**D-Alanine Carboxypeptidase from Bacillus subtilis Membranes**

I. PURIFICATION AND CHARACTERIZATION*

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**SUMMARY**

The D-alanine carboxypeptidase was solubilized from the membranes of *Bacillus subtilis* with the nonionic detergent, Triton X-100, and purified to homogeneity. The purified protein bound irreversibly radioactivity from[^14C]Penicillin G. Triton X-100, and pursued to homogeneity. The purified enzyme was assayed by a slight modification of the method of Lawrence and Strominger (1) who also demonstrated that the enzyme is inactivated by penicillin. The amino acid analysis did not indicate that the protein was unusually hydrophobic.

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Although it is generally agreed that the enzymic activity of membranes is an essential component of their biological role, the number of enzymes purified from membranes remains remarkably small. The distinguishing structural characteristics of such enzymes and the influence of their unique environment upon their activities are areas only beginning to be explored.

The presence of a penicillin-sensitive D-alanine carboxypeptidase in the membranes of *Bacillus subtilis* provided an opportunity to explore approaches to the purification of a membrane-bound enzyme, as well as to advance knowledge concerning the mode of action of penicillin. This enzymic activity was first reported by Lawrence and Strominger (1) who also demonstrated that the enzyme is inactivated by penicillin. The function of this enzyme is unknown and, although it is the major penicillin binding component of the cell, its inactivation by penicillin is not lethal (2). The enzyme catalyzes the removal of the terminal D-alanine from UDP-MurNAc-L-Ala-D-Glu-meso-Dap-D-Ala-D-Ala and related substrates to form UDP-MurNAc-tetrapeptide and D-alanine.

**MATERIALS AND METHODS**

The enzyme was assayed by a slight modification of the method of Lawrence and Strominger (1). A typical reaction mixture consisted of 180 mM sodium cacodylate, pH 6.0, 6 μM UDP-MurNAc-t-Ala-d-Glu-meso-Dap-D-[^14C]Ala-D-[^14C]Ala (2.4 × 10⁶ cpm per μmole), 35 mM ZnSO₄, and 1 μl of enzyme preparation in a total volume of 28 μl. After incubation for a given time (usually 30 min), the reaction was terminated by boiling for 2 min. The reaction mixture was spotted on sheets (20 × 20 cm) of Whatman No. 3MM paper and subjected to ascending chromatography for 3 hours in isobutyril acid-1 n NH₄OH, 5:3. If a rapid assay was desired, 5 μl of 0.2 M alanine was added to the reaction mixture just before spotting. After chromatography, the dried papers were sprayed with ninhydrin, and the alanine spots were cut out and counted. Radioautography was used when a more accurate assay was desired.

Protein was determined by the alkaline ninhydrin method of Hirs (3). Triton X-100 and other nonionic detergents interfere with alternative methods of protein determination.

**RESULTS**

**Purification of Enzyme**

The enzyme has been purified from the membrane about 100-fold to apparent homogeneity. A typical purification is presented in Table I. All operations were carried out at 4º.

**Step 1: Preparation of Membranes**—Three grams of membranes were made by grinding 250 g (wet weight) of cells of *B. subtilis* (grown as previously described (1)) with 5-mm glass beads in a colloidal mill (Micro-mill, Gifford-Wood Inc., Hudson, N. Y.) as follows. One liter of 5-mm glass beads, previously washed extensively in *aqua regia* and dried, were added to 1 liter of 100 mM Tris-HCl, pH 7.5, containing about 250 g (wet weight) of cells. Twelve drops of tri-butyl citrate were added as antifoaming agent, and the cells were ground at 4º in ice water at top speed with a vernier setting of 5 to 7 for 5 min. The mixture was then decanted into centrifuge tubes and centrifuged for 30 min at 15,000 rpm (Sorvall centrifuge, SS-34 rotor). The supernatant solution was centrifuged at top speed with a vernier setting of 5 to 7 for 5 min. The supernatant solution was decanted and saved; the pellet was reground twice more in the Micro-mill. The remaining pellet consists predominantly of cell walls. The pooled supernatant solutions were centrifuged at 3º for 1 hour at 150,000 × g. The supernatant solutions were carefully decanted, and the loose pellet was then homogenized in a Potter homogenizer with approximately 200 ml of 100 mM Tris-HCl, pH 7.5, 10⁻² M β-mercaptoethanol, to a final protein concentration of about 15 mg per ml. The membrane fraction obtained can be stored in the freezer (-20º) indefinitely.

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Purification of D-alanine carboxypeptidase

| Step                  | Volume | Total activity | Yield | Total protein | Specific activity | Purification |
|-----------------------|--------|----------------|-------|---------------|-------------------|--------------|
|                       | ml     | cpm/10^6       | %     | mg            | cpm/mg/min x 10^9 | -fold        |
| Crude membranes       | 100    | 60             | (100) | 3000          | 2                 |              |
| Triton X-100 supernatant solution | 325    | 20             | 43    | 1290          | 2                 | 1            |
| Agarose 1.5 m column | 1200   | 21             | 35    | 348           | 6.3               | 3.2          |
| ECTEOLA-column        | 95     | 4.6            | 7.6   | 10.6          | 43                | 22           |
| DEAE-column           | 124    | 3.4            | 5.6   | 2.5           | 197               | 09           |
| CaPO₄ gel             | 10     | 1.6            | 2.6   | 0.75          | 213               | 107          |

**Step 2: Solubilization with Triton X-100** — The material from Step 1 was mixed with enough Triton X-100 to bring the final concentration to 1% in detergent at a protein concentration of 10 mg per ml. This was mixed at 4°C for 30 min and then centrifuged at 120,000 x g for 1 hour at 3°C. The supernatant solution was decanted and used for the next step. The amount of solubilization obtained was pH-dependent (Fig. 1), and therefore the solubilization was carried out at pH 8.6. The presence of salt (NaCl) did not improve the amount of solubilization. The solution must be cooled to about 4°C. The enzyme was stained with Coomassie blue. Enzyme reacts irreversibly with [14C]penicillin G and was coincident with radioactivity from [14C]penicillin G bound to protein, indicating that the protein band was the carboxypeptidase (Fig. 5). The molecular weight determined on either 5 or 10% acrylamide gels in sodium dodecyl sulfate (4) was about 50,000 (Fig. 6). Gel filtration in the presence of guanidine HCl (5) gave a similar molecular weight for the single polypeptide chain (Fig. 7).

**Step 3: Calcium Phosphate Gel** — The calcium phosphate eluate was recovered by centrifugation in a Sorvall centrifuge SS-34 rotor at 10,000 rpm for 30 min and washed with 100 mM Tris-HCl, pH 8.6, 10^-3 M mercaptoethanol, 1% Triton X-100, and eluted successively with 10-mL portions of 5, 7, 10, and 20% saturated ammonium sulfate (at 4°C) in 100 mM Tris-HCl, pH 7.0, 10^-3 M mercaptoethanol, 1% Triton X-100. Fractions of 5 ml were collected. The elution profile is shown in Fig. 3. The relevant fractions were pooled and dialyzed against 10 mM Tris-HCl, pH 8.6, 10^-3 M mercaptoethanol, 1% Triton X-100 until the conductivity was again about 0.5 x 10^9 micromhos.

**Step 5: DEAE-cellulose Column Chromatography** — The material from Step 4 was charged to a 100-mL DEAE-cellulose (DE 52, Whatman) equilibrated in the same buffer as used for dialysis. After washing with 1 liter of the same buffer, the elution was carried out with a linear gradient from 1 liter of 10 mM Tris-HCl, pH 8.6, 10^-3 M mercaptoethanol to 1 liter of 100 mM Tris-HCl, pH 8.6, 10^-3 M mercaptoethanol, 0.2 M NaCl, both containing 1% Triton X-100. Fractions of 10 ml were collected. The elution profile is shown in Fig. 4. The relevant fractions were pooled and dialyzed against 10 mM Tris-HCl, pH 8.6, 10^-3 M mercaptoethanol, 1% Triton X-100 until the conductivity was again about 0.5 x 10^9 micromhos.

**Step 6: Calcium Phosphate Gel** — The pooled fractions from Step 5 were then tested by use of a 1-mL aliquot for the minimum amount of calcium phosphate gel required to absorb 90% of the total activity. Typically, 124 mL of pooled material from Step 5 were mixed with 2.5 mL of calcium phosphate gel (Bio-Rad, 0.8 g per mL of solids). After incubation at 4°C for 30 min, the calcium phosphate was recovered by centrifugation in a Sorvall centrifuge SS-34 rotor at 10,000 rpm for 30 min and washed with 100 mM Tris-HCl, pH 8.6, 10^-3 M mercaptoethanol, 1% Triton X-100, and eluted successively with 10-mL portions of 5, 7, 10, and 20% saturated ammonium sulfate (at 4°C) in 100 mM Tris-HCl, pH 8.6, 1% Triton X-100. Note that in order to form a clear solution at the higher ammonium sulfate concentrations, the solution must be cooled to about 4°C.) The enzyme was usually eluted in the fraction containing 7% ammonium sulfate and could be stored in this buffer indefinitely.

**Molecular Weight of Polypeptide Chain**

The calcium phosphate eluate showed only one protein band on polyacrylamide gel electrophoresis in sodium dodecyl sulfate stained with Coomassie blue. Enzyme reacts irreversibly with [14C]penicillin G (see following paper) and the band staining with Coomassie blue was coincident with radioactivity from [14C]penicillin G bound to protein, indicating that the protein band was the carboxypeptidase (Fig. 5). The molecular weight determined on either 5 or 10% acrylamide gels in sodium dodecyl sulfate (4) was about 50,000 (Fig. 6). Gel filtration in the presence of guanidine HCl (5) gave a similar molecular weight for the single polypeptide chain (Fig. 7).

**Amino Acid Analysis and NH₂-terminal Amino Acid**

The amino acid analysis (Table II) showed that the protein was not unusually hydrophobic; the ratio of polar to nonpolar amino acid residues (7) was 1.25, a value close to that for sperm whale

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1 The abbreviation used is: ECTEOLA-cellulose, epichlorohydrin triethanolamine cellulose.
FIG. 2 (left). Gel filtration of the carboxypeptidase. Chromatography was performed on Bio-Gel 1.5m as described in the text. The absorbance was read against buffer containing Triton X-100.

FIG. 3 (center). Elution of the carboxypeptidase from an ENZYME BOUND PENICILLIN G or Ni++ (see also 1). It is possible that UDP-MurNAc-pentapeptide is not the natural substrate for this enzyme. The high level of Zn++ required may be related to this fact, i.e. presumably the Zn++ interacts with the substrate.

Thiol Group

The enzyme was inhibited by reagents which react with thiol groups (Table IV). Substrate protected the enzyme from inactivation by one such reagent, 5,5'-dithiobis(2-nitrobenzoic acid), in an apparently competitive manner (Fig. 9), suggesting a role for a sulfhydryl group in the active site of the enzyme.

FIG. 5. Coincidence of the enzyme-bound [14C]penicillin and stained protein on gel electrophoresis in the presence of sodium dodecyl sulfate. Purified carboxypeptidase containing 14.5 μg of protein (determined with alkaline ninhydrin (3)) was incubated with 2 μg (1.6 X 10⁶ cpm) of [14C]penicillin G (35 mCi per n mole) in 100 μl of 0.5 m cacodylate buffer, pH 6.5, 2 X 10⁻² M MgCl₂ at 37° for 30 min and then dialyzed against three changes of 150 ml of Tris-HCl, pH 8.6, 1% Triton X-100 for 3 hours. The sample was then applied to a polyacrylamide gel which had been prepared without the addition of p-mercaptoethanol and then subjected to electrophoresis for 3 hours before application of the sample. After the electrophoresis with the sample was completed, the gel was removed and dried by placing it on a piece of filter paper in a Buchner funnel, covering it with a section of Saran Wrap and a rubber dental dam, and attaching the funnel to an oil pump overnight. The dried gel was then radioautographed. A sample containing [14C]penicillin but no enzyme was treated in an identical manner to locate unbound penicillin (not shown). A third gel contained enzyme and penicillin but was stained with Coomassie brilliant blue rather than radioautographed.

myoglobin (1.29). A single NH₂-terminal amino acid, valine, was found (Table III).

Metal Ion Requirement

The enzyme required 15 mM zinc ions for maximum activity (Fig. 8). The zinc could be partially replaced by Mn++, Co++,

Michaelis Constants

At 37° in the presence of Zn++, the enzyme had a Kₘ of 2.6 X 10⁻⁴ M and a rate constant (Vₘₐₓ per mole) of 1.5 X 10⁻² s⁻¹ with UDP-MurNAc-pentapeptide as substrate at pH 5.5. In the presence of Mn++, the Kₘ was 3.3 X 10⁻⁴ M and the rate constant 5.2 X 10⁻² s⁻¹.
Molecular weight of the carboxypeptidase polypeptide chain as determined by sodium dodecyl sulfate gel electrophoresis. The procedure is described under "Materials and Methods." The calibrating proteins (O) are, in order of increasing molecular weight: cytochrome c, lactate dehydrogenase, apoferritin, carboxypeptidase A, ovalbumin, glutamate dehydrogenase, catalase, bovine serum albumin, glucose 6-phosphate dehydrogenase, and γ-globulin.

The polypeptide molecular weight was also determined by gel filtration in guanidine HCl. The polypeptide molecular weight in guanidine HCl on 2% agarose columns (Bio-Gel A-15m) according to the procedure of Fish et al. (5).

A Pharmacia K 15/30 column (1.5 × 30 cm) was carefully equilibrated with 6 M guanidine HCl, buffered with 10 mM Tris-HCl to pH 8.6. Samples of calibrating proteins, or carboxypeptidase, of about 22 μg were first reduced by boiling in 100 μl of 1 M mercaptoethanol in 6 M guanidine HCl at pH 8.6, then dialyzed overnight against 6 M guanidine HCl, and then reacted with an excess of 125Iodoacetamide (1.5 × 10^6 cpn, 2.5 mCi per mmole) at pH 8.6 for 1 hour at 37°C. After about 9 hours of dialysis against 6 M guanidine HCl, the sample was mixed with glycerol to 10% final concentration and then charged to the column. An 11-cm hydrostatic pressure was maintained by a Mariette flask and 400-μl fractions were collected. These were then counted in dioxane based scintillation fluid (Aquasol, New England Nuclear).

An approximate sedimentation coefficient for the enzyme was determined by sedimentation in sucrose gradient by the method of Ames and Martin (11). A sedimentation constant of about 2.6 S was found (Fig. 15). Because the amino acid analysis failed to show any abnormality, the extremely small sedimentation constant also suggested that the protein was combined in a detergent aggregate with a much higher apparent molecular weight. This was indicated by the activity-protein profile of the agarose gel filtration column used in the purification. Most of the protein solubilized from the membrane and all of the activity of the carboxypeptidase were located approximately coincidently in a single peak eluting at about the position expected for Triton X-100 micelles (Fig. 2) (compare 8). The activity was also present as a high molecular weight species on gel filtration using Sephadex G-200 in the presence of 0.1% Triton X-100 (Fig. 13). Trailing peaks were apparently due to complex equilibrium between the aggregate, free enzyme, and detergent during filtration (9).

Molecular Weight and Interaction with Detergent

Under denaturing conditions in sodium dodecyl sulfate or guanidine HCl, the protein was observed as a single polypeptide chain with a molecular weight of 50,000. In Triton X-100 solutions, however, the enzyme appeared to be a detergent protein aggregate with a much higher apparent molecular weight. This was indicated by the activity-protein profile of the agarose gel filtration column used in the purification. Most of the protein solubilized from the membrane and all of the activity of the carboxypeptidase were located approximately coincidently in a single peak eluting at about the position expected for Triton X-100 micelles (Fig. 2) (compare 8). The activity was also present as a high molecular weight species on gel filtration using Sephadex G-200 in the presence of 0.1% Triton X-100 (Fig. 13). Trailing peaks were probably due to complex equilibrium between the aggregate, free enzyme, and detergent during filtration (9).

An approximate sedimentation coefficient for the enzyme was determined by sedimentation in sucrose gradient by the method of Hedrich and Smith (10). A sedimentation constant of about 2.6 S was found (Fig. 15). Because the amino acid analysis failed to show any abnormality, the extremely small sedimentation constant also suggested that the protein was combined in a

Fig. 6 (left). Molecular weight of the carboxypeptidase polypeptide chain as determined by sodium dodecyl sulfate gel electrophoresis. The procedure is described under "Materials and Methods." The calibrating proteins (O) are, in order of increasing molecular weight: cytochrome c, lactate dehydrogenase, apoferritin, carboxypeptidase A, ovalbumin, glutamate dehydrogenase, catalase, bovine serum albumin, glucose 6-phosphate dehydrogenase, and γ-globulin.

Fig. 7 (right). Determination of the carboxypeptidase polypeptide chain molecular weight by gel filtration in guanidine HCl. The polypeptide molecular weight was also determined by gel filtration in guanidine HCl on 2% agarose columns (Bio-Gel A-15m) according to the procedure of Fish et al. (5). A Pharmacia K 15/30 column (1.5 × 30 cm) was carefully equilibrated with 6 M guanidine HCl, buffered with 10 mM Tris-HCl to pH 8.6. Samples of calibrating proteins, or carboxypeptidase, of about 22 μg were first reduced by boiling in 100 μl of 1 M mercaptoethanol in 6 M guanidine HCl at pH 8.6, then dialyzed overnight against 6 M guanidine HCl, and then reacted with an excess of 125Iodoacetamide (1.5 × 10^6 cpn, 2.5 mCi per mmole) at pH 8.6 for 1 hour at 37°C. After about 9 hours of dialysis against 6 M guanidine HCl, the sample was mixed with glycerol to 10% final concentration and then charged to the column. An 11-cm hydrostatic pressure was maintained by a Mariette flask and 400-μl fractions were collected. These were then counted in dioxane based scintillation fluid (Aquasol, New England Nuclear).

Fig. 8. The concentration of zinc ions required for optimal activity of carboxypeptidase. Assays were performed as described under "Materials and Methods," except that the concentration of zinc ions was that indicated.
large detergent micelle, thus "floating" the enzyme in the sucrose gradient. By reacting the enzyme with dimethyl suberimidate according to the procedure of Davies and Stark (12), it was possible to demonstrate that at least some of the detergent

**Table II**

Amino acid composition of carboxypeptidase

Amino acid analysis was performed on 1-mg samples of protein (previously dialyzed against 10 mM potassium phosphate buffer, pH 7.2, for 24 hours to remove Tris and ammonium sulfate) after hydrolysis for 24 and 72 hours in redistilled 6 N HCl. Cysteine was estimated only as groups titratable by 5,5'-dithiobis(2-nitrobenzoic acid in the native protein. Tryptophan content could not be obtained because the Triton X-100 in the samples interfered both with the ultraviolet absorbance and the Spies and Chambers (6) colorimetric method. Serine and tyrosine were extrapolated to zero time, and the 72-hour value was taken for valine, isoleucine, and phenylalanine. Methionine was estimated as 50% recovery after 24 hours hydrolysis.

| Amino acids     | Amount | Residues per 50,000 |
|-----------------|--------|---------------------|
| Lysine          | 1.72   | 45                  |
| Histidine       | 0.17   | 5                   |
| Arginine        | 0.27   | 7                   |
| Aspartic acid   | 1.86   | 50                  |
| Threonine       | 1.14   | 31                  |
| Serine          | 1.06   | 29                  |
| Glutamic acid   | 1.63   | 44                  |
| Proline         | 0.61   | 14                  |
| Glycine         | 1.15   | 31                  |
| Alanine         | 1.37   | 37                  |
| Valine          | 1.28   | 35                  |
| Methionine      | 1.14   | 31                  |
| Isoleucine      | 1.04   | 28                  |
| Leucine         | 1.36   | 37                  |
| Tyrosine        | 0.62   | 14                  |
| Phenylalanine   | 0.71   | 19                  |
| Cysteine        |        |                     |
| Tryptophan      |        |                     |

* Titratable in native enzyme.
* n.d., not determined.

**Table III**

Determination of NH\(_2\)-terminal amino acid in carboxypeptidase

Enzyme (15 mg of protein eluted from calcium phosphate gel) was lyophilized and resuspended in 500 μl of 1% NaHCO\(_3\). This sample was subjected to sonication to suspend the remaining detergent. To this suspension was added 100 μl of [\(^{14}\)C]fluorodinitrobenzene with a specific activity of 31.5 mCi per mmole (New England Nuclear), and the reaction was run at room temperature for 2 hours. Then a 500-μl solution of unlabeled fluorodinitrobenzene (2.5% v/v) was added. The solution was centrifuged and the pellet washed in ethanol and ether, dried in vacuo, and hydrolyzed for 6 hours in 6 N HCl at 105°. The ether extract was chromatographed and compared to authentic derivatives in chloroform-tert-amyl alcohol-acetic acid, 70:30:3 on Silica Gel G by thin layer chromatography. The relevant spots (the NH\(_2\)-terminal amino acids and breakdown products of fluorodinitrobenzene) detected by radioautography were eluted and rechromatographed with standards in benzene-pyridine-acetic acid, 80:20:2.

A molecular weight of 53,000 was estimated from the amount of [\(^{14}\)C]dinitrophenyl-Val recovered, a value in good agreement with the value obtained by gel electrophoresis in the presence of sodium dodecyl sulfate.

| Compound                  | Chloroform-tert-amyl alcohol-acetic acid | Benzene-pyridine-acetic acid |
|---------------------------|------------------------------------------|-----------------------------|
| Authentic DNP-Val\(^{b}\) | 0.93                                      | 0.36                        |
| [\(^{14}\)C]DNP-amino acid | 0.95                                      | 0.37                        |

* DNP-Val, 2,4-dinitrophenyl-valine.

**Fig. 9.** Inhibition of the n-alanine carboxypeptidase by 5,5'-dithiobis(2-nitrobenzoic acid). A, the data were plotted by the method of Lineweaver and Burk. B, the data were plotted by the method of Dixon.
TABLE IV
Inhibition of carboxypeptidase by thiol reagents

Each inhibitor was added directly to the assay mixture and incubated as described under "Materials and Methods."

| Inhibitor                  | Counts per min |
|----------------------------|----------------|
| pCMBSA* (0.1 mM)           | 278            |
| DTNBb (20.0 mM)            | 221            |
| Iodoacetic acid (0.2 M)    | 2313           |
| None                       | 5676           |

a pCMBSA, p-chloromercuribenzenesulfonic acid.
b DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

Fig. 10. The pH dependency of the carboxypeptidase. Assays were performed as described under "Materials and Methods" with 0.5 M cacodylate buffer for pH 4.5, 5.0, 5.5, 6.0, 6.5, or 0.5 M Tris-HCl buffer for pH 7.0, 7.5. The metal concentrations were 15 mM for ZnSO₄ and 16 mM for MnCl₂.

The presence of components at multiples of 50,000 may not be due entirely to polypeptide oligomers because bound detergent could also contribute to the molecular weight (e.g. the presence of 100 moles of detergent per polypeptide chain would almost exactly double the molecular weight). Molecular sieving in polyacrylamide gels (10) in the absence of detergent yielded similar results.

DISCUSSION

The membrane-bound l-alanine carboxypeptidase solubilized in the nonionic detergent Triton X-100 was purified to homogeneity by routine methods of protein chemistry. The straightforward purification scheme demonstrated the similarity of membrane proteins to soluble proteins, the major difference apparently being that membrane proteins must be incorporated into lipid or detergent micelles either in the membrane or in solution (13). The l-alanine carboxypeptidase was pure as judged by homogeneity on sodium dodecyl sulfate gel electrophoresis, coincidence of the protein band with bound [³⁵S]penicillin G, a single NH₂-terminal amino acid, and stoichiometric inactivation by penicillin G (13). The enzyme constituted about 1% of the membrane on the basis of the 100-fold purification and, therefore, was a major membrane constituent. Although the enzyme is tightly membrane-bound and although it requires detergent for solubilization, amino acid composition was not unusually hydrophobic. The basis for these properties requires further study, e.g. the protein may contain a locus where hydrophobic amino acids are concentrated or it may be folded in such a way that its surface is predominantly composed of hydrophobic amino acids.

The purified enzyme was present in solution as a detergent enzyme complex of large molecular weight. Estimations from molecular weight of about 350,000, and reactivity with a protein cross-linking reagent suggested that some micelles might contain two or three polypeptide chains. The combination of the data obtained on sedimentation in sucrose and the gel filtration data yield an estimate of partial specific volume which would cor-
FIG. 14. Determination of the molecular weight of the n-alanine carboxypeptidase by polyacrylamide gel electrophoresis. The method of Hedrick and Smith (10) was used to determine the molecular weight of the protein by molecular sieving. A, a densitometer trace was made of the gel at 6.0% acrylamide showing the major and minor peaks. B, the log of the mobility of the carboxypeptidase and standard proteins was plotted versus the per cent cross-linkage of a series of gels (4.5, 6.0, 7.5, 8.25%, with a ratio of acrylamide to bisacrylamide of 30 to 0.8). The slopes of the lines for the standards were then plotted versus the molecular weight as shown. The two components of the carboxypeptidase (X, △) differ mainly with respect to charge. The standard proteins are (1) ovalbumin; (2) catalase; (3) phosphorylase; (4) apoferritin; (5) myoglobin; (6) α-chymotrypsin; (7) ovalbumin; (8) bovine serum albumin; (9) catalase.

FIG. 15. Determination of molecular weight of the carboxypeptidase by sucrose gradient sedimentation. Sucrose gradient sedimentations were performed according to the method of Martin and Ames (11). A, 10 μl of enzyme were applied to a 4.7-ml, 5 to 20% sucrose gradient with 100 mM Tris-HCl, pH 8.6, and 0.1% Triton X-100. Centrifugation was performed at 39,000 rpm and 5° in an International Centrifuge, model B-60, in an SB-283 rotor for 24 hours. The gradient tubes were then punctured and 40 fractions (about 150 μl per fraction) were taken. An aliquot of 15 μl from each tube was taken and assayed for enzymatic activity. Standard proteins were treated in a similar manner, but the protein was determined by alkaline ninhydrin. The proteins used as standards were (1) cytochrome c; (2) hemoglobin subunit; (3) myoglobin; (4) cytochrome c dimer; (5) chymotrypsinogen A; (6) β-lactoglobulin and hemoglobin subunit; (7) cytochrome c trimer; (8) ovalbumin; (9) calf intestine alkaline phosphatase subunit; (10) hemoglobin; (11) bovine serum albumin; (12) calf intestine alkaline phosphatase; (13) bovine serum albumin dimer. B, the presence of Triton X-100 in the sucrose gradient did not alter the sedimentation coefficient of the calibrating protein except to occasionally cause dissociation of oligomers. The sedimentation was performed for 12, 24, and 48 hours and the tube position plotted against the time of centrifugation to estimate the sedimentation coefficient more accurately. The carboxypeptidase (●) sediments close to chymotrypsinogen (X). The other standards are cytochrome c (○) and ovalbumin (△). The sedimentation coefficient obtained in this manner would be compatible with the molecular weight obtained by molecular sieving if the protein bound between 100 to 500 moles of detergent and was approximately spherical.
FIG. 16. Determination of the apposition of polypeptide chains by cross-linking. The d-alanine carboxypeptidase was reacted with dimethyl suberimidate according to the procedure of Davies and Stark (12). To 300 μl of enzyme (0.13 mg per ml, dialyzed against 0.2 M triethanolamine buffer, pH 8.5) were added 40 μl of dimethyl suberimidate in triethanolamine buffer. The mixture was incubated at room temperature for 4 hours, and then subjected to sodium dodecyl sulfate gel electrophoresis. A solution of apoferritin was treated in an identical manner. The densitometer trace is that of the carboxypeptidase and the molecular weight of the peaks are shown for carboxypeptidase and apoferritin.

respond to at least 100 moles of detergent per mole of enzyme on the average. The average protein-detergent micelle might therefore contain 3 moles of protein and 300 moles of detergent. Protein-detergent interaction therefore seemed more powerful than protein-protein binding. It can be suggested that the protein was situated in the detergent micelle as if it were incorporated into the membrane bilayer, although the details of the structure are obviously different.

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Fig. 17. Gel filtration of the purified carboxypeptidase in the absence of detergent. The experiment was performed exactly as in Fig. 13 except that Triton X-100 was omitted for the eluting and equilibrating buffers. The sample charge to the column still contained Triton X-100, however. Again the molecular weights were estimated by comparison to calibrating proteins.
d-Alanine Carboxypeptidase from \textit{Bacillus subtilis} Membranes : I.
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