Published in final edited form as:
FEMS Microbiol Lett. 2010 February ; 303(1): 76–83. doi:10.1111/j.1574-6968.2009.01863.x.

A weak DD-carboxypeptidase activity explains the inability of PBP 6 to substitute for PBP 5 in maintaining normal cell shape in *E. coli*

Chiranjit Chowdhury[1], **Tapas R. Nayak**[1], **Kevin D. Young**[2], and **Anindya S. Ghosh**[1,*]

[1] Department of Biotechnology, Indian Institute of Technology, Kharagpur, West Bengal, India, PIN-721302

[2] Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, 4301 Markham St., Slot 511, Little Rock, AR 72205-7199

Abstract

PBP 5 plays a critical role in maintaining normal cellular morphology in mutants of *E. coli* lacking multiple PBPs. The most closely related homologue, PBP 6, is 65% identical to PBP 5 but is unable to substitute for PBP 5 in returning these mutants to their wild type shape. The relevant differences between PBPs 5 and 6 are localized in a 20 amino acid stretch of domain I in these proteins, which includes the canonical KTG motif at the active site. We determined how these differences affected the enzymatic properties of PBPs 5 and 6 toward β-lactam binding and the binding and hydrolysis of two peptide substrates. We also investigated the enzymatic properties of recombinant fusion proteins in which active site segments were swapped between PBPs 5 and 6. The results suggest that the *in vivo* physiological role of PBP 5 is distinguished from PBP 6 by the higher degree of D, D-carboxypeptidase activity of the former.

Keywords

Penicillin-binding proteins; morphology maintenance; DD-carboxypeptidase; *Escherichia coli*

Introduction

Of the twelve known penicillin-binding proteins (PBPs) in *Escherichia coli*, four are D,D-carboxypeptidases (DD-CPases): PBPs 4, 5 and 6, and DacD (Holtje, 1998; Ghosh *et al*., 2008). These enzymes remove the terminal D-alanine from the pentapeptide side chains of muramic acid in peptidoglycan (PG), and the resulting tetrapeptides cannot act as donors during formation of peptide crosslinks in the cell wall (Ghuysen, 1991). Although the DD-CPases are usually the most abundant PBPs in the cell, they are not essential for bacterial survival (Denome *et al*., 1999) and the *in vivo* purposes of these seemingly nonessential and redundant enzymes are mostly unknown.

The exception to the above statement is the *E. coli* protein PBP 5, which helps maintain the normal morphology of this organism even in the absence of seven other PBPs (Nelson & Young, 2001). In the absence of PBP 5 by itself the cells exhibit small morphological aberrations, but as more PBPs are deleted the cells become greatly misshapen (Nelson & Young, 2000; Nelson & Young, 2001). PBP 5 consists of two major domains, I and II,

Correspondence: Anindya S. Ghosh, Department of Biotechnology, Indian Institute of Technology, Kharagpur, West Bengal, India. anindyain@yahoo.com.

oriented almost at right angles to one other (Davies *et al*., 2001; Nicholas *et al*., 2003). The DD-CPase active site is located in domain I and is responsible for maintaining normal cell shape (Ghosh & Young, 2003; Nelson *et al*., 2002). Domain II is composed mostly of β sheets and may lift the enzymatic domain away from the inner membrane and into the periplasm toward the peptidoglycan substrate (Ghosh & Young, 2003; Nelson *et al*., 2002). At its extreme carboxyl terminus, at the base of domain II, PBP 5 has a short 18 amino acid (Jackson & Pratt, 1987) amphipathic helix that tethers the protein to the outer face of the inner membrane (Ghosh & Young, 2003; Nelson *et al*., 2002). The closest homologue to PBP 5 from any organism is PBP 6, from *E. coli* itself. Interestingly, PBP 6, though ~65% identical to PBP 5, cannot restore normal shape to aberrant cells, as can PBP 5 (Ghosh & Young, 2003). Domain swap and mutagenesis experiments indicate that the relevant differences between the two enzymes localize to domain I, and, in fact, to a small stretch of 20 amino acids that surrounds the canonical KTG motif of the active site (Ghosh & Young, 2003; Nelson *et al*., 2002). For shorthand, we will refer to this 20 amino acid segment as the "morphology maintenance domain" (MMD) (Ghosh & Young, 2003) (Fig. 1). When PBP 6 is engineered so that its MMD is replaced by that from PBP 5, the mosaic protein (PBP 656) complements the shape defects of the *E. coli* mutants as well as wild type PBP 5 (Ghosh & Young, 2003).

Conversely, replacing the MMD of PBP 5 with that from PBP 6 (creating PBP 565) abolishes the ability to complement. PBPs 5 and 6 differ by seven residues in this peptide fragment, but only two, Asp 218 and Lys 219, seem to be necessary to confer on PBP 656 the complementation attributes of PBP 5 (Ghosh & Young, 2003) (Fig. 1). However, mutation of these two amino acids does not eliminate the ability of PBP 5 to complement shape defects, suggesting that other structural features blunt the effects of these changes in the wild type protein.

The preceding results imply that differences in the abilities of PBPs 5 and 6 to complement cell shape defects can be explained by alterations in enzymatic activity due to minor changes in the amino acid residues near their active sites. However, the DD-CPases as a group share substantial homology in their primary structures and are believed to act on PG substrates via similar mechanisms *in vitro* (Baquero *et al*., 1996). What is not known is if the MMD residues affect the DD-CPase activities of these PBPs, and whether these changes explain the difference in the physiological functions of PBPs 5 and 6. To determine more exactly how the 20 amino acid MMDs of PBPs 5 and 6 contribute to the differences in the *in vitro* DD-CPase activities of these enzymes, we compared the enzymatic characteristics of four soluble PBPs (designated as "sPBPs"): sPBP 5, sPBP 6, and the mosaic proteins sPBP 656 and sPBP 565. The variations in enzymatic activities among these proteins help explain the basis of different biochemical and physiological properties of the DD-CPase PBPs.

Material and Methods

Bacterial strains, plasmids and chemicals

E. coli BL21* (Stratagene, West Cedar Creek, TX) was used to express recombinant proteins for purification in bulk. Plasmid pT7-cPBP5 was provided as gift by Robert A. Nicholas. Plasmids pAG6-4, pAG565-3 and pAG656-1 (9), were used to amplify the genes of soluble PBP 6, PBP 565 and PBP 656, respectively. Unless otherwise specified, restriction enzymes and DNA modifying enzymes were from New England Biolabs, (Ipswich, MA) and other chemicals and reagents were from Sigma-Aldrich, (St Louis, MO).

Construction of soluble PBP variants and fusion proteins

To create genes expressing soluble PBPs, the genes encoding the respective PBPs were amplified by using oligonucleotide primers (from MWG Biotech Inc., High Point, NC) in such a way that the resulting genes would express proteins devoid of their signal peptides and carboxyl-terminal amphipathic anchors. Primer pairs used for amplifications were: (A) P1 and P2 for sPBP 6, using pAG6-4 as the template; (B) P1 and P5 for sPBP 656, using pAG656-1 as the template; and (C) P3 and P4 for sPBP 565, using pAG565-3 as the template (Table 1). The conditions for amplification with Deep Vent DNA polymerase were: 94 °C for 5 min (initial denaturation), 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min (for 30 cycles), followed by a final extension of 72 °C for 7 min. Each amplified product was cloned separately into the *Nde* I and *Hin*d III sites of pT7-7K, creating pTA6-2S (expressing sPBP 6), pTA656-2S (expressing sPBP 656) and pTA565-3S (expressing sPBP 565). Plasmids were selected by including kanamycin (50 μg mL^{-1}) in the medium and were sequenced to confirm that no mutations had arisen (sequencing was performed by MWG Biotech Inc., High Point, NC). Any sequence disparities in the constructs were removed by site directed mutagenesis and reconfirmed by sequencing.

Expression and purification of soluble PBPs

Plasmids encoding the sPBPs were transformed into *E. coli* BL21* and expressed under optimal conditions as determined beforehand (not shown). In short, for expression of sPBP5, overnight cultures were diluted 1:100 and grown at 30 °C in the presence of kanamycin (50 μg mL^{-1}) to an A_{600} of 0.2, at which point isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the cultures were incubated for an additional 12 h. For expression of all other sPBPs overnight cultures were grown at 26 °C to an A_{600} of 1.0 (stationary phase), at which time IPTG was added to a final concentration of 0.1 mM and the cultures were incubated for an additional 8 h. Cells were harvested at 5000 × g for 10 min (Beckman Avanti™ J25I, Fullerton, CA), the cell pellets were collected and resuspended in lysis buffer (400 μg mL^{-1} lysozyme, 50 mM Tris-HCl, 200 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, pH 7.5) for 5 h at 4 °C with occasional stirring. Gross cell debris was removed by centrifugation at 8000 × g (Eppendorf 5810 R, Hamburg, Germany) for 10 min at 4 °C, and membrane vesicles were removed from the resulting supernatant by ultracentrifugation at 100,000 × g for 1 h at 4 °C (Sorvall Ultra *Pro* 80, Medcompare, San Francisco, CA). Soluble PBPs were purified from this final supernatant by ampicillin affinity chromatography, as described (Nicholas & Strominger, 1988), with slight modifications. sPBP supernatants were incubated with ampicillin-coupled activated CH-Sepharose 4B (Amersham Biosciences Piscataway, NJ) for 1 h at 30 °C. The resin was washed once with 50 mM Tris-HCl, pH 7.5, containing 1 M NaCl, and then washed once more with the same buffer lacking NaCl. The resin bound PBPs were eluted with 1 M NH_2OH, 0.5 M Tris-HCl, pH 7.0 (Nicholas & Strominger, 1988). The purified PBP fractions (1.5 mL) were pooled and dialyzed against 20 mM Tris- HCl, 150 mM NaCl, pH 7.5, with three changes of buffer. Protein concentrations were determined by the Bradford assay kit (Sigma Chemical Co, St Louis, MO).

Fluorescent penicillin labeling

The activity of each purified sPBP was determined by labeling with 50 μM BOCILLIN FL(Invitrogen Inc., Carlsbad, CA) (Zhao *et al*., 1999). Reaction mixtures were incubated for 30 min at 35 °C, after which the proteins were denatured by adding 10 μL of denaturing solution to the reaction mixture and boiling for an additional 3 min. The proteins were separated and analyzed by electrophoresis through 12% SDS-PAGE gels. Labeled PBPs were visualized by washing the gel twice with deionized water and scanning immediately by using a Typhoon Trio variable imager (Amersham Biosciences, Piscataway, NJ) at an excitation wavelength of 488 nm and an emission wavelength of 526 nm.

Circular dichroism analyses

The far UV circular dichroism (Far UV CD) spectra of each soluble protein was determined by using a Jasco J-810 spectropolarimeter (Easton, MD), placing the samples in a quartz cell (path length = 0.2 cm) at 25 °C. Spectral data of sPBPs (2.5 μM) were collected with a 0.2 nm step resolution, 1 s time constant, 10 milli degrees sensitivity at a 2.0 nm spectral bandwidth, with a scanning speed of 50 nm min^{-1}. Corrected spectra were obtained by subtracting the solvent spectrum. To reduce noise and random instrumental error, an average spectrum was compiled from four successive accumulations over a range of 200–240 nm. The recorded spectra in milli degrees of ellipticity (θ) were converted to mean residue ellipticity [θ] in degree cm^2 deci mol^{-1} by using the following equation:

$$[\theta]=(\theta \times 100 \times Mr)/(c \times l \times N_A)$$

where c is the protein concentration in mg mL^{-1}, l is the path length in cm, Mr is the protein molecular mass, and N_A is the number of amino acids in the protein. Secondary structure was estimated by k2d software (Andrade *et al*., 1993).

Kinetic analysis of PBP interaction with β-lactam and peptide substrates

The kinetic parameters relating to the interaction of PBPs (E) with peptide (S) or β-lactam (S) were calculated by obeying the reaction,

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot S \xrightarrow{k_2} E-S \xrightarrow{k_3} E+\mathrm{P}$$

where, $K = (k_{-1} + k_2)/k_1$, $E \bullet S$ is the non-covalent Michaelis complex, E–S is the covalent acyl enzyme complex, and P is the released product. The rate of acyl-enzyme complex was determined by the second order rate constant k_2/K at sub-saturating concentrations of β-lactam antibiotics.

The acylation rate of sPBPs was assessed by incubating the enzymes (250 μg) for 30, 60, 90 or 120 s with BOCILLIN FLat different concentrations (25, 50 and 100 μM). Due to the poor binding of sPBP 565 with BOCILLIN FL, this protein was incubated with the substrate for longer times (1, 2, 4 and 6 h). The reaction was stopped by adding SDS sample buffer and denaturing the proteins by boiling for 5 min. Samples were analyzed by subjecting them to 12% SDS-PAGE and subsequently measuring the band intensities by densitometric scanning (UVP Gel documentation system, San Gabriel, CA) (Chambers *et al*., 1994). The second order rate constant (k_2/K) was determined by calculating the pseudo-first-order rate constant, k_a, using the following equation:

$$k_a=\frac{-\ln\{1-([\mathrm{PBP_D}]/[\mathrm{PBP_o}])\}}{t}$$

where PBP_D denotes the density (experimentally derived) of the fluorophore associated with sPBP bound BOCILLIN FLobtained by incubating for time t at a given BOCILLIN FL concentration D, and PBP_o denotes the density at which all binding sites of sPBPs were saturated with BOCILLIN FL. Values obtained for k_a were plotted against the corresponding BOCILLIN FL concentration, and k_2/K values were determined by calculating the slope.

The deacylation rate of purified sPBPs was determined by incubating proteins (50 μg) with BOCILLIN FL (50 μM) for 15 min at 37 °C. At t = 0, penicillin G was added to 3 mM, and the amount of fluorescent intensity remaining covalently bound to the protein was determined by removing the aliquots at various times (Guilmi *et al*., 2000). The labeled PBPs were quantified by densitometric scanning after separation by SDS-PAGE.

The deacylation reaction obeys the following equation:

$$\ln(PBP_t/PBP_o) = -k_3 t$$

where PBP_t is the residual acyl-enzyme concentration at time t and PBP_o is the initial concentration of acyl-enzyme complex. The deacylation constant (k_3) was determined by quantifying the fluorescence of enzyme-bound BOCILLIN FL (the acyl-enzyme complex) that decreases over time, and was determined by using the above equation.

DD-carboxypeptidase activities of each sPBP were determined for the artificial substrate N_α, N_ε-Diacetyl-Lys-D-Ala-D-Ala and for the peptidoglycan mimetic pentapeptide L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala (USV custom peptide synthesis, Mumbai, India). Each sPBP (2 μg) was mixed with varying concentrations (0.25 to 12 mM) of the respective peptides, and the reaction volume was adjusted to 60 μL with 50 mM Tris HCl, pH 8.5. The mixture was incubated for 30 min at 37 °C, at which time 140 μL of freshly prepared enzyme-coenzyme mix was added (this solution was composed of a 20:10:5:1 ratio of the following: 50 mM Tris HCl, pH 8.5; 0.3 mg mL^{-1} of FAD; 10 μg mL^{-1} of horseradish peroxidase; and 5 mg mL^{-1} of D-amino acid oxidase). This final mixture was incubated for 5 min at 37 °C. Free D-alanine generated in this reaction was detected by the method of Frere *et al* (Frere *et al*., 1976), and compared to a standard D-alanine solution by using a Multiskan Spectrum-1500 Spectrophotometer (Thermo Scientific, Nyon-1, Switzerland) set at 460 nm.

Kinetic parameters for the DD-CPase assay were deduced from the linear regression of the double reciprocal plot (Lineweaver-Burk plot) (Lineweaver & Burk, 1934).

$$\frac{1}{V} = \frac{K_m}{V_{\max}} \frac{1}{[S]} + \frac{1}{V_{\max}}$$

where, V is the reaction velocity (the reaction rate), K_m is the Michaelis-Menten constant, $V_{\max}$ is the maximum reaction velocity, and [S] is the substrate concentration. Further, to estimate the activity of the enzyme the turnover number (k_{cat}) was measured by using the following equation:

$$k_{cat} = V_{\max}/[\text{total enzyme}]$$

Kinetic data were analyzed by using the Enzyme Kinetic Module of SIGMAPLOT v 9.0 (SYSTAT Software Inc., Chicago, IL) to determine K_m and k_{cat}.

Results

Previously, we constructed recombinant fusion proteins in which MMD segments were swapped between PBPs 5 and 6, creating the mosaic proteins PBP 656 and PBP 565 (9). PBP 656 complemented the shape defects of PBP mutants, but PBP 565 did not (Ghosh & Young, 2003). Here, we investigated the properties of the fusion proteins and their wild type

counterparts to determine if enzymological differences among these PBPs might explain the disparities in their *in vivo* behaviors.

Creation and purification of soluble PBPs

To measure the biophysical and biochemical properties of PBPs 5 and 6 and their mosaic partners, it was necessary to create soluble versions of the enzymes. To do so, we constructed cloned genes that eliminated the signal peptide and C-terminal membrane anchor of each protein. Soluble PBP 5 can be prepared by deleting the C-terminal 10 amino acids that constitute the membrane-binding amphipathic helix (Pratt *et al*., 1986). Since the sequences of the C-terminal amphipathic anchors of PBPs 5 and 6 have substantial similarity (Nelson *et al*., 2002), we constructed soluble versions of these PBPs by removing 11 (PBP 565) or 15 amino acids (PBPs 6 and 656) from their carboxyl termini. In addition, we removed the 29 N-terminal amino acids that constitute the signal peptide for PBP 565 and 27 N-terminal amino acids for PBP 6 and PBP 656, so that the soluble proteins were not exported to the periplasm but remained cytoplasmic. The primer pairs used to create these soluble and truncated PBPs *via* PCR are listed in Table 1. The final proteins contained 359 amino acids (sPBP 6 and sPBP 656) or 364 aa (sPBP5 and sPBP 565). Genes encoding these soluble PBPs were cloned and the proteins were overproduced by inducing gene expression under optimum conditions of incubation time, temperature and IPTG concentration. No inclusion body was accumulated upon overexpression.

The sPBPs were purified by ampicillin-linked affinity chromatography, which generated a significant amount of product for sPBP 5 (0.4 mg mL^{-1}), sPBP 6 (0.3 mg mL^{-1}) and sPBP 656 (0.8 mg mL^{-1}). The average total amounts of these purified proteins represented approximately 3–5 mg per liter of culture. However, the concentration of purified sPBP 565 was much less, so it was necessary to concentrate this protein 200-fold by ultra-filtration (Nicholas & Strominger, 1988) to give a final concentration of 0.4 mg mL^{-1}. Molecular masses of the sPBPs, as determined by separation on 12% SDS-PAGE gels, were ~ 40 kDa (sPBP 5 and sPBP 565) and ~ 39 kDa (sPBP 6 and sPBP 656) (Fig. 2). The proteins were stable for months after storing at −80° C with 50 % glycerol and were functionally active because they all bound BOCILLIN FL(Zhao *et al*., 1999), a fluorescent version of penicillin V, even after long storage. The presence of labeled bands after SDS-PAGE indicated that BOCILLIN FL bound covalently to each sPBP (Fig. 2B), though less BOCILLIN FLbound to an equivalent amount of sPBP 565 than to the other three proteins (Fig. 2B, lane 4).

Gross secondary structures of the PBP variants are indistinguishable

It was possible that moving the MMD from one PBP to another could have altered the overall secondary structure of the recipient protein so much that one or more of its functions were disturbed. To determine if there were gross changes to the secondary structures of the mosaic PBPs, we analyzed the sPBPs by low-resolution circular dichroism (CD) spectroscopy to estimate the distribution of α-helical and β-sheet structures (Sreerama *et al*., 1999; Venyaminov & Yang, 1996). The predicted secondary structures indicated there were no substantial differences among any of the sPBPs (Table 2), suggesting that their overall folding patterns remained intact. The results eliminated this trivial explanation for the inability of PBP 6 and PBP 565 to complement shape defects *in vivo*.

The morphology maintenance domain (MMD) alters acylation and deacylation rate toward penicillin

β-lactam antibiotics bind covalently to a serine residue at the active site of PBPs, thereby inactivating the enzymes. Because β-lactams are substrate analogues of the D-alanyl-D-alanine terminus of the peptide side chain in peptidoglycan (Park & Strominger, 1957; Park, 1996), the rate of acylation by penicillin measures one facet of the enzymatic activity of the

PBPs. To determine how efficiently sPBPs bound penicillin, we assessed the interaction of each sPBP with BOCILLIN FL. The acylation rate (k_2/K) for sPBP 5 was approximately 40% of the rate observed for sPBP 6 (Table 3). The rate for mosaic protein sPBP 656 was ~70% of that for sPBP 6, which was more than 50% greater than that of sPBP 5. Thus, grafting the MMD of PBP 5 into PBP 6 decreased the penicillin acylation rate of sPBP 6, though the rate remained higher than that of wild type sPBP 5 (Table 3). This indicates that the MMD of PBP 5 is important but does not by itself determine the efficiency of acylation in the context of PBP 6. On the other hand, the acylation rate for sPBP 565 was drastically lower than that of PBP 5. Therefore, placing the MMD of PBP 6 into PBP 5 decreased the acylation rate of PBP 5 to 98% of its former value.

To understand how efficiently the sPBPs released bound penicillin from the acyl-enzyme complex (a measure of the catalytic efficiency), k_3 values were determined for each of the constructs. The acylation rate for sPBP 6 was about 10 times less than that of sPBP 5 (Table 3). However, upon grafting the stretch of amino acids that corresponds to the MMD of PBP 5 into PBP 6 (i.e., sPBP 656), the deacylation efficiency of sPBP 6 increased four-fold. In contrast, the hydrolysis of BOCILLIN FL by sPBP 565 was too slow to measure in laboratory conditions, indicating that although the PBP 5 MMD was partially efficient in influencing deacylation of the BOCILLIN substrate, the corresponding stretch of amino acids from PBP 6 had no such effect. Taken together, the influence of the PBP 5 MMD on acylation and deacylation is noteworthy, and the rates of penicillin acylation or deacylation can serve as good predictors for the ability of PBPs 5 or 6 or of their mosaic counterparts to complement morphological defects of *E. coli* shape mutants.

The DD-carboxypeptidase activity of sPBP 5 is higher than that of sPBP 6

The acylation rates as measured by penicillin binding may not reflect the true enzymatic activities of the PBPs toward their natural D-ala-D-ala peptidoglycan substrates. The conventional substrate used to assay the DD-CPase activity of PBPs is N^{α},N^{ε}-diacetyl-Lys-D-Ala-D-Ala (AcLAA) (Supplementary Fig. 1A), and the activity of PBP 5 toward this substrate is significant (Nicholas *et al*., 2003). To determine if the *in vivo* differences of the PBPs coincided with differences in their native DD-CPase activities, we measure the kinetic properties of the soluble versions of PBPs 5 and 6 and their mosaic constructs toward AcLAA. The K_m of sPBP 6 for AcLAA was 7 times lower than that of sPBP 5, indicating that PBP 6 formed the acyl-enzyme complex at a much faster rate than that of PBP 5 (Table 4).

For sPBP 656 the K_m was increased by a factor of ~3 compared to that of PBP 6, but sPBP 565 displayed no DD-CPase activity whatsoever (Table 4). These results were qualitatively equivalent to those observed for β-lactam binding among these proteins. sPBP 6 bound substrate significantly better than did sPBP 5; grafting the MMD of PBP 5 into PBP 6 reduced the affinity of sPBP 6 for its substrate, though the affinity of the mosaic protein was still higher than that of sPBP 5; and inserting the MMD of PBP 6 into sPBP 5 completely abrogated its DD-CPase activity, indicating that this active site segment of PBP 6 does not function in the PBP 5 background.

In contrast to what might be expected from the order of binding affinities, the DD-CPase activities did not correlate with higher binding of the AcLAA substrate. Instead, the turnover number (k_{cat}) of sPBP 5 was ~5 times higher than that of sPBP 6; replacing the MMD of PBP 6 with that of PBP 5 increased the k_{cat} of sPBP 656 by about 25%; but sPBP 565 remained inactive on this substrate (Table 4). Here, the degree of substrate binding was inversely correlated with the rate at which substrate was converted into product.

Only sPBP 5 acts as a DD-CPase on a pentapeptide substrate

Although AcLAA is routinely used for DD-CPase measurements, it is an artificial compound that does not exist in peptidoglycan. To analyze DD-CPase activity more appropriately, we assayed the activities of the PBPs toward a peptidoglycan mimetic pentapeptide substrate, LAla-γ-D-Glu-L-Lys-D-Ala-D-Ala (AGLAA) (Supplementary Fig. 1B). sPBP 5 exhibited significant DD-CPase activity, but sPBP 6 was inactive on this substrate (Table 4). Grafting the MMD of PBP 5 into PBP 6 produced DD-CPase activity in sPBP 656 (Table 4), indicating that this portion of the PBP 5 active site could impart to PBP 6 a measurable fraction of DD-CPase activity (about 14% that of sPBP 5). Once again, inserting the MMD of PBP 6 into PBP 5 completely eliminated the DD-CPase activity from the sPBP 565 mosaic protein (Table 4). Both the K_m and k_{cat} of sPBP 5 toward AGLAA were lower than when AcLAA was the substrate. This was in line with the behavior of sPBPs 5 and 6, in that a lower K_m for the substrate was accompanied by a reduced rate of product formation.

Discussion

PBP 5 helps maintain the normal rod shape of *E. coli* and can restore the wild type shape to *E. coli* mutants deprived of three to seven PBPs (Nelson & Young, 2000; Nelson & Young, 2001; Nelson *et al*., 2002), and the mechanism by which it does so is probably related to its D,D-carboxypeptidase activity (Ghosh & Young, 2003; Nelson *et al*., 2002; Ghosh *et al*., 2008;). However, *E. coli* also expresses PBP 6, which exhibits DD-CPase activity and is the most closely related homologue of PBP 5 (Goffin & Ghuysen, 1998; Ghosh *et al*., 2008). However, despite these resemblances, PBP 6 cannot substitute for PBP 5 in maintaining or restoring normal cell shape to PBP mutants (Nelson & Young, 2001; Nelson *et al*., 2002; Ghosh & Young, 2003). At least some of the relevant differences in the *in vivo* functions of these two PBPs lie in a short stretch of residues in and near the active site (Ghosh & Young, 2003), but it is not known how these sequence differences affect the enzymatic activities of these enzymes. Here we investigated the kinetic properties of PBPs 5 and 6 and two mosaic proteins and find that the enzymes differ in their substrate preferences and in the rates at which they remove the terminal D-alanine from these substrates. The results suggest that these differences correlate with the *in vivo* phenotypes of shape maintenance.

The DD-carboxypeptidase activity of PBP 5 is responsible for maintaining cell shape

PBP 5 is clearly a better DD-CPase than PBP 6. For example, depending on the substrate, the DD-CPase activity of PBP 5 was previously shown to be 3 to 5 times greater than that of PBP 6 (Amanuma & Strominger, 1980). In our assays, the DD-CPase activity of sPBP 5 was five times greater than that of sPBP 6 when tested against the substrate N^{α},N^{ε}-diacetyl-Lys-D-Ala-D-Ala (AcLAA). An even greater difference was observed when the enzymes were tested against the peptidoglycan mimetic substrate L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala (AGLAA), on which PBP 5 was active but to which PBP 6 may not bind covalently or else it may bind but not cleave. The failure of PBP 6 to act on this latter substrate is consistent with the observations of van der Linden and his co-workers (van der Linden *et al*., 1992). However, no DD-CPase activity on AcLAA and UDP-muramyl pentapeptide substrates was reported for either the membrane bound or the soluble form of PBP 6 (van der Linden *et al*., 1992). We speculate that sPBP 6 exhibited a low level of DD-CPase activity toward the artificial substrate, AcLAA, possibly because the active site cleft of sPBP 6 might accommodate smaller substrates like penicillin and AcLAA while being unable to bind a bulkier substrate such as AGLAA. The phenomenon of complete inertness of sPBP 6 toward pentapeptide substrate is interesting that simultaneously raises doubt whether it functions as DD-CPase *in vivo* at all.

Previously, we found that the differences between PBPs 5 and 6 in complementing shape defects *in vivo* could be narrowed down to a short stretch of 20 contiguous residues within the active site (the "morphology maintenance domain," or MMD), where the two PBPs differ from one another by only 7 amino acids (Ghosh & Young, 2003). Shape complementation was associated with the MMD from PBP 5 and not with that from PBP 6 (Ghosh & Young, 2003). Here, we assayed soluble versions of mosaic proteins in which the MMDs from the two PBPs were switched with one another and found that DD-CPase activity was associated primarily with the MMD from PBP 5 but not with that from PBP 6. That is, sPBP 656 (PBP 6 containing the MMD of PBP 5) exhibited a DD-CPase activity toward both substrates that was more like PBP 5 than PBP 6, but sPBP 565 (PBP 5 containing the MMD of PBP 6) was not active on either of two substrates. In addition to its decreased DD-CPase activity, PBP 565 also bound and hydrolyzed the β-lactam BOCILLIN FLmuch less well than any of the other proteins. These behavioral changes of sPBP 565 must not be due to the improper folding of the molecule because CD spectral analyses predict that there is no gross alteration in the composition of overall secondary structure of sPBP 565 compared to sPBP 5, except that there is 3% less β-sheet structure in the former. Although, the reason for altered behavior of sPBP 565 is not clear, a gross change in the micro-architecture of the active site cannot be ruled out. Because β-sheet structures are not usual components of active sites, the lower percentage of β-sheet structure in sPBP 565 is not likely the key feature for its altered behavior. Nevertheless, the possible changes include altering the size and volume of the substrate binding pocket that may change the affinity or activity of the chimeric protein toward specific substrates. In any case, the ability of each PBP to act as a DD-CPase correlates exactly with its ability to complement cell shape changes *in vivo*, strongly suggesting that this activity is responsible for the shape maintenance phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Robert A. Nicholas for providing the plasmid pT7-cPBP5 and for suggestions regarding the construction of soluble PBPs. We also acknowledge Rakesh Sikder for initiating the computational work. This work was supported by a grant from the Department of Science and Technology, the Government of India, to ASG, and KDY was supported by a grant R01-GM061019 from the U. S. National Institutes of Health and by the Arkansas Biosciences Institute, the major research component of the Arkansas Tobacco Settlement Proceeds Act of 2000.

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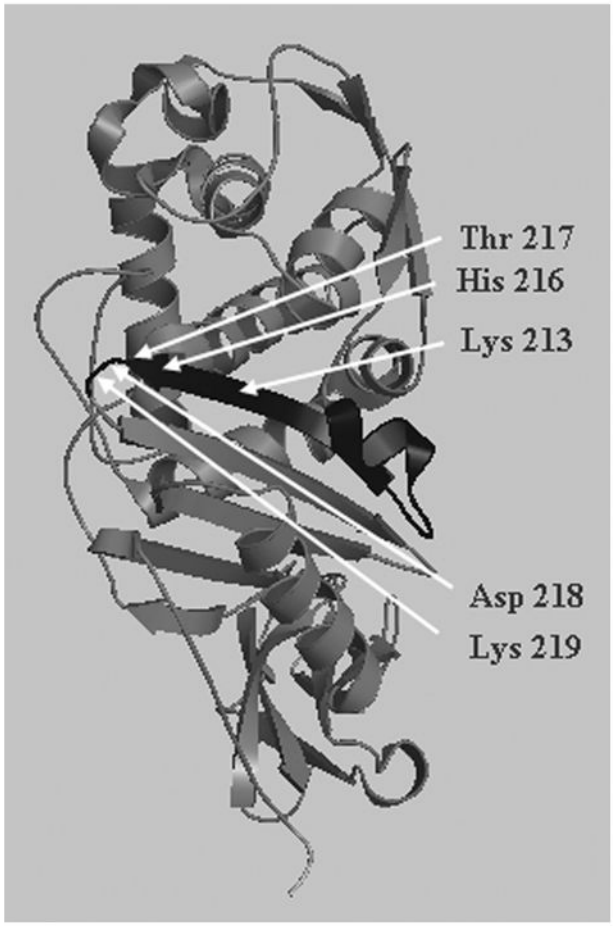

Fig. 1. Location of the "morphology maintenance domain" (MMD) of PBP 5
Top view: Arrows point to residues of sPBP 5 important for catalysis.

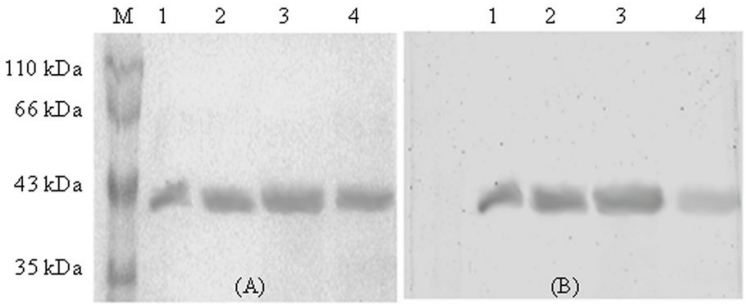

Fig. 2. SDS-PAGE analysis of purified sPBPs and their mosaics

Purified sPBPs (3 μg) were stained with Coomassie blue (A), or with BOCILLIN FL (B). Lane M, Protein molecular weight markers; Lane 1, sPBP 5; Lane 2, sPBP 6; Lane 3, sPBP 656; and Lane 4, sPBP 565.

Table 1

Primer sequences for amplifying soluble PBPs 5 and 6, and their mosaics

Primer	Primer sequence
P1	5′-CTC TCT CT**C ATA TG**G CGG AAC AAA CCG TTG AAG CGC CGA GC-3′
P2	5′-CTC TCT CT**A AGC TT**T CAT CCG CCC TCT TCC ACA TTT TCC ATC AC -3′
P3	5′-CTC TCT CT**C ATA TG**G ATG ACC TGA ATA TCA AAA CTA T -3′
P4	5′-GCA TGC **AAG CTT** TCA ATC GAT CAC CGG ATC GCC GAA GTT ACC TTC CGG GAT TTC -3′
P5	5′-GCA TGC **AAG CTT** TCA ATC CCA CAC CCG ACC AAA GAA TCC GCC-3′

Underlined sequences are complementary to the 5′ or 3′ end of the gene fragment in each construct. Boldfacing designates enzyme recognition sites: AAGCTT, *Hin*d III; CATATG, *Nde* I.

Table 2

Percentage distribution of secondary structures obtained from CD

PBPs	% of α-Helix	% of β-sheet	% of Random coil
sPBP 5	23	27	50
sPBP 6	22	27	51
sPBP 656	24	25	51
sPBP 565	24	24	52

Table 3

Kinetic parameters with BOCILLIN FL

Protein	k_2/K	k_3
	$M^{-1}\ s^{-1}$	$s^{-1} \times 10^{-5}$
sPBP 5	800 ± 50.3	18.9 ± 1.7
sPBP 6	1900 ± 65.5	1.7 ± 0.1
sPBP 656	1300 ± 45.6	6.8 ± 0.1
sPBP 565	20 ± 5.2	ND

ND = Not detected

Table 4

Kinetic parameters with peptide substrates

Protein	N^{α}, N^{ε}-Diacetyl-Lys-D-Ala-D-Ala		L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala	
	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})
sPBP 5	8.98 ± 1.79	2.70 ± 0.30	1.38 ± 0.43	1.41 ± 0.11
sPBP 6	1.28 ± 0.26	0.56 ± 0.03	ND	ND
sPBP 656	4.28 ± 1.34	0.71 ± 0.09	0.81 ± 0.21	0.20 ± 0.01
sPBP 565	ND	ND	ND	ND

ND = Not detected