Demonstration of Molecular Interactions between the Murein Polymerase PBP1B, the Lytic Transglycosylase MltA, and the Scaffolding Protein MipA of *Escherichia coli*

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Enlargement of the stress-bearing murein sacculus of bacteria depends on the coordinated interaction of murein synthases and hydrolases. To understand the mechanism of interaction of these two classes of proteins, affinity chromatography and surface plasmon resonance (SPR) studies were performed. The membrane-bound lytic transglycosylase MltA when covalently linked to CNBr-activated Sepharose specifically retained the penicillin-binding proteins (PBP’s) 1B, 1C, 5, and 3 from a crude Triton X-100 membrane extract of *Escherichia coli*. In the presence of periplasmic proteins also PBP1A was specifically bound. At least five different non-PBP’s showed specificity for MltA-Sepharose. The amino-terminal amino acid sequence of one of these proteins could be obtained, and the corresponding gene was mapped at 40 min on the *E. coli* genome. This MltA-interacting protein, named MipA, in addition binds to PBP1B, a bifunctional murein transglycosylase/transpeptidase. SPR studies with PBP1B immobilized to ampicillin-coated sensor chips showed an oligomerization of PBP1B that may indicate a dimerization. Simultaneous application of MipA and MltA onto a PBP1B sensor chip surface resulted in the formation of a trimERIC complex. The dissociation constant was determined to be about 10−10 M	extsuperscript{s}. The formation of a complex between a murein polymerase (PBP1B) and a murein hydrolase (MltA) in the presence of MipA represents a first step in a reconstitution of the hypothetical murein-synthesizing holoenzyme, postulated to be responsible for controlled growth of the stress-bearing sacculus of *E. coli*.

The cell envelope of Gram-negative bacteria is stabilized by a thin, monolayered exoskeleton consisting of the cross-linked biopolymer murein (peptidoglycan) (1, 3). By forming a bag-shaped structure the murein netting, glycan strands cross-linked by peptides, completely encloses the cell. Different models for the growth mechanism of this stress-bearing bacterial exoskeleton, called sacculus, have been put forward (2, 4, 5, 6). Despite the discrepancies in some details, the models all agree on one point, namely that murein synthases and hydrolases have to cooperate with each other to allow for safe insertion of new material into the growing sacculus. Specific protein-protein interactions between a number of enzymes involved in the metabolism of the murein sacculus have been demonstrated by affinity chromatography (7–8). This includes members of two opposing groups of enzyme specificities, murein polymerases (synthases) (9) and murein depolymerases (hydrolases) (10). The bifunctional murein transglycosylases/transpeptidases (11, 12), known as penicillin-binding proteins (PBP’s) 1A, 1B and PBP1C, and the transpeptidases PBP2 and PBP3 (13) as well as the endopeptidases PBP4 and PBP7 (14–16) and the lytic transglycosylases Slt70, MltA, and MltB (17–20) were found to interact with one another. These findings may reflect an in vivo assembly of murein synthases and murein hydrolases into a multienzyme complex that has been named a “ying yang complex” (3, 6). Such a murein synthesizing machinery has been proposed to facilitate the coordination of the action of the different enzymes involved in enlargement and seption of the murein sacculus. Formation of a multienzyme complex could be a means to secure an effective control of the potentially autolytic murein hydrolases (10, 21, 23) and to guarantee that growth of the sacculus occurs with maintenance of the specific shape of the bacterium. Here we are presenting evidence that a complex of the bifunctional transglycosylase/transpeptidase PBP1B with the lytic transglycosylase MltA can be reconstituted on a BLACORE™ sensor chip surface when a third protein, the newly discovered MltA-interacting protein, MipA, is present.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—The standard strain in this study was *Escherichia coli* MC1061 (24). In addition, the mltA deletion strain *E. coli* LT12 (ΔmltA, Cam) (19) and *E. coli* AT1325 (mer678) that overproduces PBP1B by a factor of about 4 (25) were used. The cloning vector was the pBR322 derivative pJFK118EH (26) that carries a kanamycin resistance gene and the IPTG-inducible tac promoter. Growth was routinely done in Luria Bertani medium (LB) (27) with aeration in a shaking water bath at either 30 or 37 °C. Antibiotics (50 μg/ml kanamycin or 20 μg/ml chloramphenicol) were added when needed. Absorbance (A730) readings were done in an Eppendorf photometer (Eppendorf, Hamburg, Germany) at 578 nm.

**Recombinant DNA Methodology**—Plasmid DNA was isolated by using the alkaline lysis method (28) and transformations followed the MeSO method described by Inoue et al. (29). Amplification of DNA regions was accomplished by PCR with commercially synthesized oligonucleotides (MWG Biotech) as indicated below. Reaction mixtures (100 μl) contained 10 μl of PCR reaction buffer (Stratagene, Heidelberg, Germany), 1 μl of the Kohara phage lyase, 1 μl of primer V, 1 μl of primer H, and 0.8 μl of dNTP solution to give a final concentration of 200 μM dATP, dGTP, dCTP, and dTTP. After the addition of 5 units of *Pfu* DNA polymerase (Stratagene) at 94 °C, 30 cycles of the following steps were performed: 1 min at 92 °C, 1 min at 52 °C, and 3 min at 68°C.

1 The abbreviations used are: PBP, penicillin-binding protein; LB, Luria-Bertani; RU, resonance units; PAGE, polyacrylamide gel electrophoresis; SPR, surface plasmon resonance; IPTG, isopropyl-1-thio-b-D-galactopyranoside; PCR, polymerase chain reaction.

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Molecular Interactions of Murein Hydrolases and Synthases

72 °C. The products were purified with the help of a QIAquick PCR purification kit (Qiagen, Hilden, Germany).

**Construction of Expression Plasmids**—To construct plasmids for the overproduction of MTA and of Mipa, respectively, the βRI22 derivative pFK118EH that carries a kanamycin resistance marker and the inducible expression vector pET-22 (Novagen) (1% Triton X-100) was used as a cloning vector. Amplification of a fragment with amplified by PCR from the lysate of the Kohara lambda phage 458 (38) that carries the mtaA gene (19). An appropriate restriction site was inserted by using the oligonucleotides mtaV (5′-AGAGGATCC-GAGGAGCTGTTGGAATAAGTACCC-3′) and mtaH (5′-AATGATGC-TCTACGGCCCTTTTGGTTACCTCAGATTTTGG-3′) to introduce an EcoRI and SalI restriction site. The purified and restricted PCR product was inserted into pFK118EH cut with EcoRI and SalI yielding plasmid pVW3.

**Preparation of Cell Extracts**—Cells were cooled down when an absorbance at 578 nm (A578) of 0.5−0.6 was reached and harvested by centrifugation (7,000 × g, 10 min, 4 °C). All the following steps were performed in the cold. The obtained pellet was resuspended otherwise, resuspended in 10 ml Tris- HCl, pH 8.0, and the protease inhibitor phenylmethylsulfonyl fluoride (1 mmol) and DNase (10 μg/ml) were added before the cells were broken in a French press at 16,000 pounds/square inch. Membranes were spun down (100,000 × g, 45 min), and proteins were extracted by resuspending the membranes in Triton X-100 extraction buffer (10 mM Tris maleate, pH 6.8, 10 mM MgCl2, 150 mM NaCl, 2% Triton X-100) and stirring for 12 h. Unsolubilized membrane debris was removed by centrifugation (100,000 × g, 45 min), and the supernatant containing the solubilized proteins was stored at −20 °C. Periplasmic proteins were obtained according to the method of Wirtholt et al. (30).

**Purification of MTA**—In order to obtain high amounts of MTA MC1061/pMAT was grown in a 200-liter fermenter at 37 °C in LB containing 1 M NaCl and stirred for 2 h. After centrifugation (see above) the membranes were spun down (100,000 × g) and resuspended in 1000 ml of 10 mM Tris maleate buffer, pH 5.2, and 300 ml of 1 M NaCl. Before application of the sample the column was washed at a flow rate of 20 ml/h with 350 ml of equilibration buffer. Bound proteins were eluted at a flow rate of 30 ml/h with a salt gradient from 120 ml to 1 mM NaCl in 300 ml of equilibration buffer followed by a gradient to 2 mM NaCl. MTA eluted in 10 fractions (2.5 ml each) with highest activity around 700 mM NaCl. The fractions containing the activity of MTA were pooled and concentrated to a final volume of 200 mM NaCl. The yield was 20 mg of protein with a specific activity of 7580 units/mg. One unit of MTA activity is defined as the amount of enzyme that hydrolyzes 1 μg of murein in 10 min at 30 °C with the indicated buffer conditions. The enzyme preparation (0.44 mg/ml) did not lose activity after storage for 4 months when kept at 6 °C.

**Purification of Mipa**—Mipa was isolated from the MTA deletion mutant E. coli LT2 harboring pVW3. Cells were grown in LB (10 liters) at 30 °C and induced by the addition of 0.05 mM IPTG at an A578 of 0.15. Growth was stopped when an A578 of 0.7 was reached. All following steps were performed at 4 °C. After resuspending the cells (20 g wet weight) in 10 mM Tris maleate, 10 mM MgCl2, 0.02% NaN3, pH 6.8, they were broken in a French pressure cell as described above. Membrane-associated proteins were removed by centrifugation for 1 h at 100,000 × g. The pellet (8 g wet weight) was resuspended in 50 ml of 10 mM Tris maleate, 10 mM magnesium chloride, 0.02% NaN3, pH 6.8, containing 500 mM NaCl and 2% Triton X-100 and stirred for 16 h to solubilize the membrane proteins. The extract was dialyzed against 20 mM Tris-HCl buffer, pH 8.0, containing 50 mM potassium chloride and 0.02% NaN3 prior to Q-Sepharose chromatography. A Q-Sepharose column (16 ml) was washed with 12 volumes of 1 M acetic acid and 5 volumes of deionized water before equilibration with 20 mM Tris-HCl buffer, pH 8.0, containing 50 mM sodium chloride, 1% Triton X-100, and 0.02% NaN3 at a flow rate of 10 ml/h. After applying the dialyzed membrane extract, Mipa was eluted during isoionic washing with equilibration buffer. The fractions of 10 ml were analyzed by SDS-12% PAGE (31) and stained with Coomassie Blue. The yield was 29.6 mg of purified Mipa present in the two fractions with the highest concentration of Mipa.

**Purification of the Bifunctional PBPs 1A and 1B by Moenomycin Affinity Chromatography**—PBPA1 and -1B were purified by affinity chromatography on moenomycin-agarose followed by ion-exchange chromatography. For immobilization of moenomycin to an agarose matrix, one batch of Affi-Gel 10 (Bio-Rad) was dissolved in methanol in a final concentration of about 30 mg/ml. The methanolic moenomycin solution was then mixed with Affi-Gel 10 (Bio-Rad) and incubated at 4 °C overnight. The gel was filtrated and extensively washed with methanol to remove excess moenomycin. Free functional groups of the Affi-Gel were blocked by incubation of the gel with 100 mM Tris-HCl, pH 8.0, for 3 h at 4 °C. The gel was washed with 0.02% NaN3, 1% Triton X-100, and 1 M NaCl. The moenomycin-agarose suspension. The moenomycin-agarose was added to a column (10 ml) and equilibrated with extraction buffer (10 mM Tris maleate, 150 mM NaCl, 10 mM MgCl2, 2% Triton X-100, pH 6.8). A Triton X-100 extract of E. coli AT1235 (2 g of protein; 20 mg/ml) was applied to the column at a flow rate of 10 ml/h. After a washing step with 100 ml of extraction buffer, elution of the proteins that were bound to the moenomycin column was performed with 100 mM moenomycin in extraction buffer. The fractions were analyzed for PBPs as described previously (33). Although it was not possible to separate PBPA1, -1B, and -1C from each other, almost all other proteins including other PBPs were effectively removed by this specific chromatography step. PBPA1 and PBP1B could then be separated using an additional purification by ion-exchange chromatography. According to the fractions containing PBPA1 and PBP1B were pooled, dialyzed against 10 mM sodium acetate buffer, pH 5.0, containing 10 mM MgCl2, 25 mM NaCl, 0.05% Triton X-100, and applied onto a 1-ml Highload Mono S column (Amersham Pharmacia Biotech). After washing the column with 5 ml of dialysis buffer the proteins were eluted at a flow rate of 0.5 ml/min using a linear gradient (20 ml) of 25 mM to 1 mM NaCl in the same buffer. PBPA1 eluted at a salt concentration of 120–290 mM NaCl and PBP1B at 260–480 mM (1.25 ml; about 3 μg/ml). PBPC1 was not found in the eluted fractions, probably because of precipitation during dialysis. The purified PBPs were dialyzed against HBS buffer (10 mM HEPES, 10 mM MgCl2, 150 mM NaCl, 0.05% Triton X-100), pH 7.4, and stored at −20 °C.

**Affinity Chromatography**—As a matrix to immobilize proteins,
Molecular Interactions of Murein Hydrolases and Synthases

CNBr-activated Sepharose (Amersham Pharmacia Biotech) was used. Purified proteins were coupled basically following the instructions of the manufacturer. Coupling of the proteins (between 8 and 12 mg) was done overnight at 6 °C with gentle agitation in 0.1 M sodium phosphate buffer, pH 7.0, containing 500 mM NaCl, 1% Triton X-100, and 0.8 g of activated Sepharose. After washing the gel, the coupling sites were blocked with Tris by incubation in 0.1 M Tris-HCl, 500 mM NaCl, and 1% Triton X-100, pH 8.0, overnight at 6 °C. The gel suspension was then washed alternating with 0.1 M Tris-HCl, 500 mM NaCl and 1% Triton X-100, pH 8.0, and 0.1 M sodium acetate buffer, pH 4.8, containing 500 mM NaCl and 1% Triton X-100, and finally resuspended in 16 mM Tris maleate, 10 mM MgCl2, 0.02% NaN3, pH 6.8, containing 50 mM NaCl and the same concentration of Triton X-100 as present in the extract to be applied (1% for membrane proteins, 0.28% for a mixture of membrane and periplasmic proteins). The Sepharose was filled into a 3-ml column and equilibrated with the same buffer. As control material (Tris-Sepharose) one batch of activated Sepharose was treated identically except that no protein was added.

The affinity chromatography was performed at 6 °C. After application of the dialyzed extract containing 50 mM NaCl at a flow rate of 1 ml/h (3.5 ml/h if a membrane extract containing periplasmic proteins was applied), the column was washed at 4.5 ml/h with 70 ml of 10 mM Tris maleate, 10 mM MgCl2, 0.02% NaN3, pH 6.8, containing 50 mM NaCl and 0.05% Triton X-100, pH 6.8, containing 500 mM NaCl and 1% Triton X-100. The low Triton concentration of 0.05% that is suitable for the PBPs in this fraction was chosen to allow monitoring of the eluates by UV detection. The retained proteins were eluted at 4.5 ml/h by two successive salt steps with 54 ml of 10 mM Tris maleate, 10 mM MgCl2, 0.02% NaN3, pH 6.8, containing 150 mM NaCl and 0.05% Triton X-100, and with 54 ml of the same buffer containing 1 M NaCl and 0.05% Triton X-100. A modified method was used to detect non-PBPs with high sensitivity. For this, the extract was applied with 400 mM NaCl at a flow rate of 1 ml/h. After washing the column at 4.5 ml/h with 70 ml of 10 mM Tris maleate, 10 mM MgCl2, 0.02% NaN3, pH 6.8, containing 400 mM NaCl and 0.05% Triton X-100, the retained proteins were eluted at 4.5 ml/h with the same buffer but containing 2 mM NaCl and 0.05% Triton X-100. The size of the fractions was 1.5 ml. The fractions were stored at −20 °C.

Penicillin-binding Protein Assay—PBPs were labeled using the 125I-labeled Bolton and Hunter derivative of ampicillin, prepared as described (33). Two microliters of the labeled ampicillin derivative were incubated with a 30-μl aliquot of the sample for 30 min at 37 °C. The PBP-penicillin complexes (34) were separated by SDS-10% polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography as described (33).

Immobilization of PBPs to Ampicillin-coated Sensorchips—The free amino group in the side chain of ampicillin was utilized for the immobilization of this β-lactam to a CM sensor chip via amino coupling. The coupling of ampicillin was performed according to the standard N-ethyl-N’-(3-dimethylaminopropyl)-carbodiimide hydrochloride/N-hydroxysuccinimide procedure recommended by BIAcore. The CM5 matrix of the sensor chip was activated by injecting 70 μl of a 1:1 mixture of 400 μM N-ethyl-N’-(3-dimethylaminopropyl)-carbodiimide hydrochloride and 100 μM N-hydroxysuccinimide at a flow rate of 10 μl/min. This was followed by applying 150 μl of an ampicillin solution (10 mg/ml in 100 mM sodium acetate buffer, pH 4.6). Activated functional groups on the sensor chip not saturated by ampicillin were blocked by injection of 70 μl of 1 M ethanolamine.

Immobilization of PBPs was routinely performed at the rather high temperature of 35 °C because this increased the yield of immobilization when a low concentration of protein had to be used. The binding of the PBPs to the ampicillin matrix was stopped when the desired level of about 1000–3000 RU was reached, and the chips were rinsed with 10 ml of Tris maleate buffer, pH 6.8, containing 1 mM NaCl and 2% Triton X-100. Remaining free ampicillin was digested by the injection of 120 μl of β-lactamase (50 units/ml) after washing the matrix the remaining constant.

Immobilization of MltA by Aminocoupling—Purified MltA (see above) was immobilized to BIAcore CM5™ sensor chips by direct coupling via free amino groups following the recommendations of the manufacturer. Active groups that remained unsaturated after immobilization of MltA were blocked by the injection of 70 μl of 1 M ethanolamine.

Protein-Protein Interaction Studies by SPR—Surface plasmon resonance (SPR) studies were performed with a BIAcore™ 2000 (BIAcore AB, Uppsala, Sweden). In order to determine the initial conditions for binding of the analyte to the immobilized ligand, qualitative experiments were performed prior to the quantitative analysis of the binding constants. If not stated otherwise the experiments were routinely done in HBS buffer, pH 7.4, at a flow rate of 10 μl/min. The injection volume of the analyte (about 30 μg of protein/ml) varied between 60 and 150 μl.

Regeneration of the sensor chip was achieved by injecting 30 μl of regeneration buffer (10 mM Tris maleate, 10 mM MgCl2, 1% NaCl, 2% Triton X-100, pH 6.8).

Estimation of Kinetic Parameters—The kinetic parameters of the protein-protein interactions in the BIAcore™ system were determined by equilibrium analysis. During equilibrium the following Equation 1 is valid (Rmax response at equilibrium; Rmax maximal response; Ken equilibrium association constant; C, concentration).

\[
dR/dt = k_C (R_{\text{max}} - R_{\text{eq}}) - k_R R_{\text{eq}} = 0
\]  

(Req can be obtained either directly from the sensorgrams or can be calculated by the BIAevaluation software. A graphical representation of Req against Rmax yields a straight line with the slope – Kc.

The graph is equivalent to a standard Scatchard plot. Rmax can be calculated from the intersection with the x axis.

Since the equation mentioned above is only valid if the formation of the complex on the surface of the sensorchip is kinetically controlled and not limited by transportation effects, high flow rates and low levels of immobilized ligand were used for the kinetic measurements.

The analyte was injected at a controlled injection rate of 200 μl/min to avoid individual binding events. For each set of experiments three different amounts of protein were immobilized in three of the lanes of the sensor chip; the fourth lane was used as a control and contained only a β-lactamase-digested ampicillin surface (see above). The analyte was varied in the range of 12.3 nM to 24.6 μM. Volumes of 150 μl were applied using the injekt function of the BIAcore™ system. During these experiments the temperature was kept constant at 20 °C and the flow rate at 10 μl/min.

To test that the observed binding of the analyte to the immobilized ligand was independent of mass transfer, some experiments were also performed at a higher flow rate of 40 μl/min. After injection of the analyte the dissociation phase was recorded for 30 min. During this time the system was washed with HBS buffer at a flow rate of 10 μl/min.

The evaluation of the sensorgrams was performed with the BIAevaluation program version 2.1 (BIAcore). The sensorgrams were imported into this program, and the base lines of each flow cell were normalized to zero as was done for the time at the start of the injection.

Afterwards the sensorgrams of the control lane was subtracted from the sensorgrams of the flow cells containing immobilized protein to remove the effects of unspecific binding to the ampicillin matrix. The Req values of the equilibrium were determined at a time point shortly before the end of the injection where the sensorgrams had reached equilibrium.

Additional Methods—Protein determination was according to the bicinchoninic acid method described by Smith et al. (22) using a kit from Pierce. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed the procedure published by Lugtenberg (32).

RESULTS

Specific Binding of PBPs to MltA-Sepharose—Purified MltA was coupled to CNBr-activated Sepharose and used for chromatography of various cell fractions, that is membrane proteins and a mixture of periplasmic proteins and membrane proteins. The eluates were first analyzed by PBPs. As shown by the autoradiography of an SDS-PAGE analysis of the eluates from MltA-Sepharose and from a Tris-Sepharose control column (see “Experimental Procedures”), the PBPs 1B, 1C, 2, and 3 were specifically retarded (Fig. 1). These proteins could not be eluted with 150 mM NaCl but did elute with 1 M NaCl. PBP4 was also retarded by the control column and eluted already with 150 mM NaCl. Hence we have no information whether PBP4 specifically interacts with the PBPs 1B, 1C, and 6 did not interact with MltA-Sepharose. The band below PBPs 5/6 is likely to be a degradation product of PBP3 since it is not present when PBP3 was removed from the Triton X-100 extract by immunoprecipitation. Otherwise the same PBPs were retained from a membrane extract, which was devoid of PBP3, as in the presence of PBP3 (data not shown), indicating that PBP3 is not the structural center of the complex. An interesting observation was made when a fraction of periplasmic proteins...
obtained from spheroplasts was combined with membrane proteins and then applied to the MltA-Sepharose column. It turned out that PBPA1, which was not retained when a pure membrane protein fraction was used (see Fig. 1), did specifically bind to the column and eluted at 150 mM NaCl (data not shown). Therefore, a periplasmic component exists that mediates the binding of PBPA1 to MltA-Sepharose. In addition to this periplasmic factor (that awaits further characterization), non-PBP proteins that specifically interact with MltA are also present in the membrane fraction as shown in the following paragraph.

**Specific Enrichment of Proteins Other Than PBPs by MltA-Sepharose Affinity Chromatography**—When the same aliquots of the eluates used for the PBP assay were separated by SDS-PAGE, silver staining of gels resulted in very faint bands only when the same aliquots of the eluates used for the PBP assay were separated by SDS-PAGE (10%). PBPs were visualized by autoradiography. Lane 1, sample; lane 2, flow-through; lane 3, wash fraction; lane 4, 150 mM NaCl fraction; lane 5, 1 M NaCl fraction.

![Fig. 1. PBP assay of proteins fractionated by affinity chromatography on MltA-Sepharose.](image)

FIG. 1. PBP assay of proteins fractionated by affinity chromatography on MltA-Sepharose. Purified MltA (10 mg) was coupled to CNBr-activated Sepharose (0.8 g) and filled into a column (3 ml). A Triton X-100 membrane extract of E. coli MC1061 (40 mg of protein) was applied at a flow rate of 1 ml/h, and the column was washed with 70 ml of buffer containing 50 mM NaCl (flow rate, 4.5 ml/h). Proteins were eluted with two salt steps, 150 mM and 1 M NaCl in buffer. As a control, a second sample was analyzed by chromatography on an identical column containing Sepharose that was treated with Tris instead of MltA. Aliquots (30 μl) of the pooled fractions from the eluate were incubated with 3H-labeled Bolton and Hunter ampicillin derivatives as described under “Experimental Procedures” and separated by SDS-PAGE (10%). PBPs were visualized by autoradiography. Lane 1, applied sample; lane 2, flow-through; lane 3, wash fraction; lane 4, 150 mM NaCl fraction; lane 5, 1 M NaCl fraction.

The proteins were then transferred electrophoretically onto polyvinylidene difluoride membranes (35), and the amino-terminal sequences were determined by using the method according to Edman and Begg (36). Only the 26-kDa protein (indicated by an arrow in Fig. 2, lane 4) yielded the following sequence of 13 amino acids: EGK-FXLGA(G)V(G)(N)V. This sequence was sufficient to identify the corresponding gene on the E. coli map. The gene that is identical with the recently registered gene yeaF (accession number 3025133) was named mipA to indicate that the product is an MltA-interacting protein.

**Cloning and Expression of mipA**—An open reading frame at 40 min on the E. coli map contains the sequence that matches the amino acid sequence obtained for the 26-kDa protein by Edman degradation. Upstream of the experimentally determined amino terminus Glu is an Ala-X-Ala site for the Lep signal peptidase I, and in front of this cleavage site is a typical leader sequence containing 15 hydrophobic amino acids. The sequence of the mature protein of 226 amino acids results in a theoretical molecular mass of 25,673 kDa, which is in agreement with the value of 26 kDa deduced from the SDS-PAGE. The protein consists of about 28% non-polar and 31% charged amino acids and has a calculated isoelectric point of pH 4.92. Transmembrane domains are not predicted. Homology searches revealed an open reading frame yeaT at 80 min on the map that codes for a protein that has 39% identical and 62% similar amino acids as compared with MipA. There is also a homologue, OmpV, present in Vibrio cholerae. The OmpV protein has 23% identical and 41% similar amino acids as compared with MipA and has 13 additional amino acids at the amino terminus. OmpV has been described to be a murein-associated protein and has been speculated to be related to porins (37). There is no homologue to MipA in Haemophilus influenzae or in Helicobacter pylori; however, the latter one
An inducible expression system to obtain high amounts of MipA for protein purification on a preparative scale was constructed on the basis of the pJFK118EH vector (26). The mipA gene, amplified by PCR using the Kohara lambda phage phage 331 (38) as a matrix and the oligonucleotides mip-V and mip-H as primers (see “Experimental Procedures”), was cloned behind the tac promoter to yield plasmid pWV3. Expression of MipA after induction in the presence of 1 mM IPTG resulted in cell lysis about 20 min later. This lysis was independent of the growth temperature, occurred also in the presence of 12% sucrose and 10 mM MgSO₄, and did not depend on the lytic transglycosylases MltA and Slt70 since it occurred even in a MltA deletion mutant as well as in the presence of bulgecin, a specific inhibitor of Slt70 (39). We assume that overproduction of MipA affects the membrane integrity. In accordance with this speculation, MipA was found in the membrane fraction and could be solubilized only in the presence of detergents such as Triton X-100. High concentration of salt (1 M NaCl) alone was not effective in releasing MipA from the membrane.

One-step Purification and Partial Characterization of MipA—Induction of expression of mipA from pWV3 in the presence of 0.05 mM IPTG resulted in the production of the protein in decent amounts without triggering cell lysis. These conditions were therefore used to isolate and purify MipA. Anion-exchange chromatography seemed to be a suitable purification step because of a calculated pI value around pH 4.9 for MipA. However, when a crude Triton X-100 membrane extract from the induced MltA deletion mutant LT12/pWV3 was applied onto a Q-Sepharose column, elution of MipA as monitored by SDS-PAGE started right away with the washing step. Nevertheless, this step resulted in a most effective purification of MipA since under the chosen conditions the majority of the proteins remained on the column.

Purified MipA was tested for murein hydrolase activity using the general murein hydrolase assay that measures the release of soluble products (muropeptides) from high molecular weight murein sacculi (16). No activity could be detected. Also, addition of MipA to a murein hydrolase assay with MltA did not affect the kinetics or final yield of the hydrolysis of the murein sacculi by MltA (data not shown).

Affinity Chromatography with MipA-Sepharose—To characterize the affinity of MipA to other murein-metabolizing enzymes besides MltA, the protein was covalently linked to CNBr-activated Sepharose (see “Experimental Procedures”). A crude membrane extract of E. coli MC1061 was adjusted to 400 mM NaCl and applied onto a MipA-Sepharose column. The column was then eluted with a salt step of 2 M NaCl. Western blot analysis of the eluate revealed the expected binding of MltA to the column but not of MltB (Fig. 3), indicating a very specific interaction by MipA. A PBP analysis of the fractions is shown in Fig. 4. Only one signal was found, PBPB1. The column retained no other PBPs. Thus, MipA that specifically binds to the lytic transglycosylases MltA also binds very specifically to a penicillin-sensitive murein polymerase, namely the bifunctional PBPB1.

Immobilization of PBPs to BIAcore™ Sensor Chips—Protein-protein interaction studies by surface plasmon resonance (SPR) depends on three crucial factors: the chemical nature, homogeneity, and orientation of the immobilized bait protein. Here we took advantage of the fact that penicillin-sensitive enzymes covalently bind via their active site to penicillin. Accordingly a β-lactam was used as a capturing molecule to bind PBPs to the sensor chip. Hence ampicillin that carries a primary amino group in its side chain was immobilized to a standard CM5 sensor chip by N-ethyl-N’-(3-dimethylaminopropyl)-carbodiimide hydrochloride/NHC amine coupling. About 150 RU could routinely be achieved. As a control, chips loaded with ampicillin were treated with β-lactamase that results in an inactivation of the antibiotic by cleavage of the β-lactam ring. Specific coupling of various PBPs to an ampicillin surface could be accomplished even from rather dilute protein solutions by repeated injections until the desired concentration of normally about 1000–3000 RU was reached. Interestingly, the kinetics of the binding process of PBPB1 displayed a hyperbolic curve indicating that oligomerization occurs (Fig. 5A). Thus the self-interaction of PBPB1 on the ampicillin surface was investigated in more detail.

Dimerization of PBPB1—After the binding of PBPB1 to the ampicillin surface that was followed by β-lactamase treatment, the sensor chip was thoroughly washed with 1 M NaCl and 2% Triton X-100 to dissociate any complexes that might have been formed. Then a second sample of PBPB1 was added. This resulted in another binding of PBPB1 to the immobilized...
PBP1B (Fig. 5B). When PBP1A instead of PBP1B was present as an immobilized ligand, no binding of PBP1B was observed. A calculation suggests that a dimerization of PBP1B is taking place. This conclusion is supported by earlier observations of Zijderveld and co-workers (40), who showed dimerization of PBP1B by SDS-PAGE under mild dissociating conditions of sample preparation.

Interaction of MipA with PBP1B and MltA—Affinity chromatography on MltA-Sepharose indicated a binding of PBP1B to MltA (see Fig. 1). However, direct binding of isolated MltA to a PBP1B sensor chip surface could not be observed (Fig. 8). Likewise, binding of PBP1B to MltA immobilized to a sensor chip by standard amine coupling did not occur either (not shown). One explanation for the failure to demonstrate a PBP1B-MltA interaction by SPR is that a component that is crucial for the molecular interaction of PBP1B with MltA is presented to a PBP1B covered sensor chip surface. A good candidate that could be crucial for the molecular interaction of PBP1B with MltA was the newly identified MltA-interacting protein MipA described above, which binds both PBP1B (Fig. 4) and MltA (Fig. 3). Indeed MipA was capable of binding to a PBP1B sensor chip surface (Fig. 6) but also to MltA bound to a sensor chip via amine coupling (Fig. 7). Studies with immobilized MipA could not be performed since it could not be coupled to a BIAcore CM5 sensor chip by standard amine coupling because of its low $\mu$M of about 4.92. From the binding studies at different concentrations of the analytes, the apparent kinetic constants could be obtained. Two methods were used: first determination of the $R_{eq}$ values from the curve at equilibrium and second calculation of $R_{eq}$ using the BIAevaluation software. From the Scatchard plots $R_{eq}/C$ against $R_{eq}$, the $K_D$ values were obtained. In the case of binding of MipA at concentrations between 20.0 nM and 1.72 $\mu$M to MltA a dissociation constant $K_D$ of 0.320 $\pm$ 0.005 $\mu$M could be measured (Fig. 7B), whereas a value of 0.190 $\pm$ 0.002 $\mu$M was calculated. For the binding of MipA in the range of 1.23 to 24.6 $\mu$M to PBP1B (900 RU), a $K_D$ value of 2.09 $\pm$ 0.60 $\mu$M could be measured (Fig. 6B) from the binding curves, and a value of 1.73 $\pm$ 0.06 $\mu$M was calculated.

Formation of a Trimeric Complex—The injection of a mixture of 0.46 nM MipA and 0.18 nM MltA to a PBP1B-coated sensor chip resulted in a specific signal of about 610 RU (Fig. 8). Since binding of MipA to immobilized PBP1B on the one hand gave a response of 315 RU and binding of MltA on the other hand showed only an unspecific binding of 110 RU, it can be concluded that a trimeric complex of PBP1B, MipA, and MltA has been formed on the sensor chip surface. No indication for the formation of a trimeric complex was observed with denatured immobilized PBP1B (see curve 1 in the MipA/MltA panel of Fig. 8). Binding of MipA together with MltA to a PBP1B dimer could not be studied due to an interference with the dissociation of the PBP1B dimer.

Kinetics of the Binding of MltA-MipA to PBP1B—When MltA was injected (100 $\mu$L) at a concentration of about 3 $\mu$g/ml. The flow rate was 10 $\mu$L/min. The sensorgrams of the binding of PBP1B to a sensor chip with differently modified flow cells are shown. 1, PBP1B-ampicillin; 2, PBP1A-ampicillin; 3, $\beta$-lactamase-digested ampicillin; 4, ethanolamine. Immobilization of the PBPs was done by covalent binding to ampicillin that was coupled to a CM5 sensor chip as described under “Experimental Procedures.” As controls an ampicillin surface and an ethanolamine-treated surface are included.
was presented to a PBP1B surface in the presence of MipA, a signal was produced that clearly indicated the binding of both proteins to the immobilized PBP1B (Fig. 9). Kinetic studies had to be performed at rather low concentrations of PBP1B (480 RU) on the sensor chip in order to obtain a linear relationship for the binding of the MipA-MltA complex. A series of binding studies with concentrations of equimolar mixtures of MipA and MltA ranging from 30 nM to 2.46 μM allowed the determination of a dissociation constant of $0.850 \pm 0.057$ μM (Fig. 9B). Thus, the binding of MipA-MltA to PBP1B is slightly stronger than the binding of MipA to PBP1B but weaker than the binding of MipA to MltA.

**DISCUSSION**

The murein hydrolase MltA when used as a specific ligand in affinity chromatography specifically interacted with the bifunctional PBPs 1B and 1C as well as with the transpeptidases PBP2 and -3. It is this group of enzymes that is needed to enlarge murein according to the 3-for-1 growth model (3, 6). A complex consisting of a dimer of a bifunctional PBP, a dimer of a transpeptidase PBP, a monomer of a transglycosylase, a dimer of an endopeptidase, and a monomer of a lytic transglycosylase has been postulated to be involved in growth of the high molecular weight murein sacculus (see also Fig. 10). The in vitro formation of a complex of PBP1B and MltA in the presence of the structural protein MipA is further support of the proposal that a multienzyme complex of murein synthases and hydrolases is formed in vivo. Previous experiments using Slt70 or MltB as a specific ligand for affinity chromatography pointed to a high degree of variability with respect to the
Molecular Interactions of Murein Hydrolases and Synthases

A monomer of a transglycosylase, a dimer of a bifunctional transglycosylase/transpeptidase, a dimer of a transpeptidase, a dimer of a DD-endopeptidase, and a monomer of a lytic transglycosylase. The triangle represents the structural protein MipA. The shaded small circles represent the newly synthesized murein triplet, which will be inserted into the murein sacculus upon removal of the docking strand. The hatched circles are shown as small circles in the drawing. The large circles represent the enzymes. Murein synthases are shown as hatched circles and hydrolases as shaded circles. The different specificities from the front to the background in the drawing are as follows: a monomer of a transglycosylase, a dimer of a bifunctional transglycosylase/transpeptidase, a dimer of a transpeptidase, a dimer of a DD-endopeptidase, and a monomer of a lytic transglycosylase. The triangle represents the structural protein MipA.

The presence of specific structural proteins may determine the kind of complex that is formed. The reconstitution of a complex between purified PBP1B and purified MltA was only possible in the presence of a third protein, MipA, that has affinities to both PBP1B and MltA. Hence binding of MltA to PBP1B is indirectly via MipA. Since MipA seems to lack any enzymatic activity, it may be considered a structural protein mediating the assembly of the enzymes into a complex. Scaffolding proteins are well known in the formation of multienzyme complexes such as cellulosomes (43) or the DNA replicase holoenzyme (44).

We still don’t know yet all factors (proteins) that take part in the process of the assembly of the different complexes, but the fast and sensitive SPR method should prove valuable in the identification of all components involved. Indication that periplasmic factors are needed for the specific binding of PBP1A to MltA has been presented above and has recently been obtained in our laboratory for the binding of PBP2 to PBP1C. Work is in progress to get hold of these proteins.

It has to be pointed out that the two enzymes that interact with MipA are proteins anchored to the two different membrane systems present in Gram-negative bacteria, the lipoprotein MltA resides in the outer membrane (19) whereas PBP1B is linked with its amino terminus to the cytoplasmic membrane (34, 45). If indeed MipA couples both enzymes to one another in vivo, it is likely to give rise to a membrane adhesion site as depicted in Fig. 10. Such contacts between the membranes, also called Bayer junctions, have long been known (46), although their existence has repeatedly been jeopardized (47). Consistent with our expectation PBP1B has been shown by the immune gold labeling technique to be present in membrane adhesion sites (48), and murein synthesizing activity has been found to be high in membrane fractions enriched in these zones of adhesion (49).

This is the first time that a complex consisting of a murein synthase and a murein hydrolase could be formed in vitro from isolated proteins. Thus, the core particle of the hypothetical murein synthesizing machinery, a yin yang complex combining murein polymerases and depolymerases has been reconstituted. The dissociation constant of about 0.85 µM is a reasonable value that can be compared with the binding constants of cell adhesion molecules. Employing the BIAcore™ technique, it is feasible to test all the different possibilities of combinations and sequences of the addition of the proteins to one another in order to finally establish the right assembly pathway of the multienzyme complex.

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