Purification and Properties of Penicillin-binding Proteins 5 and 6 from the dacA Mutant Strain of Escherichia coli (JE 11191)*

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Penicillin-binding proteins 5 and 6 have been purified to homogeneity from the dacA mutant strain of Escherichia coli (JE 11191). Protein 6 from the mutant strain appears to be identical to that from the wild type, but protein 5 is a mutant protein which has no D-alanine carboxypeptidase activity. Moreover, the mutant protein 5 binds, but does not release, 14C-labeled penicillin G. Correspondingly, an acyl-enzyme intermediate derived from a synthetic substrate is accumulated by the mutant protein. A comparison of the acylation site for the synthetic substrate and for penicillin G by limited proteolysis and some other properties of the mutant protein are described.

Penicillin-sensitive CPase activity of bacteria was first demonstrated in a particulate enzyme system from strains of Escherichia coli (1, 2). This enzyme catalyzes the removal of the terminal D-alanine residue of a uridine nucleotide, UDP-N-acetylmuramyl-L-Ala-γ-D-Glu-meso-2,6-diaminopimelyl-D-Ala-D-Ala, and of several other related compounds. It was suggested that CPase activity may regulate the degree of cross-linkage of the cell wall peptidoglycan by removing a terminal D-alanine residue (3), but more recent data indicate that at least one of the CPases actually catalyzes a secondary transpeptidase activity in vivo (4). An extensive characterization of the multiple CPases in E. coli was carried out by Tamura et al. (5). Three proteins were purified, each having CPase activity (CPases IA, IB, and IC). CPase IA, purified from a detergent extract of membranes, was composed of two polypeptides on SDS-polyacrylamide gel electrophoresis. Although purified separately, CPases IB (from the membrane fraction) and IC (from the supernatant solution) were indistinguishable from each other, since both contained a single polypeptide of the same molecular weight on SDS gels and showed very similar catalytic properties as CPase.

A different approach to analysis of penicillin-sensitive cell wall peptidoglycan-related enzymes of bacteria has been the study of PBPs (6, 7). Since it is hypothesized that penicillins are substrate analogues and inhibit sensitive enzymes by covalently binding to the active site of the sensitive enzymes, those proteins which specifically bind penicillins should catalyze penicillin-sensitive enzymatic reactions. The cytoplasmic membrane of E. coli contains at least 7 PBPs. Correlation between these PBPs and the CPases described above was made by a combination of genetic and biochemical studies. Two independently isolated mutants (dacB) which lack CPase IB-C activity were shown to lack PBP 4 simultaneously, indicating that PBP 4 is identical or closely related to CPase IB-C (8, 9). Based on the observed properties, such as molecular weight and interactions with penicillin G, PBPs 5 and 6 were shown to be identical with the two polypeptides found in the purified CPase IA (10). Recently, we have purified PBPs 5 and 6 each to protein homogeneity and demonstrated that each PBP catalyzes CPase IA activity independently (11).

A mutant (dacA) which showed very little, if any, CPase IA activity when assayed using a crude detergent extract of membranes was isolated by Matsuhashi et al. (12). This mutant produced a defective PBP 5 and an apparently normal PBP 6 with regard to their interactions with penicillins (13). This mutant (JE 11191) had no significant growth defects under various conditions, and, moreover, a double mutant which contained dacA and dacB mutations also grew normally. Since we had found that each of the homogeneously purified PBPs 5 and 6 of the wild type E. coli independently catalyzes CPase IA activity (11), we were interested in the enzyme activity catalyzed by each of the PBPs 5 and 6 of this dacA mutant strain. In this paper, we report the purification of PBPs 5 and 6 of this dacA mutant and describe the properties of each of these PBPs. Interactions of the purified PBP 5 of this mutant with penicillin G and with the CPase substrate were also examined. This defective PBP 5 may be particularly useful for elucidation of the mechanism of interactions of substrates and penicillins with the target enzymes.

MATERIALS AND METHODS

The procedures employed were essentially the same as described in a previous publication (11). E. coli strain JE 11191 (12), the dacA mutant of strain PA3092, was generously provided by Professor Michio Matsuhashi.

RESULTS AND DISCUSSION

Purification and CPase Activity of PBPs 5 and 6 of E. coli JE 11191 (dacA)—The isolation and properties of the dacA mutant JE 11191 had been described previously by Matsuhashi et al. (12). Purification of PBPs 5 and 6 from this strain was carried out following the method described (11). As shown below (see Fig. 4), PBP 5 of this strain does not release bound penicillin G spontaneously, but releases it upon addition of hydroxylamine (pH 7.0). This property made it possible to purify PBP 5 as well as PBP 6 of this strain by covalent penicillin affinity chromatography. As in the case of the wild type PBPs 5 and 6, PBP 6 of this mutant bound to 6-APA-CM-Sepharose, and PBP 5 did not bind to this column. The latter PBP was recovered in the flowthrough from the 6-APA-
Mutant Penicillin-binding Protein 5 of E. coli

CM-Sepharose column and then bound to 6-APA-succinylamin(2-hydroxy)propyl-Sepharose, from which it was eluted with hydroxylamine. However, in this procedure the preparation of PBPs 5 and 6 are each slightly contaminated by the other.

The hydroxylamine eluates from the penicillin-affinity columns, after dialysis, were passed through columns of DEAE-cellulose, and the flowthrough fractions were obtained. Each of the PBPs 5 and 6 was further purified by CM-cellulose column chromatography (11). Figs. 1 and 2 show the elution profiles of PBPs 5 and 6, respectively, from CM-cellulose columns as analyzed by SDS-PAGE and also the [14C]penicillin G binding and CPase activities. The elution profile of CPase activity exactly corresponded to the appearance of PBP 6. PBP 5 fractions had significant [14C]penicillin G binding activities, but no associated CPase activity could be detected. Two peptides, the first having a molecular weight of approximately 30,000 (p30) and a second which migrated at the dye front in the 10% gel, always eluted at the same position as that of PBP 5 in the CM-cellulose column (Figs. 1 and 2). The molecular weight of the latter peptide was estimated to be about 10,000 (p10) by using a 12% gel (Fig. 3C). p30 did not bind penicillin G while p10 did (Fig. 3C). In the case of the purification of PBP 5 from wild type strain, no such peptides had been found (11). These peptides could not be separated from PBP 5 by further purification on a column of hydroxylapatite, Sephadex G-50, or octyl-Sepharose 4B. However, gel filtration on Bio-Gel A-5m in the presence of 6 M guanidine HCl resulted in separation of PBP 5 from p30 and p10. The data suggest that p30 and p10 may remain associated under nondenaturing conditions since they cannot be separated from each other or from PBP 5 under these conditions.

![Fig. 1. Separation of PBP 5 from PBP 6 by chromatography on CM-cellulose.](image)

![Fig. 2. Separation of PBP 6 from the residual mutant PBP 5 by chromatography on CM-cellulose.](image)

![Fig. 3. SDS-PAGE of wild type and mutant PBPs 5 and 6.](image)

These peptides appeared in the hydroxylamine eluate from the penicillin-affinity column, and several modifications of the affinity column procedures failed to reduce the amount of these peptides in the hydroxylamine eluate. It appears that p30 and p10 are degradation products of PBP 5 and are generated during or after elution of PBP 5 by hydroxylamine from the affinity column. In support for this interpretation, the profiles of the total peptides and also of those containing [14C]benzylpenicilloyl moiety in the limited proteolytic cleavage products of the mutant PBP 5 were identical to those.
generated from the wild type PBP 5 which did not contain p30 or p10 (Fig. 7 below and Ref. 11); i.e. no additional peptides which might be attributable to p30 or p10 could be detected. Furthermore, like mutant PBP 5, p10 did not release the bound [14C]penicillin G spontaneously (data not shown).

Fig. 3, A and B, shows that each of the purified PBPs 5 and 6 of JE 11191 has the same molecular weight on SDS-gel electrophoresis as the corresponding PBP of the wild type strain PA3092, namely 42,000 for PBP 5 and 40,000 for PBP 6, and indicates the purity of the preparations employed in measurement of CPase activity. The specific activities of CPase of the purified PBPs 5 and 6 of JE 11191 are summarized in Table I in comparison with those of PBPs 5 and 6 from the wild type strain. The specific activities of PBP 5 of JE 11191 towards the natural and synthetic substrates were less than 1% of those of PBP 5 of PA3092. The specific activities of PBP 6 of JE 11191 were indistinguishable from those of PBP 6 of PA3092. These results clearly demonstrate that the dacA mutation causes the loss of CPase activity of PBP 5, but it has no effect on that of PBP 6. The previous failure to detect any CPase activity in dacA cells using crude detergent extract (12) might have been due to the fact that the CPase activity catalyzed by PBP 6 is responsible for only 7-11% of the total CPase IA activity in wild type cells (11). This residual CPase activity could be physiologically important. A deletion of PBP 6 has recently been obtained as well as a deletion of PBP 5 (14, 15). Deletion mutants of PBP 4 have previously been reported (8, 9), and thus construction of a strain lacking PBPs 4, 5, and 6 would make it possible to learn whether or not CPase activity is indispensable for cell growth.

*Interactions of the Purified PBPs 5 and 6 of JE 11191 with Penicillin G—Binding of [14C]penicillin G and its release from PBPs 5 and 6 of JE 11191 were examined using the purified materials. PBP 6 bound a stoichiometric amount of [14C]penicillin G (0.87 mol/mol of protein at saturation). The concentration of [14C]penicillin G at which 50% saturation occurred ([S]90) was 2.5 µg/ml (Fig. 4A). PBP 6 released bound penicillin G spontaneously by first order kinetics with a half-life of 35 min (data not shown). These results indicate that PBP 6 of JE 11191 is indistinguishable from the wild type PBP 6 in its interactions with penicillin G.

While PBP 5 of JE 11191 also showed an almost stoichiometric binding of [14C]penicillin G (0.67 mol/mol of PBP 5) at the [14C]penicillin G concentrations used, the binding occurred with a somewhat reduced affinity (greater than 6 µg/ml for [S]90 (Fig. 4A) compared to 3.7 µg/ml for [S]90 for wild type PBP 5 (11). Quite different from the wild type PBP 5, PBP 5 of JE 11191 showed no spontaneous release of the bound [14C]penicillin G (Fig. 4B), the half-life of the complex with PBP 5 of JE 11191 being longer than 300 min compared to 12 min for that with the wild type PBP 5. Rapid release of the bound [14C]penicillin G from PBP 5 of JE 11191, however, could be observed in the presence of neutral hydroxylamine (0.4 M) (Fig. 4B). This hydroxylamine-induced release did not occur as a first order reaction.

*Interaction of the Purified PBP 5 of JE 11191 with the CPase Substrate—As described above, PBP 5 of JE 11191 is defective in catalyzing CPase activity. It binds penicillin G covalently, but the bound penicillin G is not released spontaneously. The penicillin-sensitive CPase reaction is thought to be composed of two steps; the first step is the formation of a covalent acyl-enzyme intermediate with concomitant release of the terminal D-alanine of the substrate and the second step is the transfer of the acyl moiety to H2O and the restoration of the free enzyme. To determine which step(s) is defective in PBP 5 of JE 11191, the formation of the acyl-envelope intermediate derived from the synthetic substrate Ac2LALac was examined using the purified protein. If only the second step is defective, a stable acyl-enzyme intermediate should accumulate. Fig. 5 shows that this was the case. When PBP 5 of JE 11191 was incubated with [14C]Ac2LALac, the covalent acyl-PBP 5 complex was formed slowly. It accumulated during the incubation period and reached a plateau level after 30 min of incubation. It is clear that the novel acyl-PBP 5 complex is stable since the amount at the plateau level remained constant to 60 min of incubation (Fig. 5A). Only PBP 5, not p30 nor p10, formed a covalent acyl complex with this synthetic substrate (Fig. 5B), and the formation of the acyl complex was inhibited in the presence of penicillin G (see Fig. 6). At the plateau level, about 6% of PBP 5 formed acyl complex, based on the assumption that one PBP 5 molecule has one acylated site.

An aliquot of the substrate acyl-trapping incubation mixture was removed 10 min after the start of the incubation and analyzed by paper electrophoresis. It showed that more than 75% of the input [14C]Ac2LALac had been converted to [14C]Ac2LALac. The specific activities of CPase of the purified PBPs 5 and 6 of JE 11191 with the CPase substrate was determined in Table I.

**TABLE I**

| Strain       | PBP | Substrate | CPase | Ac2LALac | Ac2LALac | Ac2LALac |
|--------------|-----|-----------|-------|----------|----------|----------|
|              |     |           | pmol/min/µg protein | (24 µM) | (22 µM)  | (22 µM)  |
| JE 11191 (dacA*) | 5   | <0.1      | 1.1   |          |          |          |
| PA3092 (dacA*) | 5   | 18        | 161   | 92       | 156      |          |

* Assays were carried out as previously described (11).

4 UDP-MAGDAA, UDP-acetylmuramyl-L-Ala-d-Glu-meso-diaminopimelyl-d-Ala-d-Ala.
The reaction was stopped by addition of 1 ml carrier, 100 \textmu \text{M} thetatic substrate with mutant and wild type alanyl-D-lactate (49 mCi/mmol) (19) was added to start the reaction. Times indicated in the preparation of radioactivity in p30 and acid at the times indicated (zero time is approximately 5 min). For SDS-PAGE (B), samples at the times indicated in A (1 and 2) were precipitated by addition of 40 \mu\text{l} of 15% trichloroacetic acid. After centrifugation at 8000 rpm in the Sorvall SS-34 rotor for 15 min, the precipitate was then washed with 8 ml and 1 ml of the same solutions. The filters were dried in an oven and analyzed for radioactivity. For SDS-PAGE, samples at the times indicated in A (1 and 2) were precipitated by addition of 40 \mu\text{l} of 15% trichloroacetic acid. After centrifugation at 8000 rpm in the Sorvall SS-34 rotor for 15 min, the precipitate was then washed with 8 ml and 1 ml of the same solutions. The filters were dried in an oven and analyzed for radioactivity. For SDS-PAGE, samples at the times indicated in A (1 and 2) were precipitated by addition of 40 \mu\text{l} of 15% trichloroacetic acid. After centrifugation at 8000 rpm in the Sorvall SS-34 rotor for 15 min, the precipitate was then washed with 8 ml and 1 ml of the same solutions. The filters were dried in an oven and analyzed for radioactivity.

As a carrier, 100 \mu\text{l} of 1% Triton X-100 was added, and the reaction mixtures were then poured onto 2.4-cm disks of glass filter (Whatman GF/A) using a Millipore filtration apparatus. The reaction tubes were rinsed with 1 ml of 10% trichloroacetic acid twice and with 0.5 ml of 10% trichloroacetic acid at the times indicated (zero time is approximately 5 min). As a carrier, 100 \mu\text{l} of 1% Triton X-100 was added, and the reaction mixtures were then poured onto 2.4-cm disks of glass filter (Whatman GF/A) using a Millipore filtration apparatus. The reaction tubes were rinsed with 1 ml of 10% trichloroacetic acid twice and with 0.5 ml of 10% trichloroacetic acid at the times indicated (zero time is approximately 5 min).

Sorption was a result of the large amounts of PBP 5 for the substrate acyl-PBP 6 potentially significant. That most of the input \([14\text{C}]\text{Ac}_{2}\text{LA} \text{ Lac} \) was hydrolyzed to form \([14\text{C}]\text{diacetyl-L-Lys-D-Ala} \) in a short incubation period probably explains the low plateau of the acyl-PBP 5 complex. The accumulation of the acyl-PBP 5 complex was dependent on pH (Fig. 6). The optimum pH was around 9. This pH dependence is very similar to that of CPase activity of the wild type PBP 5.

Since wild type PBP 5 actively catalyses the CPase reaction, the formation of acyl-enzyme complex is transient, and the intermediate does not accumulate. Fig. 5 shows that the acyl complex with the wild type PBP 5 formed much more rapidly than that of the mutant protein, and its amount decreased with time.

These results indicate that the PBP 5 of JE 11191 has a defect(s) in an amino acid residue(s) of the wild type PBP 5 which is involved in the transfer of both the substrate acyl and penicilloyl moiety to H_2O. This, in turn, strongly suggests that penicillins covalently bind to the active site of a PBP 5 molecule. To determine whether the same amino acid residue is an acylation site for the substrate and penicillins, an acyl-PBP 5 (JE 11191) complex derived from \([14\text{C}]\text{Ac}_{2}\text{LA} \text{ Lac} \) or \([14\text{C}]\text{penicillin G} \) was partially digested with CNBr or proteases as described previously for other bacterial PBPs (16), and the digests were analyzed by SDS-PAGE and fluorography. As shown in Fig. 7, in the case of digestion with CNBr or chymotrypsin, the profiles of the acyl moiety containing peptides were identical when the substrate-derived acyl-PBP 5 and penicilloyl-PBP 5 were compared. However, different patterns were obtained in the case of the digestion with Staphylococcus aureus V8 protease.

The finding that treatment of the substrate-derived acyl-PBP 5 and the penicilloyl-PBP 5 with either CNBr or chymotrypsin yielded identical peptide maps suggest that the substrate-derived acyl moiety and the penicilloyl moiety are either on the same amino acid residue or on the same peptide bond. On the other hand, it seems more likely that the penicilloyl moiety and the substrate-derived acyl moiety are really located on the same amino acid residue in the mutant enzyme. Close examination of the cleavage patterns by S. aureus V8 protease indicates that, in the partial proteolysis of these two forms of the PBP 5, some radioactive peptides are shared and some are not. Moreover, the PBP 5 acylated by substrate is not degraded to peptides as small as those derived from penicilloyl PBP 5. If the substrate-derived acyl moiety were to shield some otherwise susceptible peptide bond from cleavage by the S. aureus V8 protease, then such a result would be obtained (compare Ref. 17) and has several precedents.

As described above, the dac4 mutation effects the desaclylation step of PBP 5 while having only a minimal effect on the acylation step (as possibly seen by the lower affinity of the mutant PBP 5 for penicilillin G (Fig. 4) or the slow

diacetyl-L-Lys-D-Ala. This conversion was probably caused by the CPase activity of PBP 6 which may exist in a trace amount in the purified PBP 5 of JE 11191 (see Fig. 1). The use of the large amounts of PBP 5 for the substrate acyl-

![Fig. 5. Accumulation of acyl-enzyme derived from synthetic substrate with mutant and wild type PBP 5.](image_url)
Mutant Penicillin-binding Protein 5 of E. coli

Fig. 7. Peptide mapping of [14C]penicillin G-labeled and of [14C]diacetyl-L-lysyl-D-alanyl-D-lactate-labeled mutant PBP 5. The experiment was carried out as described in Ref. 11, Fig. 10. A, peptide patterns detected by staining with Coomassie brilliant blue; B, peptide patterns detected by autoradiography. Odd-numbered lanes represent [14C]penicillin G-labeled mutant PBP 5, and even-numbered lanes, 14C-substrate-labeled mutant PBP 5. Lanes 1 and 2, undigested proteins; lanes 3 to 6, digestion with CNBr for 3 min or 8 min; lanes 7 to 10, digestion with S. aureus protease at 10 or 50 μg/ml; lanes 11 to 14, digestion with α-chymotrypsin at 1 or 2 μg/ml.

Fig. 8. Inhibition of PBP 5 by pCMB or by mutation. Proposed enzyme mechanisms. E, PBP 5 (wild); E', PBP 5 (mutant); S, CPase substrate; P, penicillin G.

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2 Re-examination of the sensitivity of the wild type PBPs 5 and 6 to pCMB in fact indicates that PBP 5 is far more sensitive to inhibition than PBP 6 using the separated proteins. The concentration of pCMB required for 50% inhibition was 4 μM for PBP 5 and 49 μM for PBP 6 (pre-incubation at 25 °C for 10 min).
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H Amanuma and J L Strominger

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