Penicillin-binding protein 5 can form a homo-oligomeric complex in the inner membrane of *Escherichia coli*

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Abstract: Penicillin-binding protein 5 (PBP5) is a DD-carboxypeptidase, which cleaves the terminal D-alanine from the muramyl pentapeptide in the peptidoglycan layer of *Escherichia coli* and other bacteria. In doing so, it varies the substrates for transpeptidation and plays a key role in maintaining cell shape. In this study, we have analyzed the oligomeric state of PBP5 in detergent and in its native environment, the inner membrane. Both approaches indicate that PBP5 exists as a homo-oligomeric complex, most likely as a homo-dimer. As the crystal structure of the soluble domain of PBP5 (i.e., lacking the membrane anchor) shows a monomer, we used our experimental data to generate a model of the homo-dimer. This model extends our understanding of PBP5 function as it suggests how PBP5 can interact with the peptidoglycan layer. It suggests that the stem domains interact and the catalytic domains have freedom to move from the position observed in the crystal structure. This would allow the catalytic domain to have access to pentapeptides at different distances from the membrane.

Keywords: membrane protein; homo-oligomer; penicillin-binding protein; peptidoglycan biogenesis

Introduction

Gram-negative bacteria are constrained by a macromolecular “net” called the peptidoglycan layer, which is important for protection against osmotic stress. The peptidoglycan layer is constructed from stiff glycan chains that are cross-linked with short flexible oligopeptides and is situated between the inner and outer membranes.^{1–3} During bacterial growth and cell divi-

sion, the peptidoglycan layer is continuously remodeled and turned over.⁴ This process is coordinated by a group of proteins collectively known as the penicillin-binding proteins (PBPs), as they are the targets of penicillin and penicillin-based antibiotics.⁵

The PBPs have varied functions related to peptidoglycan biosynthesis. The high molecular weight PBPs (PBP1A, 1B, 1C, 2, and 3) are transglycosylases and/or transpeptidases, whereas the low molecular weight PBPs (PBP4, 5, 6, 6B, and 7/8) are DD-carboxypeptidases and/or endopeptidases (reviewed in Refs. ^{2,6}, and ⁷). To coordinate these various biochemical activities, it has been hypothesized that the PBPs assemble into hetero-oligomeric protein complexes.^{1,8–10} Although such complexes are yet to be identified, a number of interactions have been detected, which support their existence.^{11–19} Additionally, it has been shown that many of the PBPs

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can form homo-dimers.^{19–23} Together, these protein interactions have been instrumental in extending our understanding of peptidoglycan biosynthesis.

PBP5 is a paradigm for the low molecular weight PBPs. It localizes to the sites of ongoing peptidoglycan biosynthesis,²⁴ where it cleaves the terminal D-alanine from the muramyl pentapeptide and thereby varies the substrates for transpeptidation.²⁵ In doing so, it plays a dominant role in maintaining cell shape^{25–29} and contributes to resistance of low-level β -lactam antibiotics.³⁰ The crystal structure of a soluble version of PBP5 (termed PBP5_{soluble}) shows two domains that are orientated at near right angles to each other.^{31–35} The N-terminus contains the catalytic domain (also known as domain I or the penicillin-binding domain) and the C-terminus contains the stem domain (also called domain II). The C-terminus of PBP5 also encodes a short amphipathic α -helix, which attaches the enzyme to the outer leaflet of the inner membrane and is referred to as the membrane anchor.^{36,37} The membrane anchor was not present in the crystal structures of PBP5_{soluble}, but a recent NMR structure shows a simple helix-bend-helix-turn-helix, which can enter and exit the membrane on the same side.³⁸ Intriguingly, the membrane anchor is essential for the physiological activity of PBP5, as PBP5_{soluble} cannot complement a morphological phenotype in strains where PBP5 is deleted.²⁷ The aim of this study was to better understand the context in which PBP5 functions by analyzing its oligomeric state. Our experimental data indicate that the full-length PBP5 forms a homo-oligomeric complex in detergent and *in vivo*. We have used this experimental data to build a model, which provides a foundation for future studies of structure and function.

Results

A screen for protein complexes containing PBP5

Our initial aim was to look for proteins that interacted with PBP5. Native membrane proteins were solubilized from wild-type *Escherichia coli* with a mild detergent (*n*-dodecyl- β -D-maltoside [DDM]) and then PBPs were purified using a Sepharose column loaded with 6-aminopenicillanic acid. The eluate was then analyzed by blue native-polyacrylamide gel electrophoresis (BN-PAGE), a widely used method for separating membrane protein complexes in their native state.³⁹ When coupled to a second dimension of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), individual proteins within the complexes resolved as spots in “vertical channels.” By staining with Coomassie, we could detect four protein spots, which were identified by mass spectrometry as PBP1A, PBP5/PBP6, OmpF, and LamB (Fig. 1).

As all the PBPs have membrane anchors, we determined their molecular mass by calibrating the

Figure 1. PBP5 and PBP6 resolve at a high molecular weight when analyzed by BN-PAGE. PBPs were purified from *E. coli* using an affinity column loaded with 6-aminopenicillanic acid, then separated by BN/SDS-PAGE. Proteins were stained with Coomassie dye and identified by mass spectrometry (data available in Supporting Information Table S1). The molecular mass of proteins in the BN-PAGE was calculated using membrane proteins as calibration markers. The molecular mass of proteins in the SDS-PAGE is shown to the right.

BN-PAGE with membrane protein markers (as done previously^{40–42}). The molecular mass of the spot containing both PBP5 (44 kDa) and PBP6 (43 kDa) in the BN-PAGE was calculated to be 100 ± 15 kDa (taken from the center of the spot and based on three experiments). As the spot appeared quite broad, we also calculated a molecular mass range from the edges of the spot. In the presented gel, the spot was calculated to range from 84 to 101 kDa. As all these estimates were larger than the size of the PBP5 and PBP6 monomers, we reason that PBP5 and PBP6 are oligomeric. Unfortunately, we could not resolve their oligomeric state from this experiment (this issue is addressed below). We did not detect monomeric forms of PBP5 or PBP6 in this experiment, but we cannot rule out the possibility that they were lost during the concentration step, as we used spin columns with a molecular mass cutoff of 100 kDa (chosen so that DDM micelles would not be concentrated).

The calculated molecular mass of PBP1A (94 kDa) in the BN-PAGE was 137 ± 9 kDa (taken from

Figure 2. PBP5 and PBP6 form homo-oligomers. (A) His₈-Δss-PBP5 was expressed in the cytoplasm of *E. coli* then purified by IMAC/SEC and analyzed by BN-/SDS-PAGE. In the BN-PAGE, membrane proteins were used as calibration markers. MA indicates the membrane anchor. (B) His₈-Δss-PBP6 was expressed in the cytoplasm of *E. coli* then purified by IMAC/SEC and analyzed by BN-/SDS-PAGE. Annotation as in Figure 1. See Supporting Information Table S1 for mass spectrometry data.

the center of the spot). As no other proteins were observed in the same vertical channel, we reasoned that it was monomeric in this experiment. OmpF and LamB are abundant outer membrane porins that form homo-trimers, and both these proteins are resolved at the expected molecular mass in the BN-PAGE (132 ± 11 kDa and 140 ± 3 kDa are taken from the center of the respective spots). Although LamB has no known affinity for penicillin, it is known that β -lactam antibiotics permeate through OmpF,^{43,44} which may explain why it was purified on the column.

PBP5 forms a detergent-soluble homo-oligomer

During our analysis of native PBPs by BN-/SDS-PAGE, we noted that PBP5 and PBP6 resolved at a molecular mass that was larger than a monomer. However, it was not clear whether the higher molecular mass was a result of the proteins forming homo-oligomers, hetero-oligomers or both. To gain insight into this issue, we engineered a plasmid-encoded version of PBP5, where the signal sequence was replaced by an octa-histidine purification tag [Fig. 2(A)]. Therefore, the resultant protein (His₈-Δss-PBP5) was retained on the cytoplasmic side of the inner mem-

brane and did not have access to PBP6 or any other periplasmic proteins. Cytoplasmic retention has been used previously to produce active PBP5,^{31–34} as it circumvents the toxic effects on growth that have been observed when PBP5 is over-expressed in the periplasm.⁴⁵ When we solubilized membranes with DDM and purified His₈-Δss-PBP5 by immobilized metal-affinity chromatography (IMAC) and analyzed it by size-exclusion chromatography (SEC), we noted that it was monodisperse and that it bound Bocillin-FL (Supporting Information Fig. S1), indicating that it was correctly folded. When we analyzed purified His₈-Δss-PBP5 by BN-/SDS-PAGE, we obtained one protein spot [Fig. 2(A)]. Analysis of the spot by mass spectrometry confirmed that the protein spot contained only PBP5 (PBP6 was not detected). The molecular mass of His₈-Δss-PBP5 in the BN-PAGE was calculated to be 93 ± 3 kDa (calculated from the center of the spot and based on two experiments). Because the spot was quite broad, we also calculated a molecular mass range from the edges of the spot. In the presented gel, the spot was calculated to range from 73 to 105 kDa. As the predicted molecular mass of His₈-Δss-PBP5 is 43 kDa, we reasoned that it was a homo-oligomer in the BN-PAGE.

Figure 3. Cross-linking of the PBP5 homo-oligomer *in vivo*. Single-cysteine mutants of PBP5 were radiolabeled with ³⁵S-methionine *in vivo* then oxidized or reduced by incubation with Cu(Ph)₃/DTT. Proteins were analyzed by nonreducing SDS-PAGE and detected by autoradiography. * indicates that the oxidized adduct migrated at the molecular mass expected for a homo-dimer. ** indicates that the oxidized adduct was larger than expected for a homo-dimer. Molecular weight markers are indicated to the right.

In a reverse experiment, we engineered and expressed His₈-Ass-PBP6 in *E. coli* [Fig. 2(B)]. After purification by IMAC/SEC, we noted that it bound Bocillin-FL (Supporting Information Fig. S1), indicating that it was correctly folded. When we analyzed purified His₈-Ass-PBP6 by BN-/SDS-PAGE, one protein spot was again visualized [Fig. 2(B)]. The protein spot migrated at 90 kDa in the BN-PAGE (taken from the center of the spot and no replicates) and was identified as PBP6 using mass spectrometry. We also calculated a molecular mass range from the edges of the spot. In the presented gel, the spot was calculated to range from 84 to 111 kDa. PBP5 was not detected in the same spot by mass spectrometry, and no other protein spots were detected in the same vertical channel by Coomassie staining. These data suggest that His₈-Ass-PBP6 is also a homo-oligomer.

PBP5 and PBP6 cannot form a hetero-oligomer

Although we had shown that both PBP5 and PBP6 could form homo-oligomers, it was possible that they could also form a hetero-oligomer. To address this possibility, we again used a coaffinity purification approach. In this experiment, we engineered and coexpressed His₈-Ass-PBP5 and ΔAss-PBP6. Our rationale was that if PBP5 and PBP6 could form a hetero-dimer then the untagged ΔAss-PBP6 should copurify with His₈-Ass-PBP5 following purification. When we purified His₈-Ass-PBP5 by IMAC/SEC and analyzed the eluate by BN-/SDS-PAGE, we found no evidence of ΔAss-PBP6 (Supporting Information Fig. S2). Taken together, these data indicate that PBP5 and PBP6 cannot form a hetero-oligomer when coexpressed in the cytoplasm.

In vivo cross-linking of the PBP5 homo-oligomer

To gain insight into the packing of the PBP5 homo-oligomer, we probed for residues that were at the interaction interface by expressing single-cysteine mutants and testing for disulfide bond formation *in vivo*. For this set of experiments, we cloned the gene encoding PBP5 downstream of a T7 promoter and then mutated the only cysteine codon to serine to generate a cysteine-less background (called C115S). We then used the crystal structure of PBP5_{soluble}^{31–35} to identify 18 amino acids that are randomly distributed over the surface of PBP5. We then individually mutated each residue to cysteine in the C115S background and expressed each mutant in the periplasm of *E. coli*, by induction with isopropyl-β-D-thiogalactopyranoside (IPTG) in the presence of ³⁵S-methionine and rifampicin (which inhibits the bacterial RNA polymerase but not the T7 polymerase⁴⁶). To approximate the levels of over-expression, we also labeled the cells with Bocillin-FL and scanned the SDS-PAGE for fluorescence. Densitometric quantification of the C115S band indicated that PBP5 was approximately eightfold higher than the native protein (Supporting Information Fig. S3).

When whole cells expressing the S-methionine-labeled³⁵ mutants were oxidized with 0.5 mM copper phenanthroline and analyzed by nonreducing SDS-PAGE, we could detect a series of gel shifts (Fig. 3). As each PBP5 monomer contained a single-cysteine residue, it was only possible to detect dimeric forms when the proteins were analyzed by SDS-PAGE. In all cases, the gel shifts disappeared when the cells were reduced with 0.5 mM dithiothreitol (DTT) indicating that the shift had arisen through the oxidation of cysteine (Fig. 3). For four of the single-

cysteine mutants, the size of the gel shift was consistent with the formation of a disulfide-bridged homo-dimer (Fig. 3, lanes 4, 8, 10, and 30). We did not include C115S/K64C as a positive cross-link because it did not consistently give a gel shift in replicate experiments. For another five of the single-cysteine mutants, the size of the gel shift was larger than expected (Fig. 3, lanes 6, 20, 24, 32, and 36). This could occur if the single-cysteine mutant had cross-linked with another cysteine-containing protein or if the two cross-linked PBP5 proteins ran aberrantly in the SDS-PAGE.

To determine whether our gel shifts were homo-oligomeric forms of PBP5 or cross-links with other cysteine-containing proteins, we also analyzed them by BN-PAGE (Supporting Information Fig. S4). Here, our rationale was that only intramolecular cysteine cross-links (i.e., between two PBP5 molecules) would migrate at the molecular mass of the homo-oligomer in the BN-PAGE. Surface-exposed cysteines that formed cross-links with other periplasmic proteins would yield a product that resolved at a higher molecular mass in the BN-PAGE (i.e., the detergent-soluble homo-oligomer plus the cross-linked protein). Significantly, all the single-cysteine mutants resolved at the molecular mass of the homo-oligomer when oxidized and analyzed by BN-PAGE. This observation indicates that all the cysteine cross-links that we detected (even those that were larger than predicted in the SDS-PAGE) occurred between two PBP5 molecules (Supporting Information Fig. S4). Taken together, these data suggest that nine of 18 cysteine residues tested on the surface of PBP5 were near the interface of the molecules. Importantly, we also noted that eight of 18 were unable to cross-link PBP5 (as judged by the lack of a gel shift when oxidized, Fig. 3). We conclude that these eight residues are not in close proximity at the interface of the homo-oligomer or that the sulfur of the introduced cysteine side chain is no longer surface accessible.

A putative model of the PBP5 homo-oligomer

We reasoned that a putative model of the PBP5 homo-oligomer could be attained, by juxtaposing molecules of the PBP5_{soluble} monomer in a manner that satisfied the experimental data. Although we cannot preclude other oligomeric states, the most likely interpretation of our BN-PAGE data is that PBP5 forms a homo-dimer. Therefore, we modeled PBP5 as a homo-dimer.

Intriguingly, we could not obtain a model that satisfied the cross-linking data, if we treated the crystal structure of PBP5_{soluble} as a rigid body. To overcome this problem, we treated the stem domain and the catalytic domain in isolation and omitted those cysteine cross-links that gave larger than expected gel shifts in the SDS-PAGE (i.e., N191C,

Q232C, K143C, K219C, and R194C). The isolated stem domain could be juxtaposed in only one possible manner, as it contained two positive cross-linking positions on one side (G338C and G355C) and five negative positions distributed elsewhere (K273C, K287C, K294C, Q324C, and G355C) [Fig. 4(A)]. This pattern suggested that two stem domains interact “back-to-back” to form a single pedestal [Fig. 4(B)]. This supposition is consistent with an earlier hypothesis that the stem domain could be involved in protein–protein interactions.³¹ When taken in isolation, the packing of the two catalytic domains could be reconciled with the cross-linking positions F90C and G94C, which were near the tip of the catalytic domain [Fig. 4(A)]. However, in the context of the full-length protein, the catalytic domain would have to move to a more upright position from that observed in the crystal structure of PBP5_{soluble} [Fig. 4(B)]. Such a movement would require flexibility in a linker region between the C-terminal helix of the catalytic domain and the N-terminal β -strand of the stem domain. Movement around this linker region has been speculated on previously, as it would enable PBP5 to have better access to pentapeptides in a growing glycan chain.²⁴ If full flexibility around the linker is assumed, we calculate that the catalytic site could operate between 40 and 80 Å from the membrane surface. The model we propose does not assume that the catalytic domains are always in the upright position, but rather that this position represents one extreme of the movement that is possible (as cysteine cross-links trap proteins in their nearest conformation).

In refining our model, we revisited the five cross-links that we had previously omitted because they gave unpredictable molecular masses when analyzed by SDS-PAGE (i.e., N191C, Q232C, K143C, K219C, and R194C). Intriguingly, all these cross-links could be easily explained in our model, if the catalytic domain was able to rotate slightly. This rotation would again require flexibility in the linker region between the catalytic domain and the stem domain.

Discussion

In this study, we have analyzed the oligomeric state of the membrane-anchored and physiologically active form of PBP5 using nondenaturing BN-PAGE and single-cysteine cross-linking. These approaches have enabled us to determine the oligomeric state of PBP5 in detergent micelles and in its native environment, that is, the inner membrane of *E. coli*. All experimentation indicates that PBP5 self-associates as a homo-oligomer. The most likely interpretation of our BN-PAGE data is that PBP5 forms a homo-dimer, although we cannot preclude other oligomeric states. We could not find evidence for a monomeric

Figure 4. A putative structural model of the membrane-anchored PBP5 homo-dimer. (A) Surface plot of the crystal structure of anchorless *E. coli* PBP5_{soluble} (1NZO). The native residues that were converted to cysteine are marked in green and red, depending on whether they could, or could not be used to cross-link the homo-dimer. The native cysteine at position 115 is embedded in the protein and is not marked. The active site serine 44 is marked blue. The membrane anchor was not present in the structure and was hand drawn. (B) Two molecules of PBP5_{soluble} were juxtaposed, in a manner that satisfies the single-cysteine cross-linking data (see text for details). In this model, the stem domains are constrained and the catalytic domains have some freedom to move up and rotate. This freedom is granted by flexibility in the linker region between the two domains. The C-terminal membrane anchors have been hand drawn, as they were not present in the crystal structure. The thickness of the membrane has been drawn approximately to scale. (C) Overlay of crystal structures from *E. coli* PBP5 (1NZO), *Streptococcus pneumoniae* PBP3 (1XP4), and *S. aureus* PBP4 (1TVF) (shown in white, brown, and gold respectively). In the figure, the stem domains have been superimposed to illustrate that the angle between the catalytic domain and the stem domain varies. This observation indicates that there may be a degree of flexibility in the linker region between the two domains.

form of PBP5 in these studies; however, we cannot rule out the possibility that occurs *in vivo*.

Given that PBP5 has been well studied for decades, it seems pertinent to ask why the homo-oligomer has not been noted previously. We believe that it has escaped detection because the majority of biochemical and structural analyses have been carried out on the soluble domain (PBP5_{soluble}), which

is clearly monomeric when purified.^{31–34,47} We are aware of only one published experiment where the membrane-anchored form was purified and analyzed. In that experiment, PBP5 was reported to have an apparent molecular mass of 184 kDa when purified in Triton X-100 and analyzed by SEC.⁴⁷ Although the molecular mass suggested a homo-oligomeric assembly, the authors did not address the

issue, because it was not possible to calculate the contribution of the detergent micelle to the molecular mass. Taken together, these published observations indicate that the C-terminal membrane anchor clearly plays a dominant role in either forming or stabilizing the homo-oligomer. However, we were unable to cross-link the homo-oligomer when we placed a single-cysteine residue at amino acid 365 in the membrane anchor. This may be because the position of the introduced cysteine side chain was not in an optimal position for cross-linking. Further work will be required to resolve a structural role, if any, for the membrane anchor.

Structural resolution of membrane proteins is a challenging process and they are, therefore, significantly under-represented in the protein database.⁴⁸ Indeed, our efforts to crystallize the membrane-anchored PBP5 homo-oligomer have not been successful. To gain insight into the organization of the homo-oligomer, we created a model by juxtaposing two molecules of PBP5_{soluble} in a manner that satisfied the cysteine cross-linking pattern [Fig. 4(A) and (B)]. Intriguingly, the catalytic domain acted promiscuously in our single-cysteine cross-linking experiments and our model could only be made if we allowed flexibility in a region between the stem domain and the catalytic domain. This flexibility would allow the catalytic domains to move and rotate, in a manner that is not immediately apparent in the crystal structure of PBP5_{soluble}. This movement has been speculated on previously, as it would enable PBP5 to have better access to pentapeptides in a growing glycan chain.²⁴

Has flexibility between domains been observed previously for this family of proteins? When we superimposed the structure of *E. coli* PBP5 with structures of other carboxypeptidases (*Streptococcus pneumoniae* PBP3 and *Staphylococcus aureus* PBP4), we noted that the catalytic domain was not always positioned in the same orientation relative to the stem domain [Fig. 4(C)]. A similar situation was noted when the structure of PBP6 was solved, as the angles between the catalytic and stem domains varied among the four monomers in the asymmetric unit.³⁵ Taken together, the crystal structures indicate that the angle between the stem domain and the catalytic domain is not fixed for the carboxypeptidases. These observations support our cross-linking data and subsequent model, as they independently suggest that there is flexibility in the linker region between the catalytic domain and the stem domain in PBP5.

The work presented in this study also indicated that PBP6 could form a homo-oligomer, but that PBP5 and PBP6 could not form a hetero-oligomer. This latter observation was particularly intriguing because PBP5 and PBP6 share 60% sequence identity. The model of the PBP5 homo-dimer that we present can reconcile this observation as it proposes

that the stem domains form the permanent interface between the two monomers. These domains show the least sequence homology between the two proteins²⁸ and are, therefore, likely to dictate the specificity for homo-oligomerization.

In addition to PBP5 and PBP6, there have been reports that PBP1A, PBP1B, PBP3, and PBP4 also form homo-dimers in *E. coli*.^{12,19–23} For PBP1B (and PBP2 in *S. aureus*), conformational flexibility between the glycosyltransferase and transpeptidase domains has been proposed.^{18,49} These observations are intriguing because various PBPs differ in biological function and the overall structure. The only unifying feature among the PBP family is the penicillin-binding domain. Taken together, this growing body of literature suggests that the various PBPs share a common mechanism for modifying the peptidoglycan layer.

Materials and Methods

Affinity purification of native PBPs

E. coli strain BL21(DE3) was grown at 37°C in Luria Bertani broth to the late log phase. Cells were harvested, washed in 1X phosphate buffered saline (PBS) pH 7.4, and resuspended in 5 mL of lysis buffer [1X PBS pH 7.4 with Complete Protease Inhibitor Cocktail (Roche)]. Cells were lysed using an Emulsiflex C3 (Avestin, Canada). Unbroken cells were removed by centrifugation at 9300g for 20 min and membranes were harvested from the supernatant by centrifugation at 210,000g for 2 h. Membranes were washed in 1X PBS pH 7.4.

The affinity purification protocol was adapted from Ref. 50. 6-Aminopenicillanic acid (Sigma-Aldrich) was bound to activated CH sepharose 4B (GE Healthcare) according to the manufacturer's instructions. Proteins were solubilized from membranes with 0.5% DDM, cleared by centrifugation at 210,000g for 2 h, and passed through the affinity column twice using an ÄKTA Prime purification platform (GE Healthcare). The column was washed with five column volumes of wash buffer [50 mM Tris-HCl pH 7.4, 0.1% (w/v) DDM] and eluted with five column volumes of elution buffer [50 mM Tris-HCl pH 7.4, 0.1% (w/v) DDM, 0.25M hydroxylamine]. The eluate was concentrated and exchanged into ACA₇₅₀ buffer [750 mM *n*-amino-caproic acid, 50 mM Bis-Tris, 0.5 mM disodium ethylenediaminetetraacetic acid (Na₂EDTA), pH 7.0] with 0.1% DDM using a Vivaspin 20 column (Sartorius Stedim Biotech).

Blue native-/sodium dodecyl sulphate polyacrylamide gel electrophoresis

BN-PAGE and the second dimension SDS-PAGE were carried out as described previously⁴⁰ on gels with dimensions of 14 cm × 20 cm × 1.5 mm. Mass calibration was performed using soluble proteins in the

high molecular weight calibration kit for native electrophoresis (GE Healthcare), and separately using three *E. coli* membrane protein complexes; the succinate dehydrogenase (355 kDa), the cytochrome *bo₃* (145 kDa), and the glucose dehydrogenase (85 kDa).⁴⁰

Affinity and coaffinity purification of recombinant proteins

To express His₈-Ass-PBP5 and His₈-Ass-PBP6, the *dacA* and *dacC* genes (encoding PBP5 and 6, respectively) were amplified by polymerase chain reaction (PCR) from the *E. coli* strain MG1655⁵¹ and cloned into the pGFPe vector^{52,53} using restriction endonuclease sites introduced into the oligonucleotide primers (5'*Xho*I/3'*Eco*RI and 5'*Xho*I/3'*Hind*III, respectively). In both cases, the forward primer was matched to the region coding for the mature protein, lacking the region coding for the signal sequence (i.e., 29 amino acids for PBP5 and 27 amino acids for PBP6). In addition, the forward primer contained an extension coding for an octa-histidine purification tag (His₈). As the amplified genes contained their native stop codon, the encoded proteins did not become fused to the green fluorescent protein (GFP). For coexpression of His₈-Ass-PBP5 and Ass-PBP6 as well as *dacA* and *dacC* genes were amplified and cloned into the pETDuet-1 vector (Novagen) using restriction endonuclease sites introduced into the oligonucleotide primers (5'*Eco*RI/3'*Kpn*I and 5'*Nco*I/3'*Eco*RI, respectively). In both cases, the forward primer was matched to the region coding for the mature protein and for *dacA*, it contained an extension coding for an octa-histidine purification tag. All constructs were confirmed by sequencing (BM Labbet AB, Sweden).

The plasmids described above were transformed into the BL21(DE3)pLysS strain and were grown at 37°C in Luria Bertani (LB) broth until the OD₆₀₀ reached 0.3. Expression of the recombinant proteins was induced by an addition of 0.5 mM IPTG. The cultures were grown for additional 6 h at either 25°C (His₈-Ass-PBP5) or 37°C (His₈-Ass-PBP6 and His₈-Ass-PBP5/Ass-PBP6). Cells were harvested, washed in 1X PBS pH 7.4, resuspended in 40 mL of lysis buffer (1X PBS pH 7.4 with Complete Protease Inhibitor Cocktail), and lysed using an Emulsiflex C3. Unbroken cells were removed by centrifugation at 9300g for 20 min and membranes were harvested from the supernatant by centrifugation at 210,000g for 2 h. Membranes were washed and stored in 1X PBS pH 7.4.

IMAC was carried out using a HisTrap FF 5 mL column (GE Healthcare) attached to an ÄKTA prime plus purification platform. Proteins were solubilized from membranes with 0.5% DDM and cleared by centrifugation at 210,000g for 2 h. Proteins were bound to the Ni-resin in the presence of 10 mM imidazole. The column was washed with 10 column volumes of wash buffer [1X PBS pH 7.4, 0.03% (w/v)

DDM, 40 mM imidazole] and eluted by increasing the imidazole concentration to 500 mM. Eluted proteins were concentrated using a Vivaspin 20 column.

SEC was performed using a Superdex 200 10/300 GL column (GE Healthcare) attached to an ÄKTA prime plus purification platform. Running buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.03% DDM) was passed through the column at 0.2 mL/min to elute the protein. The protein was concentrated using a Vivaspin 20 column and analyzed by BN-/SDS-PAGE as described earlier.

In vivo labeling of PBP5 with ³⁵S-methionine and cysteine cross-linking

The *dacA* was amplified by PCR from the *E. coli* strain MG1655⁵¹ using restriction endonuclease sites (5'*Xho*I/3'*Bam*HI) introduced into the oligonucleotide primers. The amplified fragment was digested with appropriate restriction enzymes (Invitrogen, Sweden) and cloned into the pGFPe vector, downstream of a T7 promoter.^{52,53} As the amplified gene contained its native stop codon, the encoded proteins did not become fused to GFP. Site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Sweden). All constructs were confirmed by sequencing. Labeling of PBP5 with ³⁵S-methionine was carried out as described previously.^{54,55} In short, the *dacA*-pGFPe plasmid was transformed into the *E. coli* strain BL21(DE3). A colony was inoculated into a 2-mL tube containing 1 mL of LB media with kanamycin (50 µg/mL) and incubated with vigorous shaking at 37°C for 16 h. The culture was back-diluted 1/20 and incubated as before until the OD₆₀₀ was between 0.3 and 0.5. Cells were harvested by centrifugation at 830g for 5 min and the cell pellet was resuspended in 1 mL of M9 minimal media, supplemented with thiamine (10 mM) and all amino acids but methionine. The cells were then incubated for 90 min to starve them of methionine. Synthesis and labeling of the plasmid-encoded PBP5, which was under the control of the T7 RNA polymerase, were initiated by incubation of the cells with 0.5 mM IPTG for 10 min. To suppress the production of native *E. coli* proteins, genomic transcription was suppressed by a further incubation with 0.2 mg/mL rifampicin for 10 min. Finally, labeling was done by incubation with 15 µCi ³⁵S-methionine for 5 min. Following the pulse-labeling reaction, the cells were resuspended in 1 mL of LB media with kanamycin (50 µg/mL) and chased for 30 min to give the radio-labeled proteins a chance to assemble.

Disulfide cross-linking of cysteine residues was carried out as described in Ref. ⁵⁶ with a few minor modifications. A 50 µL aliquot of whole cells was collected from the radiolabeling reaction, resuspended in 500 µL of 1X PBS pH 7.4, and treated with either 0.5 mM copper phenanthroline (i.e., oxidizing

conditions) or 50 mM dithiothreitol (i.e., reducing conditions). The stock solution of 50 mM copper phenanthroline was always prepared fresh, by mixing 10 μ L of 50 mM CuSO₄ with 30 μ L of 50 mM 1,10-phenanthroline. After incubation on ice for 30 min, the reactions were stopped by the addition of 10 mM *N*-ethyl maleimide and 20 mM EDTA pH 7.0. Cells were pelleted by centrifugation and resuspended in nonreducing SDS-PAGE loading buffer (i.e., without β -mercaptoethanol) and analyzed by SDS-PAGE. Alternatively, whole cells were resuspended in 1 mL of H₂O supplemented with 400 μ g/mL lysozyme and incubated at 30°C for 45 min. Crude membranes were then collected by centrifugation at 264,000g for 30 min at 4°C and resuspended in 85 μ L of ACA₇₅₀ buffer. Membrane proteins were then solubilized by the addition of 0.5% (w/v) DDM and incubation on ice for 30 min. The samples were cleared by centrifugation at 264,000g for 30 min at 4°C and the supernatant was added to 15 μ L of G250 solution [5% (w/v) Coomassie G250 in ACA₇₅₀ buffer] and analyzed by BN-PAGE (as described earlier). Gels were dried and exposed to a BAS TR2040S plate. ³⁵S-methionine-labeled proteins were detected in a Fuji FLA-3000 phosphorimager according to the manufacturer's instructions (Fuji, Tokyo, Japan).

Bocillin-FL labeling

Whole cells were resuspended in 1M 1X PBS pH 7.4 supplemented with 1.6 μ M Bocillin-FL (Invitrogen) and incubated for 30 min at 35°C. Samples were analyzed by SDS-PAGE and fluorescence was detected in a Fuji FLA-3000 phosphorimager according to the manufacturer's instructions at the wavelength of 473 nm and a Y520 filter.

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