Differences between inner membrane and peptidoglycan associated BPB1B dimers of Escherichia coli
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The rod-shaped gram-negative bacterium *Escherichia coli* has an exoskeleton to maintain its shape and to withstand differences in turgor pressure between the cytoplasm and environment. This exoskeleton, called the peptidoglycan layer, consists of glycan chains cross-linked by short peptides. The last stage of synthesis of the peptidoglycan layer takes place in the periplasm. The high-molecular-weight penicillin-binding proteins (HMW-PBPs [21, 22]) are responsible for the last stages of synthesis. The HMW-PBPs all seem to have two enzymatic activities; they can elongate glycan chains (transglycosylase activity) and cross-link the peptidoglycan-associated dimers (transpeptidase activity) [8-11, 18]. The HMW-PBP which has the highest in vitro activity is PBP1B (24), a protein of approximately 88 kDa. PBP1B exists in three different forms, which are all transcribed from the same gene, *ponB*. The forms differ in the length of the amino terminus (17). Earlier studies have revealed that many types of PBP1B dimers can occur in dimeric forms, which are detectable at a position corresponding to a molecular mass of about 140 kDa after sodium dodecyl sulfate (SDS)-gel electrophoresis (25).

In this study, we investigated further properties of the PBP1B dimers, including the nature of the bond in the dimer and the association of the dimer with the envelope. We found that the bonds of the peptidoglycan-associated dimers differ from the bonds within the inner membrane-associated dimers. The inner membrane- and the peptidoglycan-associated dimers are both linked through a disulfide bridge. However, the inner membrane-associated dimers appear to have an additional bond, which is breakable by zinc. It appeared, furthermore, that the dimer is very heat stable.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** All bacterial strains are derivatives of *E. coli* K-12. The following strains were used: CSH26 [Δ(ara-pro) ara thi] and MC4100 [F- Δ(ara-pro) Δ(galU-lac) Δ(lacYIΔ159) DE3 (pLAFR1) pLS6 (pK18Mobs) pUB110]. pBS99 was used as the PBP1B-overproducing plasmid (2). This plasmid contains the *ponB* gene and the *cat* gene responsible for chloramphenicol resistance.

**Media and growth conditions.** Cells were grown in TY medium, which contained 1% tryptone (Difco Laboratories, Detroit, Mich.), 0.5% yeast extract (Difco), and 0.5% NaCl. Liquid cultures were grown in a water bath shaker at 37°C. Plates contained TY medium with 2% Bacto Agar (Difco). When necessary, chloramphenicol (35 μg/ml; Serva, Heidelberg, Germany) was added. The cell concentration was determined from a 2-ml sample taken from a liquid culture by measuring the *A*₅₅₀ in a T-1 spectrophotometer (Gilford Instrument Laboratories Inc., Halstead, Essex, England). To the sample, 0.5 ml of 0.5% formaldehyde was added.

**Reagents and enzymes.** All chemicals were from Merck (Darmstadt, Germany) unless stated otherwise.

**Isolation of cell envelopes.** Cell envelopes were isolated by the following procedure. One liter of culture was harvested in the exponential growth phase. The pellet was resuspended in 50 mM potassium phosphate buffer (pH 7.4) containing 5 mM MgSO₄ and 1 mM phenylmethylsulfonyl fluoride and sonicated in a model B-12 Sonifier (Branson Sonic Power Co., Danbury, Conn.). Sonication was done with the large tip three times for 20 s each at 60 W. Remaining whole cells were removed by centrifugation at 18,000 × *g* for 30 min at 4°C. For the cation incubation experiments, the same procedure was followed except that the buffer contained 50 mM potassium phosphate (pH 7.4) only.

**Isolation of cytoplasmic membrane proteins.** Proteins associated with the inner membrane were solubilized from isolated cell envelopes by incubation for 30 min in 80 mM Tris- HCI (pH 8.0)–8 mM EDTA–0.5% (wt/vol) sodium laurel sarcosinate (Sarkosyl) by the method of Filip et al. (5).

**Isolation of peptidoglycan-associated proteins.** Peptidoglycan-associated proteins were isolated by centrifugation for 5 min at 10,000 rpm in a Beckman Airfuge for extraction of isolated cell envelopes at 60°C for 30 min in a buffer containing 2% (wt/vol) SDS as described by Rosenbusch (20).

**Determination of protein concentration.** The protein concentration in isolated cell envelopes was determined as described by Lowry et al. (16).

**Antibodies.** Polyclonal antibodies directed against PBP1B were described by den Blauwen et al. (4).

**Electrophoresis and immunological techniques.** SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (14). The preparation in sample buffer (25) contained 100 μg of protein per lane except for the cell fractionation samples. In the latter case, cell envelopes containing 200 μg of protein were fractionated and the soluble fraction was then brought onto the gel as one sample; the pellet was resuspended in 80 mM Tris-HCl (pH 8.0) and also applied to the gel as one sample. Western blotting (immunoblotting) was performed as described by Towbin et al. (23), with the modifications described previously (26).

**Artificial cross-linking.** Artificial cross-linking of isolated cell envelopes was performed as described by Ioannides et al. (7). DSP [diethylaminoethylpropiolate] was used at a concentration of 1 mg/ml.

**RESULTS**

**Thermal stability of the PBP1B dimer.** To study the thermal stability of the dimer in vitro, isolated cell envelopes of the PBP1B-overproducing strain CSH26(pBS99) were incubated in sample buffer at several temperatures for 10 min. They then
were cooled immediately on ice water and applied to an SDS-polyacrylamide gel. A Western blot of these samples was incubated with polyclonal antibodies against PBP1B (Fig. 1). As can be seen, the dimer is still intact at 80°C (Fig. 1, lane 6) but is dissociated at 90°C (Fig. 1, lane 7). This finding indicates that the thermostability of the dimers is very high. Fractionation experiments indicated that the peptidoglycan- and inner membrane-associated dimers are similar in heat stability (data not shown).

**Association of PBP1B with peptidoglycan.** Earlier experiments suggested that the PBP1B dimer cofractionated more easily with the cell wall than the monomeric form did (25). We therefore investigated whether it would be possible to cross-link PBP1B artificially to the peptidoglycan layer. Isolated cell envelopes were incubated with DSP and then fractionated with SDS in a peptidoglycan-containing fraction (the pellet) and a membrane fraction (the supernatant). DSP is a cross-linker which is cleavable through its disulfide bridge. A cleavable cross-linker was chosen because earlier experiments (25) indicated that disulfide cross bridges were not involved in the protein interactions inside the dimer (the following experiments, however, indicate that this view needs to be corrected). It was reasoned that this cross-linker would allow breaking of the artificial cross-linkages while leaving the dimer intact. What can be expected after a cross-linking experiment? First, proteins can become cross-linked to proteins or peptides that are functionally in their vicinity. Such cross-linked complexes are expected to produce a discrete band after SDS-PAGE. Second, proteins can become cross-linked to proteins or peptides which are nearby in the incubation mixture just fortuitously; such cross-linked complexes can vary in molecular weight and will produce a smear after SDS-PAGE.

The results of an experiment with isolated cell envelopes of CSH(pBS99) which were fractionated after cross-linking in a peptidoglycan-containing fraction and a membrane fraction are shown in Fig. 2. On the Western blot, it can be seen that labeling of the polyclonal antibodies against PBP1B occurred along the whole lane of the membrane fraction sample (Fig. 2, lane 4). It was therefore concluded that in this case the PBP1B molecules were randomly cross-linked to proteins and peptides. In the DSP-treated peptidoglycan fraction, however, no discrete bands or background smear could be observed (Fig. 2, lane 2). This we interpret to mean that the PBP1B molecules have been cross-linked in such a way that they cannot enter the gel. This is expected to happen if PBP1B cross-links to a component which is too large to enter the polyacrylamide gel. The most likely candidate for such a component is the peptidoglycan itself.

After the cross-links were broken by incubating the membrane fraction with β-mercaptoethanol, bands could be detected at two positions: 140 kDa, the position at which the PBP1B dimer runs, and 88 kDa, the molecular mass of the PBP1B monomer (Fig. 2, lane 3). Because PBP1B can occur in three different forms, multiple bands are visible at the two positions in the gel (25). After breaking of the cross-links in the peptidoglycan fraction, only one band, at 88 kDa, could be detected (Fig. 2, lane 1). This result could indicate that the dimers which have been shown to be present in the peptidoglycan-containing fraction (25) have a response to β-mercaptoethanol different from that of the inner membrane-associated dimers (Fig. 2; compare lane 1 with lane 3).

**Effect of β-mercaptoethanol on the dimers in the peptidoglycan-containing fraction and in the membrane fraction.** As shown above, it was possible to cross-link PBP1B to the peptidoglycan-containing fraction. However, it was not possible to dissociate intact dimers from these artificially cross-linked complexes. Therefore, we tested whether the dimers in the peptidoglycan-containing fraction had the same response to β-mercaptoethanol as the dimers present in the membrane fraction. Isolated cell envelopes of the PBP1B-overproducing strain CSH26(pBS99) were incubated with SDS and then fractionated. The insoluble (peptidoglycan) and soluble (membrane) fractions were each incubated with β-mercaptoethanol for 30 min at room temperature. The fraction of dimers insoluble by SDS was sensitive to β-mercaptoethanol (Fig. 3; compare lane 3 with lane 4), whereas the fraction of dimers soluble by SDS was not (Fig. 3; compare lane 2 with lane 1). Similar results were obtained when times of incubation with β-mercaptoethanol were increased to 60 min and also when cell envelopes fractionated after incubation with Sarkosyl were used (data not shown). From these experiments, it can be concluded that one or more disulfide bridges stabilize the peptidoglycan-associated dimer.

**Nature of the dimer interaction in the membrane fraction.** Because the inner membrane-associated dimers could not be
dissociated by β-mercaptoethanol (see above), it can be assumed that other bonds keep this class of dimers together.

These bonds might be additional to disulfide bridges. In that case, incubation with β-mercaptoethanol would break the disulfide bridge, but that would be masked by the remaining additional bond, and so in an SDS-PAGE experiment, a dimer would remain. In the absence of β-mercaptoethanol, the dissociation of the unknown bond alone would be masked because the disulfide bridge is still intact.

The following experiment was set up to determine the nature of the inner membrane-associated dimer interaction. First, we tested the influences of the divalent cations Mg\(^{2+}\), Ca\(^{2+}\), and Zn\(^{2+}\), and their chelator EDTA, on the membrane-associated dimer. Also, the effect of salt was tested by adding NaCl in concentrations ranging from 0.1 to 0.5 M. To avoid the effect of a possible masking of the dissociation of the bond by the presence of a disulfide bridge (as described above), the samples were incubated in sample buffer with or without β-mercaptoethanol after incubation with the agent tested. Figure 4 shows the results of incubation with zinc at 20 mM. The results revealed that zinc is able to dissociate the dimer in combination with β-mercaptoethanol. Zinc in combination with EDTA (Fig. 4, lane 5) and zinc alone (Fig. 4, lane 3) could not dissociate the dimer. In a separate experiment, it was found that EDTA alone also could not dissociate the dimer (data not shown). These results suggest that two different bonds, a disulfide bridge and a bond which is breakable by zinc, are present in the membrane-associated dimers.

The other cations tested were not able to destabilize or to stabilize dimers under all conditions tested, i.e., in a 2, 20, or 200 mM concentration (data not shown). Also, NaCl in the concentrations tested (0.1 to 0.5 M) did not have an effect on the dimer (data not shown).

**DISCUSSION**

As earlier studies revealed, PBP1B can occur in a dimeric form (25). However, no comparison has been made between the peptidoglycan-containing fraction and a membrane-containing fraction. In this study, the nature of the dimer was further analyzed.

It was found that there were two classes of dimers: (i) a peptidoglycan-associated class of dimers which can be dissociated by β-mercaptoethanol and which can be cross-linked artificially to the peptidoglycan layer, and (ii) a class of dimers associated with the membrane fraction. These dimers are likely to occur in the inner or cytoplasmic membrane, and they could be dissociated only by β-mercaptoethanol in combination with zinc. Zinc alone or β-mercaptoethanol alone could not dissociate the dimers. The disulfide bond of the two classes of dimers is expected to lie at the carboxy-terminal end of the protein, because this is the only region where two cysteines of PBP1B are located (2) (Fig. 5). Whether one or two disulfide bonds are present is not yet known.

The mechanism by which zinc is able to break the dimerization has not yet been resolved. The sequence of PBP1B was compared with sequences known to be able to bind zinc. We found that in PBP1B, there are no sequences which are known from analyses of other proteins to play a role in zinc binding (1, 6, 13, 15). Examples of zinc influencing dimerization in proteins mostly (like aspartate transcarbamoylase) involve stabilization and not, as in our case, destabilization of the dimer (12). The bond breakable by zinc must lie in the amino-terminal half of the protein, because experiments with fusion proteins (26) revealed that fusion proteins containing fewer than 299 amino-terminal amino acids were not able to form the dimer, whereas fusions containing 405 or more amino-terminal amino acids could still dimerize (Fig. 5).

It could also be shown that the interaction between the dimers is very heat resistant. Temperatures up to 80°C in the presence of SDS could not dissociate the dimer. Trimmers of the
outer membrane protein PhoE, for instance, dissociate at 65°C under similar conditions (22a). Whether there is a difference in heat stability between peptidoglycan associated- and inner membrane-associated dimers is not yet known. Nakagawa et al. (19) showed another heat-resistant property of PBP1B, namely, its binding to penicillin G. PBP1B could bind penicillin G after 10 min of incubation at 60°C (the highest temperature tested). In contrast, only 50% of PBP1A was able to bind penicillin G after 10 min at 45°C. Of the HMW-PBPs tested, PBP1B appeared to be the most heat-resistant enzyme, whereas PBP1A was the least heat stable (19).

Whether there is a difference in peptidoglycan synthesis between monomers and the two classes of dimers is a subject for further investigations. Preliminary experiments have indicated, however, that 3H-labeled meso-diaminopimelic acid becomes preferentially associated with the dimers.

REFERENCES
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