Active-Site Residues of the Transpeptidase Domain of Penicillin-Binding Protein 2 from *Escherichia coli*: Similarity in Catalytic Mechanism to Class A β-Lactamases†

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ABSTRACT: By means of amino acid sequence alignment with class A β-lactamases, the residues essential for the catalytic activity of the peptidoglycan transpeptidase of penicillin-binding protein 2 (PBP2) have been predicted to be Lys^{333}, Asp^{447}, and Lys^{544}, in addition to the acylation site residue for the acyl-enzyme mechanism, Ser^{330}. Accordingly, these residues were replaced by site-directed mutagenesis, and the resultant mutants were examined as to penicillin-binding activity and genetic complementation, which represent only the acylation step and the total reaction during transpeptidation, respectively. All the mutants at position 333 showed the complete loss of both the binding and complementation activities. Most of the mutants at position 447 retained the binding activity but lost the complementation activity, the exception being the D447E mutant, which retained both. The binding rates for various penicillins of the D447N mutant, which had lost the complementation activity, were almost identical to those of the wild type. The binding of the mutants at position 544 tended to require a higher penicillin concentration, and that of the K544H mutant required a lower pH. When the roles of the counterpart residues, Lys^{73}, Glu^{166}, and Lys^{234}, in class A β-lactamases were considered, the results suggested that Lys^{333} and Asp^{447} are essential for the acylation and acyl-transfer steps, respectively, and that Lys^{544} stabilizes the Michaelis complex through its side-chain positive charge.

Penicillin-binding proteins (PBPs)[1] (Spratt, 1975; Waxman & Strominger, 1983) are located in the cytoplasmic membrane and involved in the final steps of bacterial cell wall synthesis. PBPs exhibit penicillin-sensitive peptidoglycan transpeptidase (TP) activity or DD-peptidase (D-Ala-D-Ala carboxypeptidase) activity (Izaki et al., 1966) through an acyl-enzyme mechanism (Tipper & Strominger, 1965). The TP domain was detected in the C-terminal half of the higher molecular weight PBPs in *Escherichia coli*, whose N-terminal half carried penicillin-insensitive peptidoglycan transglycosylase (TG) (Nakagawa et al., 1984; Hedge & Spratt, 1984). β-Lactam antibiotics, such as penicillins, act as analogues of the D-Ala-D-Ala portion of the peptidoglycan precursors (Tipper & Strominger, 1965) and bind to PBPs covalently to form stable acyl-enzymes, which resembles the case of the acyl-enzyme intermediate in transpeptidation or DD-peptidation. The acylation site of various PBPs was determined biochemically to be a Ser residue in the conserved tetrad, Ser-X-X-Lys (Frère et al., 1976; Yocum et al., 1979; Nicholas et al., 1985a–c; Keck et al., 1985; Takasuga et al., 1988). This sequence is also conserved among the acylation sites of class A and C β-lactamases, which hydrolyze β-lactams through an acyl-enzyme mechanism (Joris et al., 1988).

Crystallographic studies have revealed the structural similarity of the active-site cavity around the acylated Ser residue (Ser^{70} in class A β-lactamases) among DD-peptidases (Kelly et al., 1989) and class A (Herzberg & Moult, 1987; Dideberg et al., 1987; Moews et al., 1990; Herzberg, 1991) and C (Oefner et al., 1990) β-lactamases. In all cases, the Lys residue in the tetrad, Ser-X-X-Lys, is in a position that is likely important for catalysis. This Lys residue in class A β-lactamases (Lys^{73}) was predicted to act in the acylation step (Herzberg & Moult, 1987). Another Lys or a His residue (Lys^{234} in class A β-lactamases) seemed to interact electrostatically with the C3 carboxylate of penicillins, which corresponds to the C-terminal carboxylate of D-Ala-D-Ala. This residue was conserved in the triad, Lys-Thr(Ser)-Gly or His-Thr-Gly, and had a similar position in the primary structures, as was the case for the TP domain (Joris et al., 1988). Tyr^{150} in class C β-lactamases with a hydroxyl group was considered to be an important catalytic residue, Ser^{130}, in class A enzymes sharing the corresponding position in the active-site structure (Oefner et al., 1990). In addition, we have recently verified, by site-directed mutagenesis, that Glu^{166} in class A β-lactamases acts as a catalyst for deacylation (Adachi et al., 1991).

The tertiary structure of the TP domain has not yet been reported. It is, however, expected that the spacial arrangement of the active-site residues is similar to those in DD-peptidases and class A and C β-lactamases, since the catalytic reaction of TP is analogous to those of other penicillin-interactive enzymes and the two conserved sequences, the tetrad and the triad, containing catalytically important residues actually exist in the primary structure of the TP domain.

PBP2 is a member of the higher molecular weight PBPs in *E. coli* (Ishino et al., 1986; Asoh et al., 1986). In this study,

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[1] Abbreviations: PBP, penicillin-binding protein; TP, transpeptidase; TG, transglycosylase; kb, kilobase pairs; OD, optical density; IPTG, isopropyl β-D-thiogalactopyranoside; SDS–PAGE, SDS–polyacrylamide gel electrophoresis.

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Table I: Data for the Oligodeoxynucleotides Used for Site-Directed Mutagenesis

Sequencing Primers			
name	sequence (no. of bases)	position[a]	purpose[b]
on911	5′-TTATTCCGCCTTGTTGA-3′ (17)	1949–1965	S330, K333
on912	5′-TGATTAATGACGGTATC-3′ (17)	2449–2465	K544, N527
on914	5′-TGGCAACTGCCAGGTTC-3′ (17)	2108–2122	K435–Q445, Y393, D447

Mutagenic Primers			
name	sequence[c] (no. of bases)	mutation introduced	M13 phage used
on202	5′-ACATGCCTACCCGCGAATGG*GGTGACACCATTCCGGTTGG-3′ (40)	Δ435–445	mpHA01
on251	5′-TCCCGCGTGTACAGTTA-3′ (17)	S330C	mpHA01
on261	5′-TACAGTTAGACCCTATG-3′ (17)	K333R	mpHA01
on265	5′-GTCTACAGTTCATCCCTATGTGG-3′ (23)	K333H	mpHA01
on263	5′-TCTACAGTTGAACCCTATG-3′ (19)	K333E	mpHA01
on267	5′-CGTCTACAGTTCAACCCTATGTG-3′ (23)	K333Q	mpHA01
on266	5′-GTCTACAGTTGACCCCTATGTGG-3′ (23)	K333D	mpHA01
on311	5′-CCTTCTTCTTTCAGGTGGC-3′ (19)	Y393F	mpHA01
on312	5′-CCTTCTTCTCTCAGGTGGC-3′ (19)	Y393S	mpHA01
on281	5′-ATCAGGGTGCCACCATTCC-3′ (19)	D447A	mpHA01
on282	5′-TCAGGGTGAGACCATTCCG-3′ (19)	D447E	mpHA01
on283	5′-TATCAGGGTAACACCATTC-3′ (19)	D447N	mpHA01
on285	5′-GTATCAGGGTCAGACCATTCCGG-3′ (23)	D447Q	mpHA01
on284	5′-GTATCAGGGTAAAACCATTCCGG-3′ (23)	D447K	mpHA01
on291	5′-CCGCCGAAGCCGGCAAACA-3′ (19)	D490A	mpHA01
on292	5′-CGCCGAAGAGGGCAAACAG-3′ (19)	D490E	mpHA01
on293	5′-ACCGCCGAAAACGGCAAAC-3′ (19)	D490N	mpHA01
on301	5′-TAACCGCCCTGGCGGTACGGCG-3′ (22)	N527G	mpHA02
on302	5′-TAACCGCCCTCAGGGTACGGCGC-3′ (23)	N527Q	mpHA02
on303	5′-AACCGCCCTGACGGTACGG-3′ (19)	N527D	mpHA02
on271	5′-TTGCGGCGAGGTCCGGTACC-3′ (20)	K544R	mpHA02
on272	5′-ATTGCGGCGCACTCCGGTACC-3′ (21)	K544H	mpHA02
on273	5′-ATTGCGGCGGAATCCGGTA-3′ (19)	K544E	mpHA02
on274	5′-ATTGCGGCGCAATCCGGTA-3′ (19)	K544Q	mpHA02

[a] The nucleotide sequence of the *pbpA* gene is numbered according to Asoh et al. (1986). [b] Amino acid residues at which the mutations were introduced and confirmed by sequencing using the primer. [c] * denotes the position of a deletion, and bold characters, mismatched bases.

on the basis of the sequence alignment, the residues in the TP domain of PBP2 which correspond to those constituting the active-site cavity in class A β-lactamases were replaced with other amino acids by site-directed mutagenesis. The resultant mutant enzymes were then characterized, and the role of each residue in transpeptidation was discussed.

Experimental Procedures

Construction of Plasmids and Phages. Most of the recombinant DNA techniques (Maniatis et al., 1982) and the manipulation of M13 phages (Messing, 1983) were as described previously. Site-directed mutagenesis was performed using a MUTA-GENE M13 in vitro mutagenesis kit (Bio-Rad) according to Kunkel et al. (1987), or an oligonucleotide-directed in vitro mutagenesis system (Amersham) according to Taylor et al. (1985). The oligodeoxynucleotides for site-directed mutagenesis, mutagenic primers and sequencing primers, were synthesized with an Applied Biosystems 380B DNA synthesizer. The data for the oligodeoxynucleotides and the mutations introduced in this study are summarized in Table I. The PBP2 expression plasmid, pHA103 (Adachi et al., 1987), was used for the genetic complementation test. The PBP2* expression plasmid, pHA105Δ93, was used for the penicillin-binding assay. pHA105Δ93 was constructed by replacing the ori region of the plasmid, pHA103Δ93 (Adachi et al., 1987), with that of pUC19 (Yanisch-Perron et al., 1985) to raise the copy number. In both cases, PBP2 or PBP2* should be produced under the control of the expression system of pIN-II vectors which contain the *lpp-lac* promoter (Nakamura & Inouye, 1982). The mutations planned were introduced into these expression plasmids as follows: the 1.0 kilobase pair (kb) *Eco*RV–*Kpn*I and 1.4-kb *Hpa*I–*Apa*I fragments obtained from the *pbpA-rodA* region (Asoh et al., 1983, 1986; Matsuzawa et al., 1989) were cloned into a M13 phage vector, M13mp18 (Yanisch-Perron et al., 1985), to produce mpHA01 and mpHA02, respectively. According to the position, the mutations were introduced into mpHA01 or mpHA02 by site-directed mutagenesis. The mutations were confirmed by DNA sequencing. The 0.8-kb *Hpa*I–*Nco*I or 0.6-kb *Nco*I–*Apa*I regions of both pHA103 and pHA105Δ93, which encode portions of the TP domain, were replaced by the corresponding regions of the mutant mpHA01 or mpHA02, to produce mutant pHA103s and pHA105Δ93s, respectively.

Genetic Complementation Test. E. coli TMM13, a PBP2^{ts} strain (Tamaki et al., 1980), transformed with mutant pHA103s, was cultivated to full growth in LB medium (Adachi et al., 1991) supplemented with 0.1% glucose, 50 μg/mL kanamycin sulfate, and 50 ng/mL DL-lipoic acid at 30 °C. The cells were collected, washed once with BT′ medium (1% Bactotryptone (Difco), 0.25% NaCl, 50 μg/mL adenine hydrochloride, 1 μg/mL thiamine hydrochloride, 50 ng/mL DL-lipoic acid, pH 7.0), and then spotted onto a BT′ agar plate containing kanamycin. After incubation at 42 °C for 9 h, the shape of the cells was observed under a microscope. Genetic complementation is positive (+) when the cells are rod-shaped and negative (−) when they are spherical.

Detection of Penicillin-Binding Activity (PBP Assay). The methods used were essentially those of Hedge and Spratt (1984). LB medium containing kanamycin was inoculated with 1% of an overnight culture of *E. coli* MV1184 (Vieira & Messing, 1987) harboring mutant pHA105Δ93s, followed by vigorous shaking at 37 °C. When the optical density (OD) at 660 nm of the culture reached about 0.3, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, to induce the expression of mutant PBP2*s. After shaking for 3 h at 37 °C, the culture was cooled on ice. 1.5 OD units, at 660 nm, of cells were collected by centrifu-

gation, suspended in ice-cold 10 mM sodium phosphate buffer (pH 7.0), and then adjusted to 100 μL. Twenty microliters of the resultant suspension (0.3 OD unit) was used for one assay. The cells were disrupted mildly by two cycles of freezing at −80 °C and thawing on ice and then preincubated at 30 °C for 10 min. One microliter of [^{14}C]benzylpenicillin (potassium salt, 144 or 155 μCi/mg; Amersham), at a final concentration of 50 μg/mL, or 3 μL of [^{3}H]benzylpenicillin (*N*-ethylpiperidinium salt, 65.2 mCi/mg; a gift from Dr. P. J. Cassidy, Merck Sharp and Dohme, NJ), at 7.2 μg/mL [the concentration was taken as that of the potassium salt (factor = 0.836)], was added to the mixture, followed by incubation for 10 min at 30 °C. The reaction was terminated by adding an equal volume of the SDS sample buffer (Laemmli, 1970) and quick heat treatment at 95 °C for 3 min. Proteins were then separated by 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli (1970), PBPs being detected by fluorography using Amplify (Amersham). Total protein in the sample was detected similarly by staining with Coomassie Brilliant Blue R-250. In order to determine the pH profile of the binding, the following buffers were used for suspending induced cells: 50 mM sodium acetate (pH 5.5), 10 mM sodium phosphate (pH 7.0), 50 mM Tris-HCl (pH 8.0 and 9.0), and 50 mM sodium bicarbonate–NaOH (pH 10.0).

*Purification of PBP2*s.* On the basis of the low solubility of PBP2*, which is about 0.4 mg/mL in buffer A [50 mM sodium phosphate buffer (pH 7.0), 0.5 M NaCl], the volume of the cell suspension for sonication was made relatively large, in order to recover most of PBP2* in the soluble fraction. PBP2*-overproducing cells (the wild type, and the D447E and D447N mutants) were prepared from 1 L of culture (wet weight, about 2.7 g; 1300 OD units at 660 nm) as described above. The cells were suspended in 60 mL of buffer A and then sonicated on ice. The resultant solution was adjusted to 77 mL with buffer A and then ultracentrifuged at 100000*g* for 60 min at 4 °C to remove undisrupted cells and the membrane fraction containing the other PBPs. PBP2* was purified from the soluble fraction by ammonium sulfate fractionation followed by ampicillin affinity chromatography, as previously described (Nakagawa et al., 1984; Takasuga et al., 1988), except that the protein was dissolved in or dialyzed against buffer A after each step. The yields of the purified enzyme were 2.1, 1.4, and 0.7 mg/L for the wild type and the D447E and D447N mutants, respectively. The purified enzyme in buffer A was frozen quickly in a methanol–dry ice bath, stored at −80 °C, and thawed on ice immediately before use. The protein content was determined by the method of Bradford (1976) with bovine serum albumin as a standard.

*Kinetics of Binding to and Release from Purified PBP2*s of β-Lactams.* The methods used were essentially those of Spratt (1977). The concentrations of [^{14}C]benzylpenicillin required for 50% binding were determined as follows: 9 μL of an enzyme solution containing about 1 μg of purified PBP2* in buffer A was preincubated for 10 min, and then 1 μL of [^{14}C]benzylpenicillin at various concentrations was added. The conditions for incubation, termination of the reaction, and detection of the binding activity (PBP assay) were as described above. The intensity of the band of PBP2* on the fluorogram was determined using a densitometer. That of the degradation products of PBP2* was ignored. The intensity for a much higher concentration of [^{14}C]benzylpenicillin (final, 100 μg/mL) was used as the saturation level, P. The log $[P/(P - P_x)]$ values were plotted against the concentrations of [^{14}C]benzylpenicillin, x (μg/mL), where P_x is the intensity at x μg/mL. The concentration for 50% binding where $P/(P - P_x) = 2$ was obtained by least-squares fitting to the plots. The values for nonradioactive mecillinam and cefmetazole were determined in a similar manner except that the $P - P_x$ value was directly determined by chasing a final 100 μg/mL [^{14}C]benzylpenicillin for 10 min after incubation with the nonradioactive compound. In the case of mecillinam, 0.2 μg of enzyme was used instead of 1 μg. To determine the time course of the release of bound [^{14}C]benzylpenicillin from PBP2*, about 1 μg of the protein was saturated with a final 10 μg/mL of the ligand for 30 min at 30 °C. After chasing with nonradioactive benzylpenicillin (final, 10 mg/mL) for 0, 30, 60, 90, 120, and 150 min at 30 °C, the reaction was terminated. From the log (P_t/P) against t plots, where P_t is the intensity after t min of incubation, the time for 50% release was determined.

Prediction of the Secondary Structure of the Protein. Prediction of the amphiphilic α-helical regions within the C-terminal TP domain of PBP2 was performed by the use of a combination of the two related methods reported by Schiffer and Edmundson (1967) and Eisenberg et al. (1984).

Local Homology Search. Corresponding regions within the primary structures of *E. coli* PBP2 and PBP3 to those of staphylococcal β-lactamase containing Glu^{166} were searched for by means of the SWEEP–ALIGN method as follows: the sequence of residues 164–174 in the staphylococcal β-lactamase was slid along those of the C-terminal TP domains of PBPs. At each position, a value was obtained by summing up the values for individual amino acid pairs according to the mutation data matrix of Dayhoff et al. (1978). The regions including Asp^{398} in PBP2 and Asp^{409} in PBP3, respectively, showed the highest positive values among those in which a carboxylate residue, Asp or Glu, was aligned with Glu^{166} in the β-lactamase, but they differed in the position in the primary structure from each other. Asp^{398} in PBP2 was, however, not acceptable considering the predicted secondary structure pattern and the absence of a corresponding carboxylate residue in PBP3 at this position. In contrast, Asp^{409} in PBP3 was acceptable as to the secondary structure pattern. In addition, as found with the SWEEP–ALIGN method using residues 408–419 in PBP3 as a probe, Asp^{409} in PBP3 corresponded to Asp^{447} in PBP2, sharing the same position as that of Asp^{409} in PBP3. We concluded that Glu^{166} in the staphylococcal β-lactamase corresponds to Asp^{447} in PBP2 and Asp^{409} in PBP3. By means of the same method, the positions of the tetrad, Ser-X-X-Lys, and the triad, Lys-Thr(Ser)-Gly or His-Thr-Gly, in PBP2 and PBP3 were also confirmed to be those previously reported (Joris et al., 1988).

Results

Residues of the TP Domain in PBP2 Replaced by Site-Directed Mutagenesis. Since the tertiary structure of the TP domain has not been reported, the residues constituting the active-site cavity were predicted by means of amino acid sequence alignment with class A β-lactamases, as shown in Figure 1. The positions in PBP2 of the tetrad, Ser-X-X-Lys, and the triad, Lys-Thr(Ser)-Gly or His-Thr-Gly, have already been established (Takasuga et al., 1988; Joris et al., 1988). The residue corresponding to Glu^{166} in class A β-lactamases was predicted to be Asp^{447} in PBP2 through the combination of the local homology search and secondary structure prediction described below (for details see Experimental Procedures), although Asp^{490} in PBP2 was predicted previously (Joris et al., 1988). As a result, the catalytically essential residues in class A β-lactamases, Ser^{70}, Lys^{73}, Glu^{166}, and Lys^{234}, were found to correspond to Ser^{330}, Lys^{333}, Asp^{447}, and

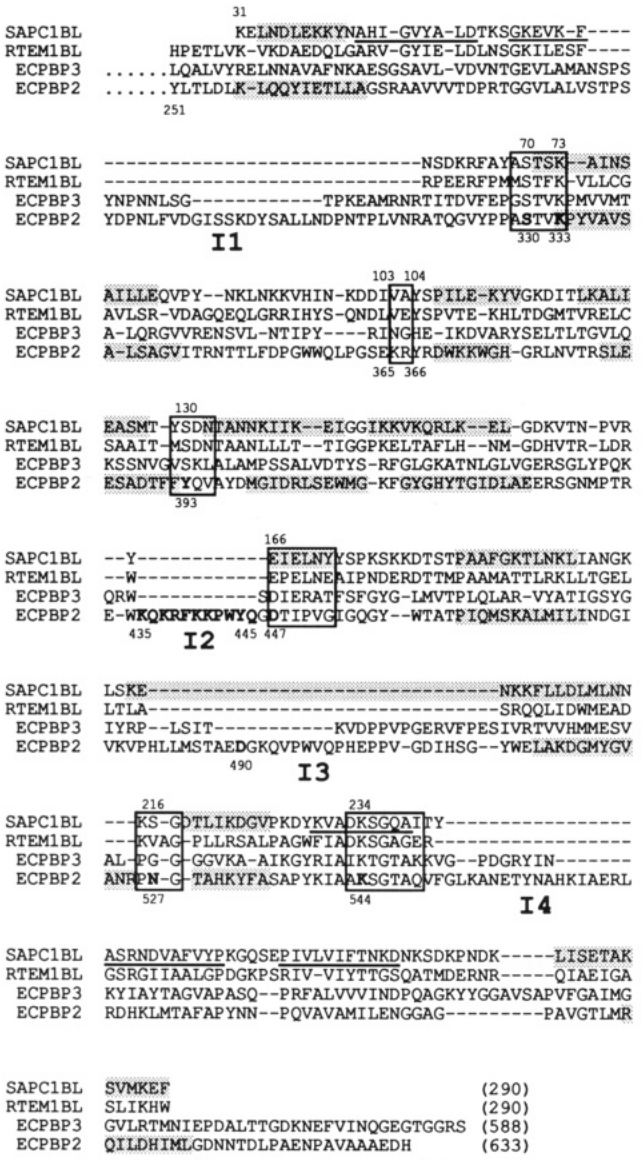

FIGURE 1: Amino acid sequence alignment of class A β-lactamases and the TP domains of *E. coli* PBP2 and PBP3. SAPC1BL, β-lactamase from *S. aureus* PC1 (Ambler, 1975); RTEM1BL, RTEM-1 β-lactamase (Sutcliffe, 1978); ECPBP2 and ECPBP3, the TP domains of *E. coli* PBP2 (Asoh et al., 1986) and PBP3 (Nakamura et al., 1983), respectively. The numbers above and below the sequences are the residue numbers of SAPC1BL (Ambler, 1980) and ECPBP2, respectively. The residue numbers of the C-terminus are indicated in parentheses. The shadowed and underlined regions in SAPC1BL denote α-helices and β-strands, respectively, according to a crystallographic study (Herzberg & Moult, 1987), and the shadowed regions in ECPBP2, predicted α-helices. Six boxes show the sequences corresponding to those constituting the substrate-binding cavity in SAPC1BL. I1–I4, relatively long inserted sequences in ECPBP2 and/or ECPBP3. Bold characters denote the residues of ECPBP2 replaced or deleted in this study.

Lys^{544} in PBP2, respectively. These residues, as well as Asp^{490}, were replaced by site-directed mutagenesis to characterize their roles in catalysis.

Furthermore, the α-helical regions of the class A β-lactamase from *Staphylococcus aureus* PC1 determined in a crystallographic study (Herzberg & Moult, 1987) and the predicted α-helical regions in the TP domain of PBP2 were aligned, revealing four relatively long inserted regions (Figure 1, I1–I4) in PBP2. The sequence of the TP domain in *E. coli* PBP3, which is the most homologous to that of PBP2 (Asoh et al., 1986), is also aligned in Figure 1. The spatial arrangements of the secondary structures of DD-peptidases and class A and C β-lactamases are quite similar, although the sequence homology among them is not so high (Kelly et al., 1986; Samraoui et al., 1986; Oefner et al., 1990). It was, therefore, expected that the TP domains fold similarly. Among the residues which potentially constitute the cavity represented by the boxes in Figure 1, Tyr^{393} and Asn^{527} were also altered. Tyr^{393} corresponds to Ser^{130} in class A β-lactamases. One of the inserted regions in the alignment, I2 (residues 435–445), was deleted, which might be independent of the penicillin-binding activity. All the mutations obtained are shown in Table I. In this paper, K333R denotes the mutation by which Lys^{333} was replaced with Arg, for example. The deletion from Lys^{435} to Gln^{445} is called Δ435–445.

It is true that, except for the positions of the conserved tetrad and triad, the basis for the alignment is weak because of the low sequence homology. However, the results of the mutation analyses, which are independent of the alignment, should reflect the residues' roles in catalysis.

Genetic Complementation Test with the Mutant PBP2 Genes. PBP2 is involved in the division and morphogenesis of rod-shaped *E. coli* cells (Spratt, 1975; Ogura et al., 1989) through its TG and TP activities. A temperature-sensitive mutant strain of PBP2, *E. coli* TMM13, grows as spherical cells at 42 °C (Tamaki et al., 1980). In this study, the morphological recovery of TMM13 at 42 °C by mutant PBP2s expressed from an expression plasmid, pHA103, was used to test their overall TP activity, since all the mutations obtained were located in the TP domain.

The results are shown in Figure 2D. The result for the acylation-site mutant, S330C, previously reported (Takasuga et al., 1988), was used as a negative control. All the mutants at positions 333 and 544, including replacements with Arg and His having a positive charge on their side chains, lost the complementation activity, indicating that Lys at these positions is essential for complementation. All the mutants at position 447 except for the D447E mutant lost the genetic complementation activity. The positive results for the D447E mutant indicate that a carboxylate side chain is essential for complementation. For position 393, the Y393F mutant retained the complementation activity, indicating that a side chain with a hydroxyl group is not essential for this function. In contrast, the Y393S mutant lost the complementation activity. All the mutants at positions 490 and 527, including replacements with Ala, retained the complementation activity, indicating that the side chains of Asp^{490} and Asn^{527} are not involved in this function of PBP2. The Δ435–445 mutant lost the complementation activity.

*Penicillin-Binding Activity of Mutant PBP2*s.* The penicillin-binding activity of mutant PBP2s represents only the acylation step including the formation of the Michaelis complex during the TP reaction, since penicillins bind covalently to PBPs as substrate analogues in a mechanism-based manner, and the resultant complex resembles an acyl-enzyme intermediate. For this assay, the mutations were introduced into PBP2* because of the higher production from the expression plasmid than that of intact PBP2 (Adachi et al., 1987; Adachi et al., unpublished results). PBP2* is a water-soluble form of PBP2 obtained through a deletion of the membrane-spanning region (Adachi et al., 1987). All the mutant PBP2*s obtained in this study were overproduced up to about 2% of the total cellular protein under the control of the *lpp-lac* promoter with IPTG induction (Figure 2A).

The penicillin-binding activity of all the mutant PBP2*s was examined with radioactive benzylpenicillin as a substrate under the standard conditions, as follows: in 10 mM sodium phos-

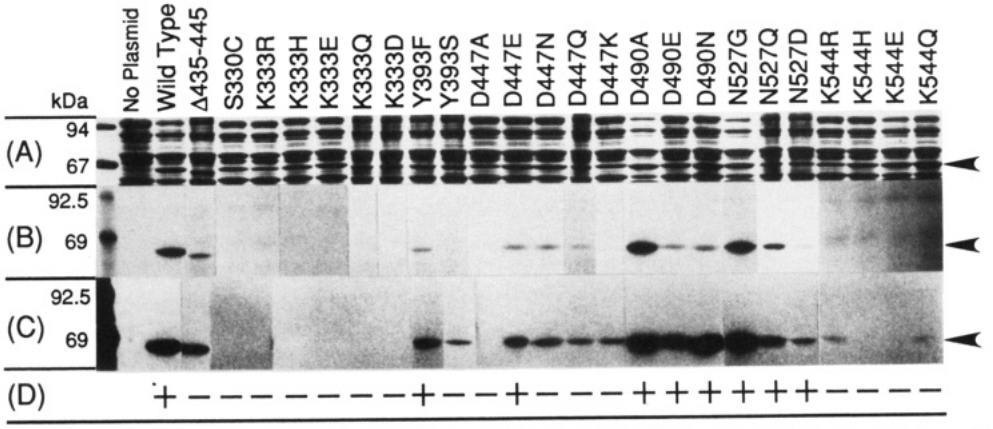

FIGURE 2: Penicillin-binding activity and genetic complementation activity of mutant PBP2s. (A–C) Total protein from 0.3 OD unit, at 660 nm, of cells overproducing mutant PBP2* was separated by 10% SDS–PAGE. Mutant PBP2* was detected by Coomassie Brilliant Blue staining (A) and PBP assay, in which the protein was labeled with a final 7.2 μg/mL (B) or 50 μg/mL (C) of radioactive benzylpenicillin for 10 min at 30 °C at pH 7.0. Arrowheads indicate the position of PBP2*. (D) Results of the genetic complementation test.

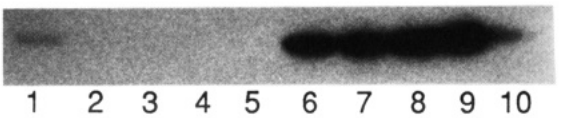

FIGURE 3: pH profiles of penicillin-binding activity of the K544H mutant (lanes 1–5) and the wild-type (lanes 6–10) PBP2*s. The PBP assay was performed as in Figure 2C (50 μg/mL benzylpenicillin) except for the pH, as follows: pH 5.5 (lanes 1 and 6), 7.0 (lanes 2 and 7), 8.0 (lanes 3 and 8), 9.0 (lanes 4 and 9), and 10.0 (lanes 5 and 10).

phate (pH 7.0) for 10 min at 30 °C (Figure 2B,C). Two concentrations of benzylpenicillin, 7.2 (Figure 2B) and 50 μg/mL (Figure 2C), were used to examine the affinity for the substrate. In some cases, the pH profiles of the binding were examined in 50 μg/mL benzylpenicillin, as shown in Figure 3.

Even with the lower concentration, the wild type showed the binding activity. In contrast, the acylation-site mutant, S330C, did not bind even with the higher concentration, confirming the previous results with the membrane-bound S330C mutant (Takasuga et al., 1988). All the mutants at position 333 were completely inactive, including the replacements with Arg and His, suggesting that Lys^{333} is essential for catalysis. For the following mutants, binding activity with the higher penicillin concentration was also not detected at various pHs from 5.5 to 10.0: S330C, K333R, K333H, and K333Q (data not shown).

For the mutants at position 544, decreased binding affinity for the substrate was observed. Under the standard conditions, the K544R and K544Q mutants showed the binding activity only with the higher concentration. For the K544R mutant, slight binding could be observed with the lower concentration when the incubation time was prolonged to 60 min (data not shown). The K544H mutants did not show the activity at pHs higher than 7.0, but did exhibit it at a lower pH, pH 5.5 (Figure 3). The K544E mutant did not exhibit binding activity even with the higher concentration.

Most of the mutants at position 447 retained the binding activity. The D447N and D447Q mutants, without a carboxylate side chain, retained the binding activity even with the lower penicillin concentration (Figure 2B), as well as D447E, which retained the complementation activity. In spite of the different characteristics of Lys from those of Asp, the D447K mutant also retained the penicillin-binding activity, although only with the higher concentration (Figure 2C). The D447A mutant lost the penicillin-binding activity (Figure 2B,C).

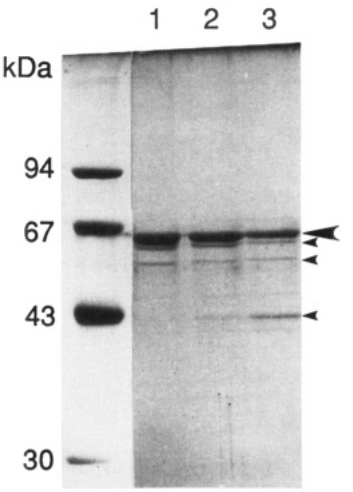

FIGURE 4: Purified PBP2*s detected by Coomassie Brilliant Blue staining of the 10% SDS–PAGE gel. Lanes 1–3: The wild type (1.1 μg) and the D447E (1.2 μg) and D447N (0.6 μg) mutants, respectively. The large arrowhead indicates the position of PBP2*, and the small ones indicate those of the degradation products of PBP2*s.

All the mutants at positions 490 and 527 and the Y393F mutant showed the binding activity even with the lower penicillin concentration, as expected from the complementation activity. The Y393S mutant showed the binding activity only with the higher concentration (Figure 2C). As expected from the alignment in Figure 1, the Δ435–445 mutant showed the binding activity even with the lower concentration. The fact that this mutant lost the complementation activity may suggest that the sequence of residues 435–445, rich in basic side chains, N-KQKRFKKPWYQ-C, is involved in the determination of the specificity of PBP2 activity in morphogenesis and cell division (Begg et al., 1990). This sequence is a unique insertion in PBP2, not existing in PBP3 (Figure 1).

Binding Activity of the D447N Mutant toward Various Penicillins. In order to determine whether the loss of complementation activity of the 447 mutants resulted from a defect in the acyl-transfer step in transpeptidation, the penicillin-binding activity of the D447N mutant was analyzed and compared with those of the wild type and the D447E mutant. For this purpose, the wild-type and mutant PBP2*s were purified mainly by ampicillin affinity chromatography, utilizing their retained penicillin-binding activity, as shown in Figure 4. Like the wild type, the mutants were purified, although the degradation products of PBP2* were produced in a relatively larger amount in the cases of the mutants. Using the

Table II: Concentrations of β-Lactams Required for Binding to Half of the Purified PBP2*s in 10 min at pH 7.0

β-lactam	purified PBP2* (μg/mL)		
	wild type	D447E	D447N
[^{14}C]benzylpenicillin	1.3	1.8	4.1
mecillinam	0.04	0.06	0.04
cefmetazole	>100	>100	>100

purified enzymes, the binding affinity for [^{14}C]benzylpenicillin, mecillinam, and cefmetazole was measured by determining the concentration for binding to half of the enzyme in 10 min at 30 °C. Mecillinam binds specifically to PBP2 and cefmetazole to all *E. coli* PBPs but PBP2. The results are shown in Table II. The binding specificity of the D447N mutant was almost identical to those of the wild type and the D447E mutant, although the value for benzylpenicillin was a little larger. The value of the wild-type PBP2* for benzylpenicillin was also similar to that of intact PBP2 in the membrane fraction (Spratt, 1977), showing that the deletion of the membrane-spanning region did not affect the binding activity. In addition, the half-life of [^{14}C]benzylpenicillin-bound PBP2* was determined to be 210, 74, and 48 min for the wild type and the D447E and D447N mutants, respectively. The retained large value for the D447N mutant comparable to that for the D447E mutant confirmed that the stability of the acyl-enzyme of this mutant was not reduced significantly. These results suggested that the loss of the complementation activity of the D447N mutant did not result from a defect in acylation.

Discussion

Lys^{333}. Lys^{333} is located in the tetrad, Ser-X-X-Lys, together with the acylation site, Ser^{330}, and therefore corresponds to Lys^{73} in class A β-lactamases. On the basis of crystallographic studies (Herzberg & Moult, 1987; Moews et al., 1990), the positively charged side-chain ammonium group of Lys^{73} in class A β-lactamases is proposed to facilitate direct proton transfer from Ser^{70} to β-lactam nitrogen in the acylation step. All the replacements of Lys^{333} in PBP2, with Arg, His, Glu, Gln, or Asp, resulted in the complete loss of both the penicillin-binding activity and the genetic complementation activity (Figure 2). These results suggested that Lys^{333} participates in the acylation step. At present, we propose that Lys^{333} functions in acylation, acting like Lys^{73} in class A β-lactamases, as mentioned above. For the class A β-lactamase from *Bacillus cereus*, the K73R mutant showed greatly reduced but considerable activity in vivo (Madgwick & Waley, 1987) and in vitro (Gibson et al., 1990), supporting the importance of the positive charge. Therefore, the fact that the K333R and K333H mutants in PBP2 failed to bind penicillin under any conditions may indicate a definite requirement for a Lys residue at this position. For class C β-lactamases, mutation analyses of Lys^{67}, the counterpart of Lys^{73} in class A enzymes, also showed that a positive charge at this position is important (Tsukamoto et al., 1990). In this case, however, the hydroxyl group of Tyr^{150} is proposed to participate in acylation (and deacylation) and to act as a general base catalyst, Lys^{67} supporting this Tyr^{150} function (Oefner et al., 1990). The roles in acylation of the Lys residues in class A enzymes and TP of PBP2 may be different from those in class C enzymes.

Lys^{544}. Lys^{544} is located in the triad, Lys-Thr(Ser)-Gly or His-Thr-Gly, and therefore corresponds to Lys^{234} in class A β-lactamases. On the basis of the tertiary structure of the active-site cavity (Herzberg & Moult, 1987; Oefner et al., 1990; Kelly et al., 1989) and a study on mutants for position 234 of class A β-lactamases (Ellerby et al., 1990), it is likely that the side chain of Lys or His in the triad in class A and C β-lactamases and DD-peptidases interacts electrostatically with the C3 carboxylate of penicillins. This interaction was proposed to stabilize both the ground- and transition-state binding of class A β-lactamases (Ellerby et al., 1990). It was therefore expected that Lys^{544} in PBP2 interacts electrostatically with the carboxylate of penicillins and also with the corresponding C-terminal carboxylate of D-Ala-D-Ala in transpeptidation. The penicillin-binding activity of the mutants at position 544 supported this idea. The binding of the mutants tended to require a higher penicillin concentration than that of the wild type, and the positive charge of the side chain seemed to be preferred for the binding, compared to a neutral or negative one (Figures 2 and 3). The importance of the positive charge was also supported by the reduced binding activity of the wild-type PBP2* with Lys^{544} at pH 10.0 (Figure 3, lane 10). Similar results were obtained for the wild-type DD-peptidase (Varetto et al., 1987). Genetic complementation of all the 544 mutants was negative (Figure 2D). This can be explained by the reduced rate of transpeptidation in vivo due to their low affinity for the D-Ala-D-Ala substrate.

Asp^{447}. On the basis of the alignment and mutation analysis, we propose in this study that the carboxylate residue, Asp^{447}, in PBP2 corresponds to Glu^{166} in class A β-lactamases and participates in the acyl-transfer step during transpeptidation. Glu^{166} in class A β-lactamases, highlighted by a crystallographic study (Herzberg & Moult, 1987), is an active-site residue acting as a catalyst in deacylation. We have recently shown that the replacement of Glu^{166} with Ala, Gln, or Asn resulted in the complete loss of the hydrolytic activity and in the accumulation of the acyl-enzyme intermediate, indicating that Glu^{166} is not essential for acylation but for deacylation (Adachi et al., 1991). In addition, the E166D mutant showed greatly decreased but detectable activity, suggesting that the side-chain carboxylate is essential for deacylation (Gibson et al., 1990; Adachi et al., 1991). Corresponding results were obtained for the mutant PBP2s at position 447 (Figure 2). Among the mutants, only the D447E one retained the complementation activity, suggesting that the carboxylate side chain at position 447 is essential for transpeptidation. The other mutants except for the D447A one showed the binding activity, including the D447K mutant with a positive charge on the side chain. In addition, the D447N mutant, which had lost the complementation activity, showed almost identical binding rates and substrate specificity to the wild type as for the acylation by penicillins (Table II), suggesting that a carboxylate at position 447 is not essential for acylation but for acyl transfer in transpeptidation. The results for D447A contradicted this idea. However, it is possible that the active site of the D447A mutant did not have a normal conformation. Alternatively, Asp^{447} participates in both steps, and Gln, Asn, and Lys mutants can be functional only in the acylation step, although this is less conceivable. In transpeptidation, the carboxylate should act as a general base catalyst which attacks an acceptor amino group instead of a water molecule in the β-lactamase reaction of class A enzymes.

Tyr^{393}. In class C β-lactamases, the hydroxyl group of conserved Tyr^{150} was proposed to act as a general base catalyst, and to participate in both acylation and deacylation (Oefner et al., 1990). Superimposition of the active sites showed that the hydroxyl group of Tyr^{150} in the class C enzymes corresponds to that of conserved Ser^{130} in class A enzymes (Oefner et al., 1990). The highly resolved structure also suggested that Ser^{130} may participate in the catalysis (Herzberg, 1991). In our alignment, Tyr^{393} in PBP2 corresponds to Ser^{130} in class

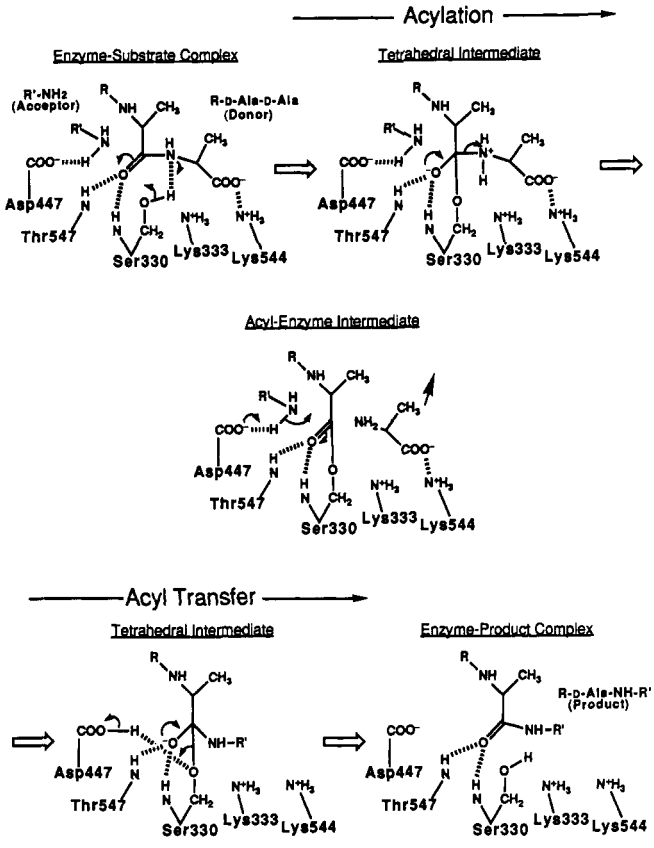

FIGURE 5: Schematic drawing of a possible catalytic mechanism for peptidoglycan transpeptidase in *E. coli* PBP2.

A enzymes. Our results for the Y393F mutant showed that the hydroxyl group at this position is not essential. The reduced activity of the Y393S mutant, however, may suggest that this residue constitutes the wall of the cavity. Recently, mutation analysis suggested that Ser^{130} in class A β-lactamases is involved in maintaining the structure of the active site rather than in catalysis (Jacob et al., 1990).

A Possible Catalytic Mechanism for the Transpeptidase in PBP2. Our results strongly suggest that the spatial arrangements of catalytically essential residues are similar in the active sites of class A β-lactamases and the TP domain of PBP2, both containing an important carboxylate residue. It is not clear at present whether such an essential carboxylate residue exists in the structures of DD-peptidases or class C β-lactamases. On the basis of the proposed catalytic mechanism for class A β-lactamases (Herzberg & Moult, 1987; Adachi et al., 1991), a possible catalytic mechanism for transpeptidase in PBP2 is presented in Figure 5. In addition to the functions of the residues mentioned above, tetrahedral intermediates and associated transition states occurring during acyl transfer to and from Ser^{330} were predicted, which could be stabilized by the oxyanion hole comprising the two main-chain NH groups of Ser^{330} and Thr^{547}, according to the common feature of class A (Herzberg & Moult, 1987; Murphy & Pratt, 1988) and C (Oefner et al., 1990) β-lactamases and DD-peptidases (Kelly et al., 1989). For more detailed elucidation of the catalytic mechanism, accurate kinetic studies on the TP reaction, which are impossible at present, and an X-ray crystallographic study on PBP2 are required. We are now trying to crystallize PBP2* for this purpose.

Supplementary Material Available

Three figures showing the complete construction schemes for the plasmids and phages used in this study (3 pages). Ordering information is given on any current masthead page.

Registry No. Asp, 56-84-8; Lys, 56-87-1; Ser, 56-45-1; Tyr, 60-18-4; transpeptidase, 9059-29-4; β-lactamase, 9073-60-3.

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Solution Structure of Phosphorylase Kinase Studied Using Small-Angle X-ray and Neutron Scattering†

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ABSTRACT: Small-angle X-ray and neutron scattering have been used to characterize the solution structure of rabbit skeletal phosphorylase kinase. The radius of gyration of the unactivated holoenzyme determined from neutron scattering is 94 Å, and its maximum dimension is approximately 275-295 Å. A planar model has been constructed that is in general agreement with the dimensions of the transmission electron microscope images of negatively stained phosphorylase kinase and that gives values for the radius of gyration, maximum linear dimension, and a pair distribution function for the structure that are consistent with the scattering data.

Phosphorylase kinase (PhK)[1] is a large complex molecule (approximately 1.3×10^6 Da) that consists of 16 subunits, made up of four identical copies of four different polypeptides denoted α, β, γ, and δ, with molecular weights 138 422, 125 294, 44 637, and 16 680, respectively [for a review, see Pickett-Gies and Walsh (1986)]. The γ-subunit contains the catalytic site. The δ-subunit is identical to calmodulin, confers Ca^{2+} sensitivity to the enzyme, and is the primary mode whereby the enzyme is regulated by Ca^{2+} in response to neural and hormonal signals. Both the α- and β-subunits are regulatory and are phosphorylated by either the cAMP-dependent

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[1] Abbreviations: AFM, atomic force microscopy; c, protein concentration; d_{max}, maximum linear dimension; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; I_0, forward scatter or scattered intensity at zero scattering angle; LQD, low-Q diffractometer; PhK, phosphorylase kinase; R_g, radius of gyration; STEM, scanning transmission electron microscopy; STM, scanning tunneling microscopy; TEM, transmission electron microscopy.

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