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NOTES

Antibody to the D-Alanine Carboxypeptidase of *Bacillus subtilis* Does Not Cross-React with Other Penicillin-Binding Proteins

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The fact that antibody to D-alanine carboxypeptidase of *Bacillus subtilis* does not cross-react with other penicillin-binding proteins suggests that these proteins are not precursors or multimers of the enzyme.

Multiple penicillin-binding proteins (PBPs) have been found in all bacteria examined so far (9). Some of these proteins have enzymatic activity, and there is evidence that some of them are involved in important cellular processes (4, 7). The question remains, however, of the relationship of these various proteins to one another. In *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli*, those proteins with no identifiable activity are of higher molecular weight than the PBPs that have been found to be enzymes (2, 8; J. W. Kozarich, and J. L. Strominger, J. Biol. Chem., in press). Thus, there is a possibility that some of them may be precursors or multimers of the lower-molecular-weight enzymes. One approach to testing this hypothesis is to determine the immunological relatedness of the PBPs by using antibody prepared against the individual proteins. We report here an investigation of the relationship of the *B. subtilis* PBPs to one another, using antibody to PBP V.

D-Alanine carboxypeptidase accounts for at least 80% of the protein that binds penicillin in *B. subtilis* (2). It is the smallest one of the five PBPs and the only one that has a known enzymatic activity, but it has been shown not to be the penicillin killing site (1). Rabbit antibody was prepared against the carboxypeptidase by using protein purified as described (3). The purified immunoglobulin G (IgG) gave one precipitin line in agar with pure carboxypeptidase (data not shown) and could totally abolish the carboxypeptidase activity (Fig. 1). The IgG also gave a reaction of identity between the pure carboxypeptidase and a mixture of all five PBPs, and no spurs or secondary lines were observed.

The other four PBPs were separated from carboxypeptidase (PBP V) by eluting the mixture from a column of diethyaminoethyl-cellulose (Whatman) with a 0.1 to 0.3 M NaCl gradient. Carboxypeptidase was eluted first, and a mixture of the other proteins appeared in subsequent fractions. This column chromatography succeeded in reducing the amount of carboxypeptidase to 2% of the total protein in the mixture (calculation based on enzyme activity), but it did not separate PBPs I to IV from each other (Fig. 2B). In addition, a sixth protein of slightly lower molecular weight than PBP V has been isolated (Fig. 2A to C). However, it does not bind ^{14}C-labeled penicillin G (data not shown), nor does it cross-react with antibody to the carboxypeptidase (Fig. 3). Possibly, it is the result of degradation of one of the PBPs since its appearance in different preparations is variable.

Immunoelectrophoresis of a preparation of PBPs I to IV containing 2% carboxypeptidase gave a single precipitin arc attributable to the carboxypeptidase contaminant (Fig. 3). Coomassie brilliant blue staining of the agarose after electrophoresis (not shown) revealed that PBPs I to IV diffused into the agarose but did not migrate from the center well, whereas carboxypeptidase protein moved toward the anode and aligned precisely with the single precipitin arc. The low-molecular-weight contaminant migrated much further toward the anode (2.5 cm beyond the carboxypeptidase), where no reaction with the antibody was observed.

It appears, then, that the major antibody produced against pure carboxypeptidase does not recognize any of the other four PBPs. This

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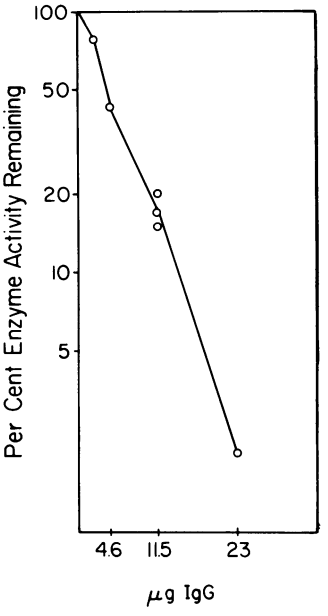

FIG. 1. *Antibody inactivation of D-alanine carboxypeptidase. For the antiserum preparation, after an initial bleeding to obtain preimmune sera, each of three male New Zealand white rabbits received 0.5 mg of pure carboxypeptidase in Fruend complete adjuvant (Difco) injected in the footpads. After 4 weeks, each rabbit received 0.5 mg of enzyme in saline subcutaneously. A second boost of 0.5 mg of protein was given 4 weeks later. Ten days later 30 to 50 ml of blood was collected from the ears of each animal, and sera were prepared and titrated (11). One rabbit had no detectable antibody and its serum was discarded. IgG fractions were isolated by ammonium sulfate precipitation and used for this study. For the enzyme assay, antibody was diluted in 0.05 M tris-(hydroxymethyl)aminomethane buffer, pH 7.5, and mixed with the enzyme. They were incubated together at 37°C for 30 min before substrate was added. The assay was performed as previously described (1). Similar inhibition was observed with IgG preparations from both rabbits. A control assay showed no inhibition with the preimmune IgG. Protein was determined by the method of Lowry et al. (5).*

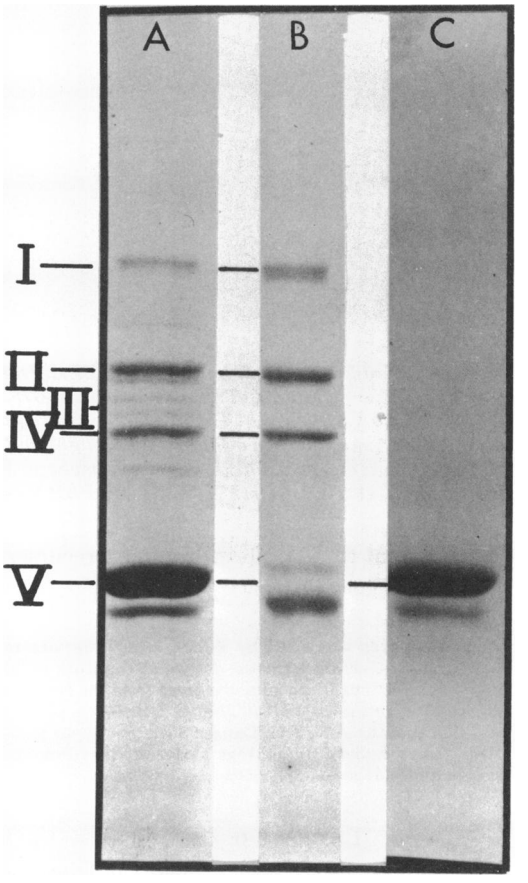

FIG. 2. *Sodium dodecyl sulfate-polyacrylamide gel of PBPs. Slab gel electrophoresis was performed as described previously (4) with the upper stacking gel containing 3% acrylamide and the running gel containing 7.5% acrylamide. The gel was stained with Coomassie brilliant blue and destained with 5% methanol and 7.5% acetic acid. (A) PBPs from B. subtilis Porton isolated by affinity chromatography (2). (B) PBPs I to IV with 2% PBP V purified as described in the text. (C) Pure PBP V (D-alanine carboxypeptidase) prepared by the method of Blumberg and Strominger (3). This enzyme has a molecular weight of 50,000 (10).*

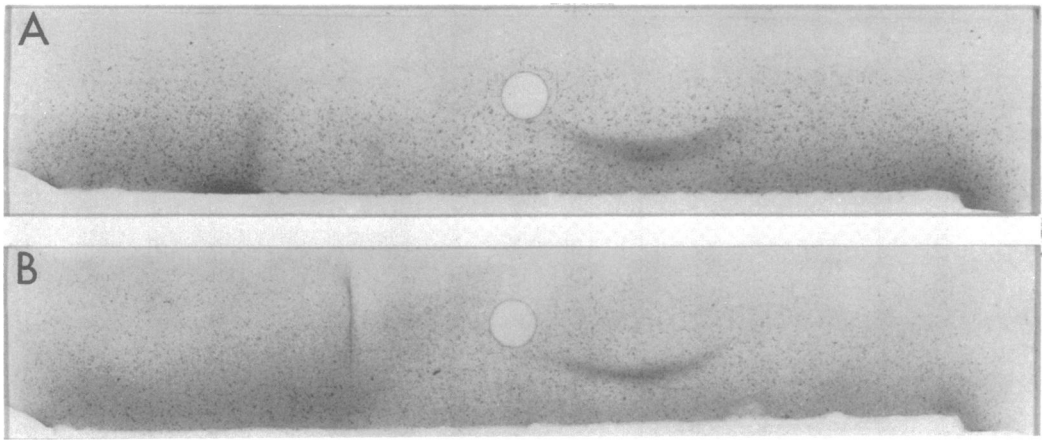

FIG. 3. *Immunoelectrophoresis of the PBPs. Protein in the center well was subjected to electrophoresis in agarose (Fisher) with 1% Triton X-100 in 0.075 M tris(hydroxymethyl)aminomethane-barbital, pH 8.8 (Gelman), at 6 V/cm for 5 h. The anode is on the right in this figure. After electrophoresis, 150 μl of undiluted rabbit IgG (15 mg/ml) was added to the trough, and the precipitin reaction was allowed to develop for at least 24 h at room temperature in a moist atmosphere. The agarose slide was then stained as described (6). (A) PBPs I to IV, 11.2 μg, with 2% (0.224 μg) PBP V (see Fig. 2B). (B) Pure PBP V, 0.224 μg (see Fig. 2C).*

suggests that these proteins are not precursors or multimers of the enzyme.

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