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Identification of Two Penicillin-Binding Multienzyme Complexes in *Haemophilus influenzae*

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Dansyl-labeled penicillin, reversed-phase chromatography, and peptide mapping have been used to detect, separate, and study penicillin-binding proteins (PBPs) and PBP multienzyme complexes of *H. influenzae.* The cross-linking of proteins in the multienzyme complex was accomplished with the aid of cyanogen, a salt-bridge specific cross-linking agent. The chromatographic profile of the PBPs clearly showed a dramatic change in the number and identity of peaks after treatment of the bacterial cells with cyanogen. The disappearance of all seven peaks corresponding to the PBPs was accompanied by the emergence of two new peaks with molecular weights between 400 kDa and 600 kDa. The results hint at the existence of two penicillin-binding multienzyme complexes, each containing subunits that interact via salt-bridges. Chromatographic active site peptide mapping of PBPs and PBP complexes was used to determine the identity of PBPs involved in each complex. It is postulated that one multienzyme complex containing PBP 2 may be involved in cell elongation while the other complex containing PBP 3 may be responsible for cell division. © 1999 Academic Press

In Gram-negative bacteria, the cell envelope is enclosed by a murein biopolymer, the peptidoglycan, which is made of glycan strands cross-linked by peptides. The glycan strands are composed of the aminosugars N-acetylglucosamine and N-acetylmuramic acid linked together by β-1,4 glycosidic bonds. The lactyl group on the N-acetylmuramic acid provides a point of attachment for peptides, which are responsible for the cross-linked net structure of the murein. In *Haemophilus influenzae,* the peptide bound to the N-acetylmuramic acid is composed of L-alanine, D-glutamic acid, meso-diaminopimelic acid, and D-alanyl-D-alanine (7).

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Forming peptide bonds or hydrolyzing them are essential parts of cell wall synthesis and are catalyzed by a number of enzymes, among which are the penicillin-binding proteins (PBPs). The existence of PBPs was first confirmed in 1972, and in the following years they were shown to catalyze one or more of the transglycosylation, transpeptidation, carboxypeptidation, or endopeptidation reactions in the bacterial cell (3–6). It is logical to assume that PBPs and other morphogene products work in close association with one another in synthesizing and maintaining the bacterial cell wall. Recent studies have been hinting that some of these proteins may interact as members of multienzyme complexes, allowing better efficiency for incorporation of newly synthesized cell wall or the degradation and removal of old cell wall (8–10).

Most recently, protein-protein interaction studies by affinity chromatography have demonstrated that murein hydrolases and synthases can in fact interact with one another (11, 12). However, there has been no *in vivo* evidence for existence of such a complex, even though the most widely accepted model for cell wall enlargement proposes that the transpeptidase and transglycosylase enzymes that enlarge the wall, probably exist together as a complex, and that incorporation and cleavage occur coordinately (13, 14). Here we are presenting evidence that may reflect an *in vivo* assembly of PBPs with murein synthase and murein hydrolase activity in a multienzyme complex. Two such complexes were detected by selective cross-linking of interacting proteins with cyanogen, which is a salt-bridge specific cross-linking agent (15, 16). The PBP components of each complex have been identified by chromatographic active site peptide mapping as PBP 1a, PBP 3, PBP 4, and PBP 6 in one complex and PBP 1b, PBP 2, PBP 4, and PBP 5 in another complex.

MATERIALS AND METHODS

Cell culture. Haemophilus influenzae Rd strain KW20 (Gram-negative) was purchased from ATCC (#51907). The cells were grown in brain-heart infusion broth supplemented with the required cofactors, NAD (10 μg/mL) and hemin (10 μg/mL). Bacterial growth was

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monitored at 650 nm with a Spectronic 20 spectrophotometer. Bacteria were maintained at −80°C in a medium containing supplemented broth and glycerol.

Preparation of fluorescent label. Dansyl chloride was coupled to 6-aminopenicillanic acid (6-APA), following the previously described procedure (17), with some modification. A 26 mL solution containing equal volumes of 3% $NaHCO_3$ and acetone was prepared. The pH was adjusted to 9.0 before the addition of 6-APA (0.27 g). Dansyl chloride (0.50 g, 1.5 molar excess), dissolved in 2 mL of acetone, was added to the above solution. The solution was allowed to stir for 3 hours, with constant monitoring of pH, which was kept between 8.5 and 9.0 using 3 M NaOH. The final solution was washed with ether and the pH of the aqueous layer was adjusted to 2.5 with 2 M HCl. The solution was saturated with NaCl before extraction with ethyl acetate. The combined extracts were dried over $MgSO_4$, and filtered. The ethyl acetate was removed, and the product was crystallized from ethyl acetate/hexane. The vacuum-filtered product (150 mg) was dried, and stored at −20°C. The fluorescent penicillin, dansyl 6-APA was separated from the crude sample by HPLC. A C_4 Microsorb reversed-phase column (5 μ, 300 Å, 4.6 × 250 mm) was employed using gradient elution (95:5 water:acetonitrile to 0:100 water:acetonitrile in 20 min) and a flow rate of 1.0 mL/min. The elution profile was based on absorption of eluate at 320 nm. The peak containing the fluorescent penicillin was identified by addition of penicillinase II (from *B. cereus*); then eluted, collected and lyophilized.

Labeling of penicillin-binding proteins. Bacterial cultures (100 mL) were allowed to reach the late exponential phase of growth (A_{650} = 0.9). Bacteria were placed on ice and then centrifuged at 4°C and 4,600 × *g* for 6 min to harvest the cells. The pellets were resuspended and washed twice with 10 mM sodium phosphate buffer (pH 7.0) and then again resuspended in 40 mL of the buffer. To label the penicillin-binding proteins, dansyl 6-APA (10 mg), dissolved in 50 mM phosphate buffer (pH 7.0), was added to the bacteria and the mix was incubated for 30 min at room temperature. 6-APA (10 mg) was added instead of dansyl 6-APA in the control experiment. The cells were then centrifuged and washed as before.

Cross-linking of proteins. After washing the cells and resuspending them in 6 mL of buffer, six 100 μL aliquots were removed and placed in 1.5 mL microcentrifuge vials. The head-space of each vial was swept with a 20% stream of cyanogen (C_2N_2), and the sample was incubated at room temperature: vial 1 (no cross-linking agent), vial 2 (0.5 min treatment), vial 3 (2 min treatment), vial 4 (10 min treatment), vial 5 (30 min treatment), vial 6 (60 min treatment). After treatment with cross-linking agent, the cyanogen gas was swept out of each vial with a stream of argon gas.

Extraction of proteins. Cells were lysed and membrane proteins solubilized by adding a 15% SDS solution (7 μL) to each vial and incubating the vial at room temperature for 30 min. The lysed bacterial cells were centrifuged at 16°C and 18,400 × *g* for 30 min. The extremely viscous bottom layer was discarded while the top layer containing solubilized proteins was removed and maintained at −80°C. Alternatively, the proteins in the top layer were precipitated with acetone (2) and maintained at −80°C.

Reversed-phase chromatography. Precipitated proteins from 60 μL of detergent solubilized sample were redissolved in 60 μL of 10 mM phosphate buffer. Detection and purification of PBPs and PBP complexes was carried out on a C_4 Microsorb reversed-phase column (5 μ, 300 Å, 4.6 × 250 mm) using gradient elution (95:5:1:0.1 water: acetonitrile:isopropanol:TFA to 0:100:1:0.1 water:acetonitrile:isopropanol:TFA in 45 minutes) at a flow rate of 0.65 mL/min. The elution profile was based on the fluorescence of the eluate and monitored with an HP1046 fluorescence detector (λ_{ex} = 228 nm, λ_{em} = 528 nm). Individual peaks were collected in glass vials and lyophilized.

Size-exclusion chromatography. Individual peaks eluted from the reversed-phase column and lyophilized were redissolved in 20 μL of 10 mM phosphate buffer containing 0.1% SDS. Size-exclusion chromatography was carried out using a Rainin Hydropore-5 SEC column (5 μ, 300 Å, 4.6 × 250 mm) and isocratic elution (10 mM phosphate buffer with 0.1% SDS) at a flow rate of 0.3 mL/min. The elution profile was monitored as for reversed-phase chromatography. In order to determine the molecular weights and identify the PBPs, a calibration curve was constructed using 5 protein standards: carbonic anhydrase (29 kDa), albumin (66 kDa), β-amylase (150 kDa), alcohol dehydrogenase (200 kDa), and thyroglobulin (669 kDa).

Proteolytic digestion of PBPs and PBP complexes. Individual PBPs and cross-linked PBPs, purified by reversed-phase chromatography, were dissolved in 80 μL of 0.1 M NH_4HCO_3 (pH 7.5) and placed in a boiling water bath for 2 min. To each sample was added 25 μL of a TPCK-treated trypsin solution (1 mg/ml in 0.1 M HCl). The mix was incubated at 37°C for 1.5 hr, after which another 20 μL aliquot of the trypsin solution was added and the solution was incubated for an additional 1.5 hr.

Chromatography of tryptic digests and construction of chromatographic tryptic maps. The tryptic digests were chromatographed on a C_{18} Microsorb reversed-phase column (5 μ, 100 Å, 4.6 × 150 mm) using gradient elution (95:5:1:0.1 water:acetonitrile:isopropanol: TFA to 65:35:1:0.1 water:acetonitrile:isopropanol: TFA in 50 min) at a flow rate of 0.70 mL/min. The elution profile was based on the fluorescence of the eluate (monitored as was previously described). The exact times for elution of major peaks were recorded, and a tryptic chromatographic map was constructed.

RESULTS

Purification and Characterization of Fluorescent Probe

Separation of the fluorescent probe (dansyl 6-APA) from crude product was facilitated through reversed-phase chromatography. The fluorescent penicillin peak was identified by the addition of a penicillinase which resulted in disappearance of one major peak, namely that of the fluorescent penicillin. IR (neat) 3022, 2975, 1789, 1570, 1462, 1336, 1146 cm^{-1}; ^{1}HNMR (250 MHz, CD_3CN) δ 1.49–1.56 (6H, d), 2.86 (6H, s), 4.08 (1H, s), 4.38–4.45 (1H, d), 5.05–5.13 (1H, d), 5.47 (1H, s), 7.26–7.30 (1H, d), 7.56–7.74 (2H, dd), 8.22–8.30 (2H, dd), 8.56–8.60 (1H, d).

Detection of Penicillin-Binding Multienzyme Complexes

Without the use of cross-linking agent, seven peaks corresponding to the penicillin-binding proteins were observed when comparing the reversed-phase HPLC chromatogram of labeled cells to those of the controls (Fig. 1a). Generally, peak ratios differed as a function of time of cell harvest. The fourth peak was consistently the largest when cells were harvested at A_{650} = 0.9. The addition of isopropanol to the mobile phase enhanced protein recovery from the column. In order to identify each peak, it was collected, lyophilized, and then analyzed by size-exclusion chromatography. Cross-linking experiments were done using cyanogen (C_2N_2). This compound affords a technique for detection of specific interactions between subunits of an enzyme complex, by covalently linking only those pro-

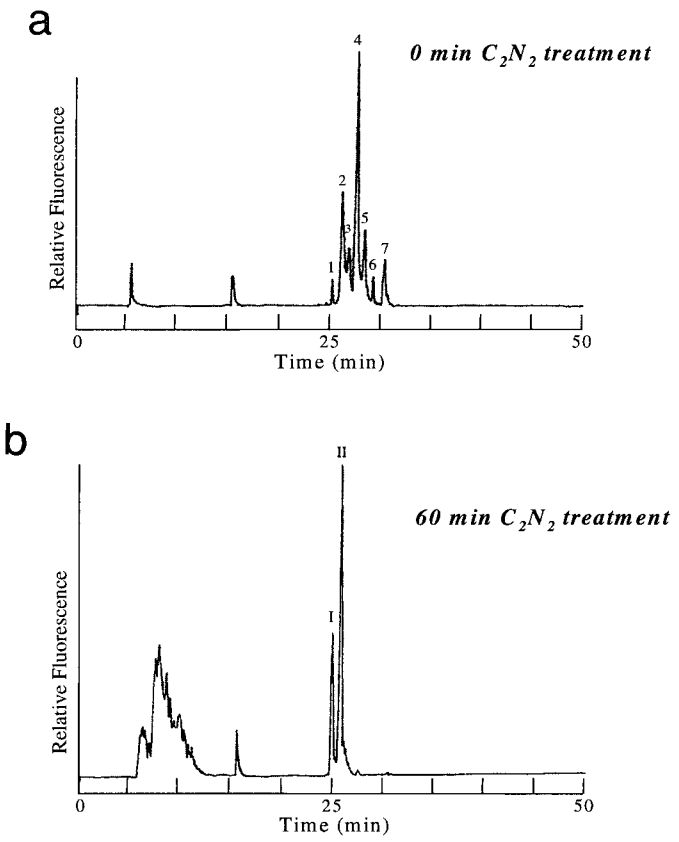

FIG. 1. Reversed-phase HPLC profile of labeled proteins, showing the penicillin-binding proteins without the use of cross-linking agent (a), and after treatment with cross-linking agent (b). The chromatogram (b) shows the disappearance of seven peaks representing the PBPs and appearance of two new peaks (with different elution times) after treating the cells with cyanogen.

teins with salt-bridges at their interfaces. As almost all protein-protein interactions studied so far have been shown to involve salt-bridges which seem to play a major role in protein binding and complex formation (19, 20), this cross-linking agent was believed to be quite useful in demonstrating possible interactions between the different PBPs. After treatment of the bacterial cells with cyanogen, the chromatographic profile of the PBPs clearly showed a dramatic change in the number and identity of peaks (Fig. 1b). The disappearance of all seven peaks corresponding to the PBPs (with molecular weights between 30 kDa and 90 kDa) was accompanied by the emergence of two new peaks (with molecular weights between 400 kDa and 600 kDa). The results hinted at the existence of two murein-synthesizing multienzyme complexes, each containing subunits that interact via salt-bridges.

Molecular Weight of PBPs and PBP Complexes

Molecular weights for PBPs and cross-linked PBP complexes were determined by size-exclusion chromatography, using the constructed standard calibration curve (Table 1). Calculated PBP molecular weights closely matched values determined from the genome (1) and those found by SDS–PAGE (18). The obtained molecular weights for the complexes indicated that they are comprised of a number of penicillin-binding proteins. The smaller peak (Peak I) seemed to contain heavier or a higher number of components than the larger peak (Peak II).

Identification of PBP Subunits of the Complexes

A small pore-size C_{18} column gave the best results for peptide separation. One major peak, indicating the active site peptide, bound to the fluorescent probe, was seen for every PBP. The complexes contained multiple peaks containing a number of active sites. Due to the high resolution and the existence of only one major active site peptide for each PBP, it was assumed that one could compare the tryptic map of the complexes to those of the PBPs in order to determine the PBP components of each complex with very little ambiguity. Using this simple technique, which we refer to as active site peptide mapping, it was found that complex I was comprised of PBP 1a, PBP 3, PBP 4, and PBP 6, while complex II was made up of PBP 1b, PBP 2, PBP 4, and PBP 5 (Fig. 2). The existence of PBP 5 in complex I and PBP 1a in complex II could not be determined unambiguously. Both complexes contained hydrolases and synthases.

DISCUSSION

Using the combination of liquid chromatography, protein cross-linking, and active site peptide mapping, we have identified two multienzyme complexes, each composed of a number of PBPs with polymerase and depolymerase activity. Clearly, these findings support recent speculations regarding the close association of hydrolases and synthases in assembling the cell wall. Located close to one another as part of a multienzyme

TABLE 1

Molecular Weights for PBPs and PBP Complexes

Peak[1]	Molecular weight (kDa)[2]	PBP
1	73.7	2
2	76.7	1b
3	88.1	1a
4	43.1	5
5	36.8	6
6	59.3	3
7	49.6	4
I	598	Complex I
II	458	Complex II

[1] From RP-HPLC column.
[2] As determined by size-exclusion chromatography.

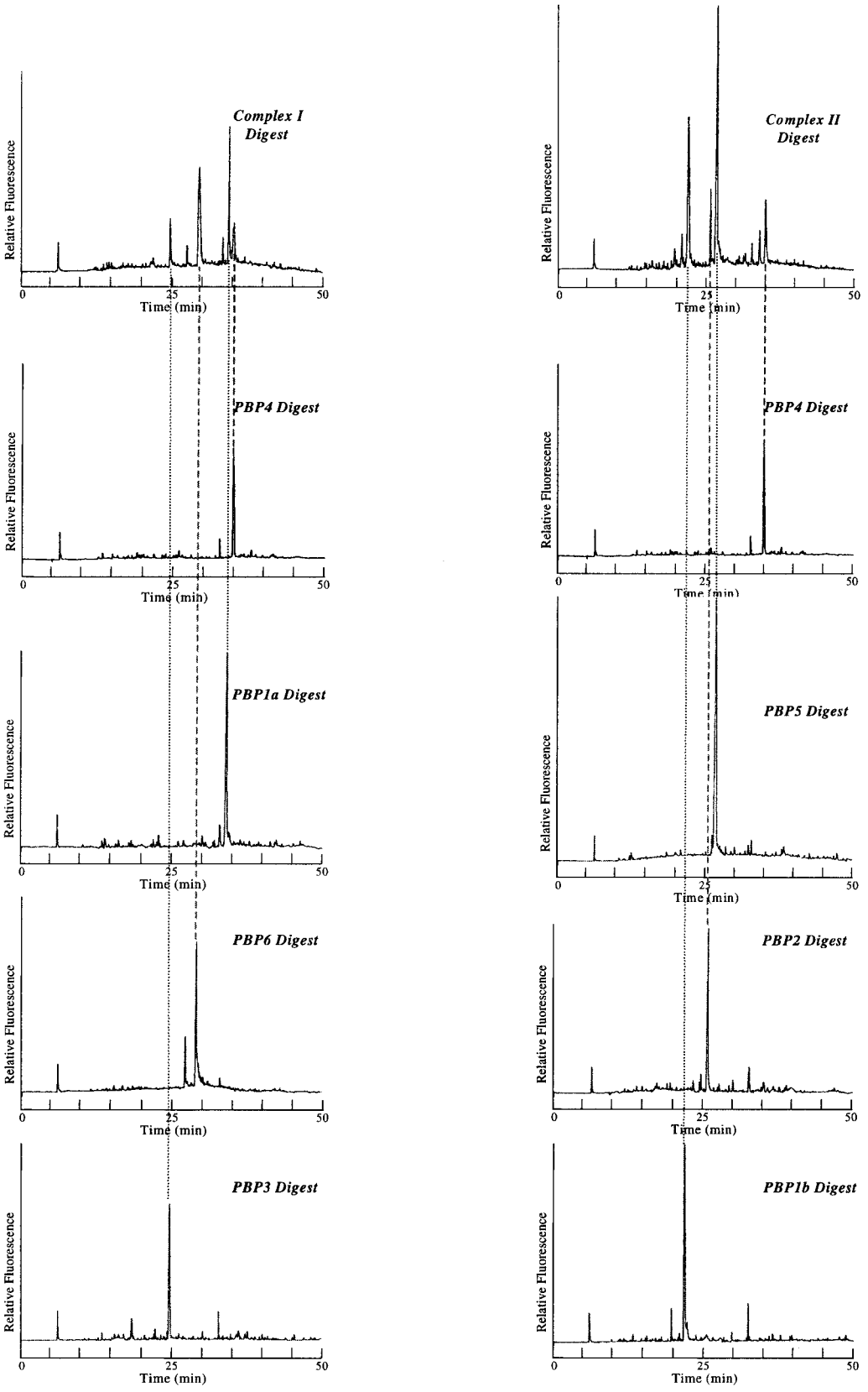

FIG. 2. Comparison of the active site tryptic maps of individual PBPs with those of the complexes. Broken lines connect active site peptide peaks for each complex to the corresponding peak for individual PBPs. Elution times of labeled active site peptides of complex I match those of PBPs 1a, 3, 4, and 6. Elution times of labeled active site peptides of complex II match those of PBPs 1b, 2, 4, and 5.

complex, these enzymes are able to carry out the task of inserting new strands in a secure and efficient manner.

If we assign the function of each PBP of *Haemophilus influenzae* based on sequence homology with *E. coli* (1), it becomes apparent that the two complexes identified here contain enzymes with similar functions. While complex I contains PBP 1a, complex II holds its functional equivalent, PBP 1b. Similarly, PBP 3 is in complex I, while the other transpeptidase, PBP 2, is in complex II. On the other hand, while no role is foreseen for an enzyme with carboxypeptidase activity as part of a multienzyme complex by any of the proposed models, the results obtained here indicate that at least one of the complexes (complex II) contains the carboxypeptidase PBP 5.

While PBP 2 has been found to be essential for formation of rods, PBP 3 has been implicated as a necessary component for the process of cell division (2, 22). In this study, the finding that one complex contains PBP 2 while the other has PBP 3 indicates that there may be two different types of cellular machinery for cell elongation and cell constriction. This seems logical since the two processes are most probably very different as far as requirements for insertion of new peptidoglycan are concerned.

It is important to keep in mind that in identifying the subunits of the two murein-synthesizing multienzyme complexes, only the penicillin-binding proteins were targeted in this study. Other studies have indicated that certain non-penicillin-binding proteins such as lytic transglycosylases may also be involved in the complex (12, 21). The fact that the obtained molecular weights for the two complexes are significantly higher than the sum of the PBP molecular weights which constitute those complexes, may provide further evidence for involvement of other proteins. In addition, it could mean that certain PBPs exist in numbers greater than one within each complex (8). It is therefore logical that in continuing this work, to focus on finding ways to identify possible non-penicillin-binding members, in addition to the number of PBPs in each multienzyme complex. Furthermore, extending this study to other organisms particularly *E. coli*, which has been studied much more extensively than *H. influenzae*, would undoubtedly improve our knowledge of involvement of PBPs and other proteins in bacterial cell wall synthesis.

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