidases of other organisms with the similarities and differences discussed.

In 1972, Wickus and Strominger (1, 2) proposed a reaction sequence for the incorporation of free DD- and meso-diaminopimelate into the peptidoglycan synthesized by membrane particles of *Bacillus megaterium* KM. This reaction sequence in the presence of concurrent peptidoglycan synthesis involved the replacement of the terminal d-alanine of the disaccharide-pentapeptide of the peptidoglycan by diaminopimelate. Consequently, n-alanine was released. Some of the incorporated DD-diaminopimelate resulted in a peptide cross-linkage unique to *B. megaterium* (1), whereas the remaining portion of the incorporated DD- and meso-diaminopimelate resulted in no cross-linkage. These authors suggested that this activity was due to enzyme(s) similar to the carboxypeptidase(s)-transpeptidase(s) of other organisms (3).

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‡ Recipient of Research Career Development Award GM 70194 from the National Institute of General Medical Sciences. The abbreviations used are: pentapeptide, L-Ala-D-γ-Glu-meso-Apm-d-Ala-d-Ala; tetrapeptide, L-Ala-D-Glu-meso-Apm-d-Ala; tripeptide, L-Ala-D-Glu-meso-Apm; disaccharide-peptide, GlcNAc-MurNAc pentapeptide, tetrapeptide, and tripeptide in an unknown ratio, Apm, α,ε-diaminopimelate.

Taku et al. (4, 5) have also studied the incorporation of diaminopimelate into the peptidoglycan synthesized by toluene-treated cells and membrane particles of *B. megaterium* 899. They found that toluene-treated cells of *B. megaterium* could utilize the externally added nucleotide precursors UDP-GlcNAc and UDP-MurNAc-Ala-Glu-Meso-Ala-Ala to synthesize peptidoglycan and that these cells could also incorporate free diaminopimelate into the cell wall. Proteins could be extracted from these toluene-treated cells by a high concentration of LiCl. The LiCl-treated cells synthesized peptidoglycan and incorporated free diaminopimelate less efficiently than before the extraction. The loss of these synthetic activities could be restored by adding a crude extract of the LiCl-soluble proteins to the LiCl-treated cells. Of the several extracted proteins, one was purified to electrophoretic homogeneity and was identified as the N-acetylglucosaminyltransferase involved in peptidoglycan synthesis and its addition stimulated this synthesis in the LiCl-treated cells (6, 8).

In this communication, we examine the LiCl-extractable enzyme able to incorporate free diaminopimelate into previously formed cell walls. The diaminopimelate used was a mixture of LL, DD, and meso-isomers. We report here the purification and initial characterization of this enzyme which we demonstrate to be a diaminopimelyl-D-alanyl-carboxypeptidase-transpeptidase. We propose to call this enzyme carboxypeptidase-IIW since it is wall-bound and has a mode of action similar to that of the diaminopimelyl-D-alanyl-carboxypeptidase-II of other organisms, which also removes the terminal alanine from the MurNAc-tetrapeptide of peptidoglycan. In addition, most bacteria possess a D-alanyl-D-alanyl-carboxypeptidase-I which cleaves the terminal alanine from the MurNAc-pentapeptide of peptidoglycan (3).

The study of the mode of action of this enzyme should give additional information about the variety of ways free diaminopimelate can be added to the peptidoglycan of the cell wall. As described below, carboxypeptidase-IIW has both significant differences and similarities with other carboxypeptidases-transpeptidases. Comparisons among all these enzymes will be discussed.

**MATERIALS AND METHODS**

**Bacterial Strains**—*B. megaterium* 899 (7) and *Bacillus subtilis* Marburg (8) have been described previously from our laboratory. *Micrococcus lysodeikticus* ATCC 4698 and *Bacillus cereus* T were obtained from J. S. Anderson, Department of Biochemistry, University of Minnesota. *Escherichia coli* coln ML 308-225 was obtained from J. P. Reeves, Department of Physiology, The University of Texas Health Science Center. *Bacillus licheniformis* 6348 was obtained from C. S. Forsberg, National Institute of Medical Research, Mill Hill, London, United Kingdom.

**Chemicals**—Mixed isomers of LL-, DD-, and meso-[14C]diaminopimelate (0.3 to 2 Ci/mmol) were purchased from Amersham/Searle. Other radioactive amino acids were purchased from Amersham/
Searle or New England Nuclear. Hydroxylapatite (Bio-Gel HTP) and Affigel-10 were obtained from Bio-Rad. Sephadex G-100 was obtained from Pharmacia. Authentic disaccharide-peptide (monomer) was the kind gift of Dr. M. Schwartz of the Max Planck Institute, Tubingen, Germany. Other chemicals were purchased from Sigma Chemical Co. Cell walls and nucleotide precursors, both radioactive and nonradioactive, were included in the accompanying miniprint. 2

Assay for Carboxypeptidase-IIW—Two separate assays were used. The binding assay was particularly useful when the enzyme concentration was low so that a large aliquot was needed for the assay. Various volumes of enzyme solution (up to 100 ml) were mixed with walls purified from 1.5 x 10^6 cells and suspended in 0.7 ml of 0.1 M Tris-HCl, pH 7.4, 0.01 M MgCl2, and 1 mM 2-mercaptoethanol. After 15 min incubation at 0-4 °C, the complex of enzyme bound to walls was centrifuged for 2 min at 12,000 x g. The supernatant solution was then discarded and the pellet was resuspended in an assay incubation of 20 ml containing 0.25 mM [3H]diaminopimelate (0.5 µCi), 0.01 M MgCl2, and 0.15 M Tris-HCl, pH 8.0. At the end of 60 min incubation at 30°C, the reaction mixture was precipitated with 1 ml of 5% trichloroacetic acid. The precipitate was collected on a Gelman (0.45 µm) membrane filter, washed with 10 ml of 5% trichloroacetic acid at room temperature, and counted in a liquid scintillation spectrometer. The direct assay used the same assay incubation and analysis except the final volume was increased from 20 ml and the enzyme was added directly to the reaction mixture without the prior binding step. Again 0.25 mM [3H]diaminopimelate (0.5 µCi) was present in each incubation. The amount of dianminopimelate incorporated by this assay was 2.5 times more than that incorporated by the binding assay. One unit of activity was arbitrarily defined as 1 pmol of diamino-

Purification of Carboxypeptidase-IIW

The enzyme has been purified 106-fold to a single major band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The details of the purification including Figs. 1 to 3 are presented in the accompanying miniprint. In brief, the enzyme was extracted from toluene-treated B. megaterium cells using LiCl. Then the enzyme was purified using hydroxylapatite, Sephadex G-100 gel filtration, and affinity chromatography steps. Before choosing the LiCl extraction of toluene-treated B. megaterium cells as the source of the enzyme, initial studies were performed to determine the best method for extracting the enzyme. When fresh cells were extracted by 1.5 M LiCl with and without prior toluene treatment, it was observed that the enzyme could be extracted without toluene pretreatment. Carboxypeptidase-IIW had a specific activity 8-fold higher in these extracts than in LiCl extracts of cells that were also toluene-treated. However, the yield of the enzyme in these extracts was 5-fold less compared to extracts of toluene-treated cells. Moreover, the proteins in these extracts could not be concentrated by a simple ammonium sulfate precipitation step.

Cells were sonicated to obtain membrane particles and wall preparations as described elsewhere (13). The endogenous content of carboxypeptidase-IIW in cell walls was measured by the incorporation of diaminopimelate into freshly isolated native walls. This incorporation was approximately 3 times higher than that of membranes mixed with purified walls. However, only 4% of the wall-bound enzyme could be extracted by 1.5 M LiCl. This was observed both for native walls with associated endogenous enzyme or purified walls bound with externally added enzyme. The inefficient extraction of carboxypeptidase-IIW from enzyme bound to purified walls suggested that this enzyme was difficult to remove by salt extraction rather than the alternative of the bacteria possessing more than one dianminopimelate-incorporating enzyme.

The facts that no more than 4% of the enzyme located in the walls could be extracted by LiCl and that toluene treatment resulted in 5-fold increase in the yield of enzyme extracted, suggested that carboxypeptidase-IIW extracted by the above method was mainly a membrane-bound enzyme.

The affinity step took advantage of the observation that the enzyme bound to walls and hence was likely to bind to wall fragments. In fact, the enzyme bound to one of the wall fragments obtained by lysozyme digestion, namely the cross-linked disaccharide-peptide (monomer) of the peptidoglycan. Hence, the enzyme was attached to an affinity column containing this monomer. A high salt concentration was used to elute the enzyme because the enzyme could be extracted from the cells and walls using the same conditions (5). The main reason for attaching the enzyme to disaccharide-peptide (monomer) linked to immobile beads rather than undegraded walls was that recovery of the enzyme bound to intact walls was no more than 4%. In addition, intact walls have several other components besides the disaccharide-peptide (monomer) (3). Thus, some proteins besides carboxypeptidase-IIW could have bound to these components, leading to a less specific purification.

Purification Summary

The results of the above four purification steps are summarized in Table I. Minimal loss of activity was observed when the enzyme was maintained at pH 6.5, even though the optimal pH for the assay of the enzyme activity was 8.0.
Hence, sodium phosphate buffer, pH 6.5, was used during the purification whenever feasible. When the final preparation from the affinity chromatography step was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 4), one major and one minor band were observed. The major band had a molecular weight of 60,000 (Fig. 5B). The enzyme from the affinity chromatography step was also applied to a Sephadex G-100 column for molecular weight determination and a value of 56,000 was obtained. The fact that a single component of similar molecular weight was found for the enzyme using both an enzymatic (Fig. 5A) and a protein (Fig. 5B) measurement, indicated that the enzyme was purified close to electrophoretic homogeneity during the three chromatographic steps employed. The similarity in molecular weight suggested that carboxypeptidase-IIW is a monomeric enzyme which does not dissociate into subunits in the presence of sodium dodecyl sulfate and a reducing agent.

**Table I**

Summary of purification of carboxypeptidase-IIW

| Step | Total protein | Total activity | Specific activity | Purification fold | Yield % |
|------|---------------|----------------|-------------------|-------------------|--------|
| Step 1. Concentrated LiCl extract | 911 | 48,100 | 53 | 1.0 | 100 |
| Step 2. Hydroxylapatite chromatography | 32 | 25,300 | 791 | 15 | 52 |
| Step 3. Sephadex G-100 chromatography | 6 | 11,100 | 1,850 | 35 | 23 |
| Step 4. Affinity chromatography | 1.2 | 6,730 | 5,610 | 106 | 14 |

**Properties of Carboxypeptidase-IIW**

**Assay Conditions**—Unless otherwise stated, experiments described below were performed using the partially purified enzyme from Step 2. Its specific activity ranged from 600 to 900 units/mg of protein. The direct assay described under “Materials and Methods” was employed.

The incorporation of diaminopimelate into cell walls by the enzyme was linear with respect to time up to 90 min (Fig. 6A). The assay also was linear with respect to enzyme concentration until a plateau was reached (Fig. 6B). The maximum amount of diaminopimelate that could be incorporated into walls was 1.5 x 10^6 mg of protein. The enzyme concentration being in the range of 10 mM (Fig. 7). Higher concentrations were inhibitory. Table II shows that Mg^2+ ions could be replaced by Mn^2+ or Ca^2+ ions but not by Cu^2+ or Zn^2+ ions.

The presence of Mg^2+ ions stimulated the activity of the enzyme approximately 2- to 2½-fold with the optimal concentration being in the range of 10 mM. This is contrary to the data published earlier which states that the activity is insensitive to heat to some extent (4). Probably the heat sensitivity of the enzyme depends upon the exact inactivation conditions, the buffers used and the degree of purity of the enzyme.

The pH curve for the enzyme assay was determined using various buffers ranging from pH 3 to pH 10. The optimum pH range was 7.5 to 8.2 (Fig. 8).

The incorporation of diaminopimelate into walls could be...
The incorporation of diaminopimelate into walls by carboxypeptidase-IIW approached a maximum at a concentration of 12 mM diaminopimelate in the typical assay mixture (Fig. 9), using enzyme from Step 4 of the purification with specific activity of 6,000 units/mg of protein. In both the direct and the binding assay, a much lower concentration of diaminopimelate (0.25 mM) was used in order to increase the specific radioactivity of [3H]diaminopimelate and hence the sensitivity of the assay. Since the amount of diaminopimelate incorporated by the enzyme at these suboptimal concentrations of substrate was very low (23 pmol in Fig. 9) in the direct and the binding assay, the possibility existed that the compound incorporated into walls was not diaminopimelate but rather some contaminant. This possibility was ruled out by analysis of the walls which had incorporated [3H]diaminopimelate. These walls were hydrolyzed in 4 N HCl for 18 h after complete removal of all unused substrates by centrifugation and washing. The hydrolyzed material was subjected to paper chromatography in isobutyric acid:1 M NH₄OH (5:3, v/v) for 18 h and the region of the paper corresponding to diaminopimelate was cut out and counted. All of the radioactivity incorporated saturated by an excess of walls. The plateau was reached at walls obtained from 1.5 x 10⁶ cells when the direct assay with 0.25 mM diaminopimelate and 35 μg of partially purified enzyme from Step 2 was used. The total hexosamine content of this amount of walls was determined following acid hydrolysis (14), and was found to be approximately 40 nmol. The $K_m$ value for walls could not be determined, in part because the walls are large insoluble complexes with the enzyme being bound to these complexes. Additionally, only one of the components of the wall, the disaccharide-tetrapeptide (monomer) was used as the substrate (see below). The amount of this component in the walls was not known.

**Table II**

| Divalent cations | [3H]Diaminopimelate incorporated (cpm) |
|------------------|--------------------------------------|
| None             | 364                                  |
| MgCl₂            | 885                                  |
| MnCl₂            | 837                                  |
| CuCl₂            | 167                                  |
| ZnCl₂            | 154                                  |
| CaCl₂            | 692                                  |

Partially purified enzyme from Step 2 (37 μg) was assayed by the direct assay except that MgCl₂ was omitted and replaced by 10 mM of the divalent cations shown.

**Fig. 6.** Dependence of carboxypeptidase-II assay on time and enzyme concentration. *Frame A,* partially purified enzyme from Step 2 (32 μg) was assayed by the direct assay as a function of time. *Frame B,* partially purified enzyme was assayed by the direct assay as a function of enzyme concentration.

**Fig. 7.** Effect of magnesium ion concentration on the incorporation of diaminopimelate into purified walls by carboxypeptidase-IIW. Partially purified enzyme from Step 2 (25 μg) was assayed by the direct assay at various magnesium ion concentrations.

**Fig. 8.** Dependence of carboxypeptidase-IIW assay on pH. The various buffers used were: O, sodium citrate; D, sodium cacodylate; ●, sodium phosphate; ■, Tris/maleate; and A, glycine/NaOH. Partially purified carboxypeptidase-IIW from Step 2 (37 μg) was assayed by the direct assay at various pH values in buffers at 0.15 M.

**Fig. 9.** Dependence of the carboxypeptidase-IIW assay on diaminopimelate concentration. Purified enzyme from Step 4 (2.5 μg, 16 units) was assayed with various concentrations of diaminopimelate by the direct assay except that 1.0 μCi of [3H]diaminopimelate was used instead of 0.5 μCi. *Inset,* Lineweaver-Burk plot of the same data.
into walls by the enzyme was found in the diaminopimelate position. From the Lineweaver-Burk plot of the result of Fig. 9 (inset), the K_m value for diaminopimelate was about 2 mM. The turnover number for carboxypeptidase-IIW for diaminopimelate incorporation was 0.075 mol/mol/min from this figure. However, the K_m is a maximum estimate and the turnover number is a minimum estimate because the diaminopimelate concentration curve was constructed using only the concentration of walls that were purified from 1.5 x 10^9 cells. Besides, as discussed below, the enzyme has a hydrolytic activity perhaps more efficient than the diaminopimelate-incorporating activity.

**Effect of Sulphydryl Inhibitors on Carboxypeptidase-IIW**—
Treatment of the enzyme with either 3 mM N-ethylmaleimide or 3 mM iodoacetamide resulted in the loss of approximately 60% of the activity while one-tenth this concentration of inhibitors had no substantial effect. Dithiothreitol and 2-mercaptoethanol at concentrations of 0.6 mM and 2.0 mM, respectively, stimulated the activity of the enzyme to 141% and 139% of the value without the reducing agent (Table III). Hence, during all purification procedures 1 mM 2-mercaptoethanol was included. The inactivation of the enzyme by sulphydryl inhibitors appeared to be partially reversible. Pretreatment with N-ethylmaleimide did not prevent subsequent stimulation by dithiothreitol, although full stimulation was not achieved. These observations suggested the requirement of free sulfhydryl group(s) for optimal activity of the enzyme.

**Alanine Release**—Since carboxypeptidase-IIW can bind to walls and incorporate diaminopimelate in the absence of an exogenous energy source, it was surmised that a peptide bond was being broken to obtain energy necessary for the incorporation of diaminopimelate. Other enzymes of this nature have already been reported (see review by Blumberg and Strominger (33)). To test this possibility, cell walls radioactively labeled in the d-alanine positions of the peptidoglycan were incubated with the enzyme. Upon chromatography of the reaction mixture, free radioactive alanine was observed to be released (described more fully below and in Table IV). When the same experiment was carried out using cell walls labeled with radioactive diaminopimelate at the third position of the pentapeptide of the peptidoglycan, no free radioactive amino acid or small peptide was released (data not shown). This

**TABLE III**

| Treatment                      | Per cent relative activity |
|--------------------------------|---------------------------|
| None                           | 100                       |
| 2-Mercaptoethanol, 0.6 mM       | 139                       |
| 2-Mercaptoethanol, 0.06 mM      | 91                        |
| Dithiothreitol, 2.0 mM          | 141                       |
| Dithiothreitol, 0.2 mM          | 102                       |
| N-Ethylmaleimide, 3.0 mM        | 32                        |
| N-Ethylmaleimide, 0.3 mM        | 80                        |
| Iodoacetamide, 3.0 mM           | 29                        |
| Iodoacetamide, 0.3 mM           | 95                        |
| N-Ethylmaleimide, 3.0 mM, and dithiothreitol, 2.0 mM | 87                        |
| N-Ethylmaleimide, 0.3 mM, and dithiothreitol, 0.2 mM | 83                        |

**TABLE IV**

| Substrate                        | Concentration (mM) | [14C]Alanine released | % input |
|----------------------------------|--------------------|-----------------------|--------|
| Substrates with pentapeptide only|                    |                       |        |
| UDP-MurNAc-pentapeptide          | 0.08               | 0.43                  | 24     |
| MurNAc-pentapeptide              | 0.16               | 0.00                  | 00     |
| Peptidoglycan made in vitro with penicillin | 0.03 | 0.50                | 17     |
| GlcNAc-MurNAc-pentapeptide       | 0.08               | 0.00                  | 00     |
| Substrates with tetrapeptide     |                    |                       |        |
| UDP-MurNAc-tetrapeptide          | 0.08               | 0.37                  | 72     |
| MurNAc-tetrapeptide              | 0.23               | 0.00                  | 00     |
| Peptidoglycan made in vitro without penicillin | 0.06 | 9.50               | 80     |
| GlcNAc-MurNAc-pentapeptide + GlcNAc-MurNAc-tetrapeptide | 0.18 | 7.30                 | 63     |
| Cell walls                       | -                  | 5.30                  | 10     |

*Carboxypeptidase-I of E. coli could remove 50% of the [14C]-alanine from this substrate (see "Materials and Methods").

* In monomer equivalents.

* Membrane particles of E. coli containing carboxypeptidase-II could remove essentially all the [14C]-alanine from this substrate.

* Prepared as a mixture from peptidoglycan made in vitro without penicillin.

* Walls from 1.4 x 10^9 cells were used in the incubation.

suggested that the tripeptide of the peptide moiety of peptidoglycan was not used as a substrate by the enzyme. The fact that only free alanine was released suggested that the enzyme has a hydrolytic activity on the tetrapeptide or the pentapeptide containing 1 or 2 d-alanine residues, respectively. Since carboxypeptidase-IIW also incorporated diaminopimelate, this amino acid must be replacing the terminal or the subterminal d-alanine of the peptide moiety if the enzyme acted as a transpeptidase.

**Transpeptidation in Organic Solvents**—The alanine release experiments described above indicate that the enzyme has a hydrolytic as well as transpeptidase activity. That both activities are due to the same enzyme was demonstrated by an experiment of a type described by Zeiger et al. (15). In a nonaqueous environment where the hydrolysis is limited by the absence of water molecules, the release of d-alanine should be dependent on the presence of diaminopimelate. Therefore, cell walls labeled with ^14C at the d-alanine positions were incubated with carboxypeptidase-IIW in varying concentrations of a mixture of aqueous and nonaqueous solvents. The amount of [14C]alanine released and [3H]diaminopimelate incorporated was measured. The diaminopimelate-incorporating activity of the enzyme decreased with increasing proportions of the nonaqueous solvent. At 55% H_2O, the activity of carboxypeptidase-IIW was 14% of that of 100% H_2O (data not shown). Fig. 10 shows that within the limits of discernible activity of the enzyme, the hydrolytic activity is partially dependent on the presence of diaminopimelate. Even in the presence of H_2O alone, slightly more alanine was released in the presence of diaminopimelate than in its absence. Complete dependence of hydrolytic activity on the presence of diaminopimelate was not obtained at any of the conditions used and may require complete removal of the water from the
assay. Moreover, the enzyme was progressively inactivated as the H₂O is replaced. Therefore, it was difficult to study alanine release in the absence of H₂O.

These observations support the conclusion that carboxypeptidase-IIW is capable of carrying out both the alanine release in the absence of HzO. In this respect it resembles the carboxypeptidases of other organisms studied (3).

**Substrate Specificity of Carboxypeptidase-IIW**—Various compounds generally used as substrates for carboxypeptidases were tested for their suitability as substrates for carboxypeptidase-IIW (Table IV). The enzyme released very little alanine from UDP-MurNAc-pentapeptide, MurNAc-pentapeptide, or disaccharide-pentapeptide. The enzyme also did not hydolyze peptidoglycan made in the presence of 100 μg/ml of penicillin G which contains uncross-linked polymer with predominantly disaccharide-pentapeptide units (9). All the above compounds are generally substrates used by carboxypeptidases-I (3, 9, 16–18). The enzyme also failed to remove alanine from UDP-MurNAc-tetrapeptide and MurNAc-tetrapeptide, which are generally substrates used by carboxypeptidases-II (19, 20). Therefore, the enzyme is dissimilar to the previously reported carboxypeptidases-I and -II. Moreover, carboxypeptidase-IIW failed to demonstrate any endopeptidase activity as evidenced by its failure to convert bis(disaccharide-peptide) (cross-linked dimer, see “Materials and Methods”) to disaccharide-peptide (monomer) (data not shown). However, the enzyme was able to use as substrate, cell walls, peptidoglycan made in vitro, and disaccharide-peptide (monomer). Fig. 2 shows the release of [¹⁴C]alanine by the enzyme on incubating the enzyme with a monomer preparation of GlcNAc-MurNAc-tripeptide-D-[¹⁴C]Ala-D-[¹⁴C]Ala and GlcNAc-MurNAc-tripeptide-D-[¹⁴C]Ala. Under several conditions, Frere et al. (21) were able to demonstrate cross-linking activity of disaccharide-transpeptidase of Streptomyces R61. However, our enzyme failed to cross-link under similar conditions.

**Product Characterization**—The disaccharide-peptide (monomer) used in Table IV and Fig. 11 were made from peptidoglycan synthesized in vitro from UDP-MurNAc-tripeptide-D-[¹⁴C]Ala-D-[¹⁴C]Ala. The radioactive monomeric material was a mixture of disaccharide-tetrapeptide and disaccharide-pentapeptide in an unknown ratio. It is technically difficult to separate these two kinds of monomers cleanly. But if the disaccharide-tetrapeptide is the actual substrate for carboxypeptidase-IIW and if there is a replacement of its terminal D-alanine by diaminopimelate, then the disaccharide-tetrapeptide of the composition, GlcNAc-MurNAc-tripeptide-D-[¹⁴C]Ala should yield a product GlcNAc-MurNAc-tripeptide-[TH]₂Apm when the substrate is incubated with the enzyme and free [¹⁴H]diaminopimelate. This product should therefore contain [¹⁴H]diaminopimelate and no D-[¹⁴C]alanine. This same mechanism predicts no reaction product containing both [¹⁴H]diaminopimelate and D-[¹⁴C]alanine. This will not be true for other reaction schemes where the disaccharide-pentapeptide, GlcNAc-MurNAc-tripeptide-D-[¹⁴C]Ala-D-[¹⁴C]Ala is converted to GlcNAc-MurNAc-tripeptide-p-[¹⁴C]Ala-[¹⁴H]Apm, with the product containing both [¹⁴H]diaminopimelate and D-[¹⁴C]alanine. These two reaction schemes are the most plausible ones where diaminopimelate incorporation is accompanied by the release of D-alanine. To distinguish between these possibilities, disaccharide-peptide labeled with [¹⁴C]alanine at the terminal positions were incubated with carboxypeptidase-IIW and [¹⁴H]diaminopimelate. At the end of the incubation period, the reaction mixture was boiled and the products of the reaction were analyzed. The first step involved chromatography on a Dowex 1-formate column to remove the salts in the reaction mixture. These salts would have interfered with subsequent electrophoresis. The Dowex column was then eluted with increasing concentrations of ammonium formate. The bulk of the unused diaminopimelate eluted first. These fractions which contained [¹⁴H] but had no trace of [¹⁴C] radioactivity were discarded. The unused disaccharide-peptide eluted later and the reaction product containing [¹⁴H]diaminopimelate eluted after that by virtue of the extra carboxyl group on the incorporated diaminopimelate. However, the two kinds of monomers were poorly separated. The radioactive eluate consisting of unreacted disaccharide-peptide monomers, the product of carboxypeptidase-IIW activity and the remaining unused diaminopimelate not separated by the Dowex fractionation, were combined and lyophilized to remove the formate completely. This material was subjected to paper electrophoresis at pH 3.5 whereby the reaction product was well separated from the two substrates, namely disaccharide-peptide and any residual diaminopimelate.

That disaccharide-tetrapeptide was the substrate for carboxypeptidase-IIW was demonstrated by the data in Fig. 12A.
The reaction product (Peak I) in fact had $^3$H and no $^{14}$C as predicted, and also migrated toward the anode 5 cm less than the unused disaccharide-peptide it should be nearer the cathode since the reaction product containing $[^3$H]diaminopimelate and $[^14$C]alanine positions. The enzyme was insensitive to penicillin even at this very high concentration. This penicillin resistance is in close parallel to the penicillin insensitivity of the carboxypeptidase-II of other organisms that use the tetrapeptide moiety and in contrast to the penicillin sensitivity of carboxypeptidases-I that use the tetrapeptide moiety are normally penicillin-insensitive. Therefore, the penicillin sensitivity of carboxypeptidase-IIW was examined.

**Pencillin Insensitivity of Carboxypeptidase-IIW**—The studies described so far indicated that the enzyme acted on the tetrapeptide moiety of the peptidoglycan. In other organisms, carboxypeptidase-IIW that act on the tetrapeptide moiety are normally penicillin-insensitive. Therefore, the penicillin sensitivity of carboxypeptidase-IIW was examined.

Carboxypeptidase-IIW was incubated with cell walls and with peptidoglycan labeled at the $\alpha$-alanine positions in the presence of 500 µg/ml of penicillin G. As seen in Table V, the enzyme was insensitive to penicillin even at this very high concentration. This penicillin resistance is in close parallel to the penicillin insensitivity of the carboxypeptidase-IIW of other organisms that use the tetrapeptide moiety and in contrast to the penicillin sensitivity of carboxypeptidases-I that use the pentapeptide moiety. Taku et al. (4, 5) also observed the penicillin insensitivity of diaminopimelate incorporation into preformed cell walls of toluene-treated and membrane particles of *B. megaterium*.

**Incorporation of Various Amino Acids by Carboxypeptidase-IIW**—Since carboxypeptidases-transpeptidases in general can incorporate a variety of $\alpha$-amino acids, the enzyme

**TABLE V**

| Substrate                      | $[^{14}$C]Alanine released |
|--------------------------------|---------------------------|
|                                | + Penicillin | - Penicillin |
| Peptidoglycan made *in vitro*  | 4.8          | 5.3          |
| Cell walls                     | 1.7          | 1.9          |

**TABLE VI**

| Amino Acid | Amount incorporated (nmol) |
|------------|---------------------------|
|            | µmol                      |
| D-Alanine  | 8.2                       |
| L-Alanine  | 11                        |
| Glycine    | 30                        |
| L-Leucine  | 11                        |
| L-Proline  | 24                        |
| D-Lysine   | 15                        |
| D-Serine   | 5.2                       |
| DD-, LL-, meso-diaminopimelate | 11                     |
| No competitive amino acid      | 33                       |

*Competition for incorporation of diaminopimelate into cell walls by other amino acids. Enzyme (32 units) was incubated with 0.25 mm $[^{14}$C]diaminopimelate (1 µCi) and 12.0 mm various amino acids in the diaminopimelate incorporation assay.

*Incorporation of various amino acids by carboxypeptidase-IIW into cell walls. Enzyme (15.6 units) was incubated with 2.5 nmol of various radioactive amino acids (1 µCi) under the condition of diaminopimelate incorporation assay.
was tested for its ability to incorporate amino acids besides diaminopimelate into walls. Competition experiments revealed that many D-amino acids could compete with diaminopimelate for incorporation into walls but none of the L-amino acids tested were active (Table VI). The incorporation of radioactive diaminopimelate does not decrease in proportion to the competing nonradioactive diaminopimelate because the assay used a suboptimal amino acid concentration. Therefore, more total diaminopimelate was incorporated when the concentration of this amino acid was increased (Fig. 9). This same phenomenon might explain the high residual diaminopimelate incorporation when other D-amino acids were used in the competition. Besides the ones listed in this table, other L-amino acids tested were histidine, lysine, methionine, serine, glutamic acid, threonine, and valine.

Table VI also shows the direct incorporation of some of the amino acids besides diaminopimelate into the walls. The results are in agreement with those of the competition experiment. From these data, we presume that of the three isomers found in the racemic mixture of diaminopimelate, only the D- or the meso-diaminopimelate, or both, are incorporated into walls while the LL isomer is not.

**Effect of Carboxypeptidase-IIW on Walls Purified from Various Bacteria**—It was not known whether disaccharide-peptide was the only component in the wall that was necessary for optimal enzymatic activity. Therefore, purified walls from various sources of bacteria were incubated with the enzyme and the incorporation of diaminopimelate was measured (Table VII). Of these bacteria, only *M. lysodeikticus* does not have meso-diaminopimelate in the third position of the peptide moiety (22). Walls from all the bacterial species tested have the same disaccharide-tetrapeptide but they differ otherwise in their cell wall composition (22-25). The fact that the enzyme failed to incorporate diaminopimelate into these walls, with the possible exception of *B. cereus*, suggested that other components besides the disaccharide-tetrapeptide can affect carboxypeptidase-IIW activity. However, since the amount of uncross-linked disaccharide tetrapeptide was not determined for the various bacteria tested, it is conceivable that the differences in carboxypeptidase-IIW activity might have been due to variations in the quantity of this substrate.

**DISCUSSION**

In this communication we report the purification and characterization of the enzyme carboxypeptidase-IIW that incorporates diaminopimelate into purified cell walls of *B. megaterium* 899 in the absence of an exogenous energy source. This enzyme, purified 106-fold, has a molecular weight of 56,000 by Sephadex G-100 filtration and 60,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It probably requires sulfhydryl group(s) for optimal activity and also has a requirement for divalent cations. The $K_v$ value for diaminopimelate has a maximum value of 2 mM (Fig. 9).

Our data showed that purified carboxypeptidase-IIW could incorporate diaminopimelate into previously formed cell wall and could release alanine. The release of alanine was partially dependent on the presence of diaminopimelate especially when the water in the assay was partially replaced. Substrate specificity studies indicated, among other things, the requirement of the tetrapeptide moiety of the peptidoglycan for enzymatic activity. Product analysis studies indicated that diaminopimelate replaces the terminal D-alanine of the disaccharide-tetrapeptide. On the basis of these observations, we propose the mode of action of carboxypeptidase-IIW (Product A', Fig. 13).

This enzyme activity is different from the diaminopimelate-incorporating activity in the membrane particles of *B. megaterium* KM as described by Wickus and Strominger (1, 2) (Fig. 13). The latter activity is penicillin-sensitive as would be expected from the substrate being the pentapeptide of peptidoglycan (3). Carboxypeptidase-IIW, on the other hand, is penicillin-insensitive similar to carboxypeptidase-II of other organisms that act on disaccharide-tetrapeptide (19). Moreover, the diaminopimelate-incorporating activity by the membrane particles has been reported to be dependent on concurrent pep tidoglycan synthesis (1). Nevertheless, the diaminopimelate-incorporating activity of carboxypeptidase-IIW is independent of such synthesis (4).

Available evidence suggests that the diaminopimelate incorporation by membrane particles is due to carboxypeptidase-IIW in addition to the penicillin-sensitive activity described above. For example, Wickus and Strominger have noted that 13% of the diaminopimelate incorporated into peptidoglycan was independent of peptidoglycan synthesis (1). This activity could well have been due to carboxypeptidase-IIW. The finding that most of the diaminopimelate-incorporating activity was dependent on peptidoglycan synthesis suggests that carboxypeptidase-IIW activity contributed only a small portion of all the diaminopimelate incorporated by membrane particles. In agreement with this possibility, Taku et al. (4, 5) have observed that toluene-treated cells and membrane particles of *B. megaterium* 899 have 3-fold higher diaminopimelate-incorporating activity in presence of peptidoglycan synthesis than in its absence. The relatively low activity of carboxypeptidase-IIW in the membranes might explain why Wickus and Strominger did not observe the Product A' of Fig. 13 in their chemical analyses of the reaction product of diaminopimelate incorporation into peptidoglycan. Moreover, Products A and A' might have been difficult to resolve using the solvent and the chromatographic systems they employed.

In being penicillin-insensitive and in having a substrate specificity for the tetrapeptide moiety of the peptidoglycan, carboxypeptidase-IIW is similar to other carboxypeptidasess-II. However, carboxypeptidase-IIW is also significantly different from carboxypeptidases-II of other organisms. Whereas the carboxypeptidases of *E. coli* (19) have no other specificity besides the tetrapeptide moiety of the peptidoglycan, our enzyme requires the GlcNAc moiety of the peptidoglycan for activity. That this enzyme differs from carboxypeptidase-II is evident from the fact that membrane particles of *B. megaterium* also have a carboxypeptidase-II that is similar to the carboxypeptidase-II of *E. coli* (data not shown). The specificity of carboxypeptidase-IIW for the GlcNAc moiety could explain in part the high affinity of this enzyme for the cell wall. However, this enzyme fails to utilize walls derived from other organisms that contain meso-diaminopimelate in their peptidoglycan and therefore have the disaccharide tetrapeptide of composition identical with that of *B. megaterium* as one of their wall components. This suggests that other wall...
FIG. 13. Mode of action of enzymes adding free diaminopimelate to peptidoglycan. The scheme giving Product $A'$ is from experiments in this communication. The scheme giving Products $A$ and $B$ is from Wickus and Strominger (1).

associated materials such as teichoic acids may affect carboxypeptidase-IIW activity. Further evidence suggesting that this enzyme binds to walls by recognizing more than just the disaccharide-peptide (monomer) is seen in the affinity chromatography step of the purification of the enzyme. The enzyme could be dissociated from disaccharide-peptide (monomer) by 1.0 M NaCl, yet no more than 4% of the wall-bound enzyme could be dissociated by 1.5 M LiCl.

Since carboxypeptidase-IIW can attach diaminopimelate to a peptide side chain in vitro, it functions as a transpeptidase. No such transpeptidation has been reported for any of the carboxypeptidases-II of other organisms studied. Moreover, the enzyme under study is capable of using diaminopimelate as an acceptor, an amino acid that is a natural acceptor of transpeptidation in this organism. Localization experiments suggested that though the enzyme is found both in the membranes and in the walls, the endogenous activity of the enzyme is higher in the walls than in the membranes. From these facts, it is conceivable that this enzyme might also act as a cross-linking enzyme in vivo. However, a variety of attempts to demonstrate cross-linkage by the enzyme have failed (data not shown).

Carboxypeptidase-IIW is similar in many but not all respects to other carboxypeptidases-transpeptidases, especially the carboxypeptidases-II reported previously. These enzymes function much more efficiently in hydrolysis than in transpeptidation (17). Comparison of the amount of alanine released from walls purified from $1.5 \times 10^8$ cells and diaminopimelate incorporated by the same amount of walls by an identical amount of enzyme show this to be true for carboxypeptidase-IIW also. In that case carboxypeptidase-IIW could explain the presence of some of the disaccharide-tripeptide commonly found in large quantities in $B. megaterium$ cell walls (29). It can be calculated that there are approximately 5 nmol of disaccharide-tripeptide present in the walls derived from $1.5 \times 10^8$ cells. This amount of cells has 1.8 pg of carboxypeptidase-IIW, which under the experimental conditions employed can release 3 to 4 nmol of alanine from the above amount of cell walls. Thus the carboxypeptidase-IIW could well account for all the disaccharide-tripeptide present in the cell. However, these disaccharide-tripeptides can also arise from the activity of a carboxypeptidase-II similar to those of other bacteria. The presence of carboxypeptidase-II and of disaccharide-tripeptide has been noted in a variety of bacteria (26-28). It has been suggested that in $E. coli$ the cleavage of the tetrapeptide might control the peptidoglycan lipoprotein cross-linkage and the association of the outer membrane with the peptidoglycan during septum formation (29, 30).

It has been noted that 15% of the diaminopimelate in the $B. megaterium$ cell walls is of the $D$ isomer (25, 31). The meso-diaminopimelate found in the third position of the peptide moiety of the peptidoglycan is incorporated into the peptidoglycan precursor UDP-MurNAc-pentapeptide in the cytoplasm. How DD-diaminopimelate is incorporated into cell wall is not known. However, since carboxypeptidase-IIW can recognize the $D$-asymmetric center of an amino acid and since this enzyme can incorporate diaminopimelate, it is conceivable that this enzyme is at least partially responsible for the
existence of D-diaminopimelate in the B. megaterium cell wall. How this Ddiaminopimelate would be made available outside the cell wall in the living organism is not known. Even the need for the D-diaminopimelate is obscure.

Although the physiological role of carboxypeptidase-IIW is not clear, the facts that the enzyme will only function after wall synthesis is completed and does not use the wall precursor, UDP-MurNAc-tetrapeptide suggest that it is a wall-modifying enzyme.

Since carboxypeptidase-IIW can remove the terminal D-alanine from the disaccharide-tetrapeptide portion of the peptidoglycan with the simultaneous replacement of D-alanine with diaminopimelate or a variety of other D-amino acids or can remove the D-alanine by hydrolysis, it is conceivable that this enzyme can also be assayed by its ability to release D-alanine or by its capacity to incorporate D-amino acids besides diaminopimelate into the cell wall. Carboxypeptidase-IIW was assayed using its diaminopimelate transpeptidase activity rather than its hydrolitic activity because the incorporation could be analyzed simply by filtering the walls after the incubation. An assay of the enzyme by its hydrolitic activity would entail a more time-consuming chromatographic assay.

Therefore, the assay of carboxypeptidase-IIW by its hydrolitic activity would entail a more time-consuming chromatographic assay.

Although the carboxypeptidases of Bacillus steatorhombus (17, 32) and E. coli (20) also exhibit transpeptidase activities, only a few D-amino acids can act as acceptors, notably D-serine, D-alanine, and glycine. However, diaminopimelate cannot act as an acceptor, at least in the case of B. steatorhombus (17, 32). The similarities between various Bacillus species suggested that besides carboxypeptidase-IIW, B. megaterium might also have enzyme(s) like the carboxypeptidases of the other organisms. In this case, the use of D-amino acids besides diaminopimelate might have led to the co-purification of these enzyme(s) along with carboxypeptidase-IIW. During the purification we obtained no evidence that this enzyme activity was due to more than one enzyme, when [3H]diaminopimelate was used in the assay. Therefore, the use of this amino acid led to the specific purification of carboxypeptidase-IIW.

The inability of the carboxypeptidase of B. steatorhombus to use diaminopimelate as an amino acid acceptor in a transpeptidation reaction has led to the speculation (17) that this enzyme does not function in vivo as the transpeptidase which generates the typical peptide cross-bridges with D-alanine linked to meso-diaminopimelate. In this case, the transpeptidation in vitro is presumed to be an artifact with the B. steatorhombus enzyme replacing the water in its more usual hydrolytic activity with a D-amino acid when the D-amino is present at very high concentrations (17). Our enzyme differs significantly from this enzyme in that it can use diaminopimelate as an acceptor for transpeptidation. In this respect, this enzyme closely resembles the purified Dd-carboxypeptidases-transpeptidases of Streptomyces R61 and R39 which also incorporate diaminopimelate into many natural and synthetic substrates of which the best is N,N-diacetyl-L-Lys-D-Ala-D-Ala (18, 33).
Supplemental material

Purification and characterization of a carbonic anhydrase: The enzyme was purified from human urine by B. Banerjee and D. Sen. Biochim. Biophys. Acta 233: 457-462 (1971). The enzyme was also purified from urine by A. F. Shanks and J. S. Chen. J. Biol. Chem. 245: 3918-3923 (1970). The enzyme was further purified by H. L. H. and D. C. R. J. Biol. Chem. 245: 3918-3923 (1970). The enzyme was also purified from urine by A. F. Shanks and J. S. Chen. J. Biol. Chem. 245: 3918-3923 (1970). The enzyme was further purified by H. L. H. and D. C. R. J. Biol. Chem. 245: 3918-3923 (1970). The enzyme was also purified from urine by A. F. Shanks and J. S. Chen. J. Biol. Chem. 245: 3918-3923 (1970).
Figure 1. Purification of carboxypeptidase-IIW by hydroxyapatite column chromatography. Concentrated SDS extract from D. subterraneus was dialyzed and chromatographed on a hydroxyapatite column. Enzyme activity was measured by the binding assay (see text).

Figure 2. Purification of carboxypeptidase-IIW by Sephadex G-200 column chromatography. Material pooled from column of Figure 1 was concentrated, chromatographed, and assayed for enzymatic activity by the binding assay (see text).

Figure 3. Purification of carboxypeptidase-IIW by affinity chromatography. One-third of the pooled and concentrated material from the column of Figure 2 was chromatographed on a affinity column (see text) containing dithiobalactone-peptide (O-nitro) (see Materials and Methods). The enzyme was assayed by the binding assay.
Purification and characterization of a carboxypeptidase-transpeptidase of Bacillus megaterium acting on the tetrapeptide moiety of the peptidoglycan.  
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