Substitution of Lysine 213 with Arginine in Penicillin-binding Protein 5 of Escherichia coli Abolishes D-Alanine Carboxypeptidase Activity without Affecting Penicillin Binding*

Kiran T. Malhotra and Robert A. Nicholas‡

From the Department of Pharmacology, University of North Carolina, Chapel Hill, North Carolina 27599-7365

Penicillin and other β-lactam antibiotics exert their lethal effect by binding to and covalently inactivating the enzymes that are involved in the synthesis of peptidoglycan at the bacterial cell surface (1). These cell wall synthesis enzymes, called penicillin-binding proteins (PBPs), can be detected by incubation of bacterial membranes with radiolabeled penicillin G, followed by SDS-polyacrylamide gel electrophoresis and fluorography (2). In Escherichia coli, there are at least seven proteins that can be detected in this manner, and biochemical and genetic analysis indicates that these PBPs fall into two distinct groups. The high molecular weight PBPs (PBPs 4A, 1B, 2, and 3) are essential for cell viability and are thought to be bifunctional enzymes that catalyze both penicillin-insensitive transglycosylase reactions and penicillin-sensitive transpeptidase reactions utilizing the lipid-linked disaccharide pentapeptide (3–6). The low molecular weight PBPs (PBPs 4–6) catalyze either secondary transpeptidase activity (PBP 4) (7) or D-alanine carboxypeptidase activity (PBPs 4–6) (8–10). Although they make up close to 90% of the [3H]penicillin G binding to cell membranes, the low molecular weight PBPs do not appear to be essential for cell viability.

Another group of enzymes, called β-lactamases, also interacts with penicillin and other β-lactam antibiotics. The production of these enzymes is the major clinical cause of resistance to β-lactam antibiotics (11). β-Lactamases are known to catalyze the hydrolysis of β-lactam antibiotics through an acyl-enzyme mechanism that is very similar to the mechanism of β-lactam antibiotic interaction with PBPs. When the primary structures of several PBPs and β-lactamases became available, however, the sequence homology between the two groups of enzymes was found to be quite low. The greatest homologies are found within the region of the active-site serine residues. Within this small region, up to 60% homology can be observed in some cases, but it quickly decreases as larger and larger regions are compared. The lack of significant sequence homology between PBPs and β-lactamases brought into question the evolutionary relatedness of these two groups of enzymes. The relatedness was strongly suggested, however, when the crystal structures of either Bacillus licheniformis 749/C β-lactamase (12) or Bacillus cereus β-lactamase I (13) were compared to the crystal structure of the low molecular weight β-lactamase from Streptomyces R61 (14). The similarities of the secondary structures of these enzymes were quite striking, even though the two enzymes differed by 8 kDa and showed very little sequence homology.

The evolutionary relationship between these two groups of penicillin-interactive enzymes was further investigated using homology searches and amino acid alignments, using the Streptomyces R61 D-alanyl-D-alanine peptidase as a reference, of all of the known sequences of PBPs and β-lactamases (15). This method identified several regions, called boxes, that consist of strict identities or homologous amino acids. These boxes, whose importance is highlighted by the known structures of several members of the penicillin-interactive family, are situated in or near the active site of these enzymes and catalyze the acyl-exchange reactions that occur during turnover. One of these conserved regions, called Box II, is the well-characterized Ser-X-X-Lys tetrad that contains the active-site serine nucleophile. Another interesting region of homology, called Box VII, is a conserved triad Lys-Thr-Gly, Lys-Ser-Gly, or His-Thr-Gly that occurs within the carboxyl-terminal half of the primary sequence of these enzymes. Kelly et al. (16), from the crystallographic analysis of β-lactam antibiotics bound to the Streptomyces R61 DD-peptidase, have suggested that the binding of substrate is facilitated by the conserved triad of Box VII and that the histidine residue...
contributes to the initial binding. Because these hypotheses have not been tested at the biochemical level, however, the role of the basic residue within the Box VII region of PBPs has not been confirmed.

We chose to investigate the role of the conserved lysine (Lys213) of Box VII in the catalytic mechanism of E. coli PBP 5 to gain a better understanding of the role of this amino acid in the molecular interactions of a PBP with both β-lactam antibiotics and peptide substrate. PBP 5 is a low molecular weight PBP with a M, of 41,300 that is known to catalyze the major D-alanine carboxypeptidase activity in vivo (17, 18). It is initially synthesized with a signal sequence that is cleaved off during translocation to the periplasm (19). The carboxy-terminal 15 amino acids have been shown to mediate the association of the protein with the membrane as removal of these amino acids results in the synthesis of a soluble periplasmic protein (20). PBP 5 is unique in that it displays the highest β-lactamase activity of all of the E. coli PBPs; at pH 7 and 30 °C, the half-life of the acyl-enzyme complex is ~10 min (21, 22). The D-alanine carboxypeptidase activity that it catalyzes can also be easily assayed in vitro. We chose the method of site-saturation mutagenesis to investigate the role of the basic residue in the Box VII region of a soluble form of PBP 5 in which Lys213 was mutated to 18 other amino acids and the properties of these mutants were investigated.

EXPERIMENTAL PROCEDURES

Materials—All restriction enzymes and DNA-modifying enzymes were from Pharmacia LKB Biotechnology Inc. [153CIPenicillin G (155 μCi/mg) was from Amersham Corp. [3H]Penicillin G (57.8 mCi/mg) was the generous gift of Dr. Patrick Casey (Merck Sharp and Dohme). Oligonucleotides were synthesized on a Milligen/Biosearch Cyclone Plus DNA synthesizer. The strains used were JM103 and M13 vectors, CI236 (dut-, ung+) for the production of uracil-containing M13 phage, and NK5830 (F' lacpro/arg Dlux proXNal rif' recA-56 sia ara thy') for the overexpression of cloned genes of PBP 5 mutants. Sequencing was performed with the Sequenase II kit from United States Biochemical Corp.

Construction of Soluble PBP 5 Expression Vector—A soluble form of PBP 5 (sPBP 5) was constructed as described previously (22). The truncated dacA gene encoding sPBP 5 was reconstructed in the expression vector pTTQ18 (Amersham International) by standard cloning techniques (25). Since pTTQ18 uses ampicillin resistance as its selectable marker, which is detrimental to the detection and purification of PBPs, the vector was modified by digesting with DraI, which removes a 700-bp fragment from within the ampicillin resistance gene, and replacing this with the 293-bp SalI-BglII fragment within the dacA gene which was synthesized (26). The vector was then transformed into JM103 and the recombinant plasmid, called pKM1325, was also constructed to be a derivative, the 293-bp SalI-BglII fragment from pMSG (Pharmacia) was digested with HindIII (Pharmacia) back in its place. The final construct, which contains the dacA gene encoding sPBP 5, the kanamycin resistance gene, and the lacF gene, was designated pWT3K. A derivative of pWT3K, called pKM1355, was also constructed to be a recipient of the mutated fragments generated below. In this derivative, the 239-bp SalI-BglII fragment within the dacA gene was replaced with the 1325-bp SalI-BglII fragment from pMSG (Pharmacia), which allowed the unambiguous detection of reconstructed dacA mutants by restriction screening (see below).

Site-saturation Mutagenesis—The 1230-bp EcoRI-HindIII fragment from pWT3K was cloned into M13mp19, and the recombinant M13 phage was used to make uracil-containing single-stranded DNA as described by Kunkel (24). Site-saturation mutagenesis was performed by first using an oligonucleotide to loop-out the AAA lysine codon, as described by Sharp and Li (26) were selected. The single-stranded DNA from each selected mutant was sequenced through the SalI-BglII fragment to ensure that no other mutation had occurred during mutagenesis. The replacement of each codon was confirmed by the 50% decrease of the half-life of the acyl-enzyme complex is ~10 min (21, 22). The D-alanine carboxypeptidase activity that it catalyzes can also be easily assayed in vitro. We chose the method of site-saturation mutagenesis to investigate the role of the basic residue in the Box VII region of an otherwise identical form of PBP 5 in which Lys213 was mutated to 18 other amino acids and the properties of these mutants were investigated.

Expression of sPBP 5 Mutants—Cells harboring the sPBP 5 mutant genes in pKM3K were grown at 37 °C in 50 ml of 2 X YT medium (23) in the presence of 50 μg/ml kanamycin. When the absorbance at 600 nm reached 0.5, protein synthesis was induced by adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.5 mM and incubating with shaking for an additional 2 h. The cultures were subjected to osmotic shock as described by Kustu et al. (27) and the shock fluids were concentrated 10-fold on an Amicon ultrafiltration device fitted with a PM-10 filter. The final protein concentrations of shock fluids were 2–5 mg/ml as estimated by the protein assay of Lowry et al. (28).

[3H]Penicillin G Binding and Immunoblotting of sPBP 5 Mutants—Aliquots of each shock fluid (20 μg) were incubated with [3H]penicillin G at a final concentration of 40 μg/ml for 20 min at 30 °C, and the labeled proteins were visualized by SDS-PAGE and fluorography. The final protein concentrations of shock fluids were 2–5 mg/ml as estimated by the protein assay of Lowry et al. (28). Aliquots (20 μg) of osmotic shock fluids were separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose using a semidry blotting apparatus. After blocking with nonfat dry milk, the nitrocellulose was incubated first with antiserum raised against sPBP 5' and then with alkaline phosphatase-conjugated goat anti-rabbit Fab fragment (Sigma). The sPBP 5 proteins were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (29). The antisem was produced by injecting SDS-polyacrylamide gel elusion of sPBP 5' subcutaneously into New Zealand white rabbits as described by Harlow and Lane (30).

Determination of Affinities of sPBP 5 and sPBP 5-K213R for [153CIPenicillin G—The affinities of sPBP 5 and sPBP 5-K213R for [153CIPenicillin G were measured essentially as described by Ghysen et al. (31). Aliquots (15 μl) of either the purified protein (1.5 μg) or osmotic shock fluids (20 μg) were incubated with 5 μl of serial dilutions of [153CIPenicillin G (80–1.25 μg/ml final concentration) for 20 min at 30 °C. Following incubation, SDS-PAGE sample buffer was added, and the samples were separated on a 12% SDS-polyacrylamide gel. The gel was treated with ENHANCE, dried, and exposed to film. The amount of radioactivity present in the proteins was estimated by densitometric scanning of the film with a Hoefer Scientific Instruments GS 300 densitometer.

Determination of Rate of Hydrolysis of Bound [153CIPenicillin G by sPBP 5 and sPBP 5-K213R—Hydrolysis assays were performed essentially as described previously (22). Aliquots of purified proteins (6 μg, 1 μg/time point) or osmotic shock fluids (120 μg, 20 μg/time
point) in 25 mM Tris-HCl, pH 7.3, were incubated with [14C]penicillin G at a final concentration of 40 μg/ml at 30 °C. After 20 min, nonradioactive penicillin G was added to a final concentration of 4 mg/ml, and the incubation was continued. Aliquots were removed at various times, and the amount of radioactivity remaining in the sPBP 5 proteins was determined by SDS-polyacrylamide gel electrophoresis, fluorography, and densitometry as described above.

**RESULTS**

**Box VII Sequences of PBPs and Site-saturation Mutagenesis**—Alignment of the sequences of E. coli PBPs, the Streptomyces R61 DD-peptidase, and several β-lactamases in the region of Box VII shows the conserved character of the triad (Fig. 1). This triad can in fact be KTG, KSG, or HTG. We chose to investigate the apparent role of the lysine residue (Lys<sup>213</sup>) of the Box VII region in the catalytic mechanism of sPBP 5. A soluble form of sPBP 5 was chosen for mutational analysis since it can be separated from endogenous PBP 5 by osmotic shock, and it is known that removal of the carboxy-terminal membrane anchor of PBP 5 does not effect its activity (22). The entire sPBP 5 coding sequence was cloned into M13mp19 to generate a template for mutagenesis. Site-saturation mutagenesis was performed as described under “Experimental Procedures,” and mutant genes containing random codon insertions specifying all 20 amino acids at the former position of Lys<sup>213</sup> were identified by sequencing.

**Subcloning of sPBP 5 Mutants into Expression Vector**—For the subcloning of sPBP 5 mutant genes into an expression vector, we employed a strategy that allowed us to clone a small fragment of the PBP 5 mutant gene into an altered plasmid that recreated the entire gene including the mutation. The expression vector pKM1325 contained the wild-type sPBP 5 gene, except that the 293-bp Sall-BglII fragment that encompasses the Box VII region (Fig. 2) was replaced with a 1325-bp Sall-BglII fragment from an unrelated plasmid (pMSG). This strategy allowed us to subclone a small sequence fragment from the mutagenesis (all 20 mutants were sequenced through the Sall-BglII fragment to ensure that only the intended mutation was present) without the problem of contamination of the original fragment. The final constructs are shown in Fig. 2. Nineteen of these plasmids (the tyrosine mutant was lost during the subcloning process and was not pursued) were transformed into NK5830, and the overproduction of sPBP 5 mutants in the periplasm was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside.

**Expression and Activity of sPBP 5 Mutants**—Following the induction of the sPBP 5 variants, the periplasmic fraction was isolated from each culture using osmotic shock and was used to assess both the activities and the levels of expression of each mutant. To assess the [14C]penicillin G binding activities of the sPBP 5 variants, osmotic shock fluids were preincubated with [14C]penicillin G for 10 min at 30 °C and analyzed by SDS-PAGE and fluorography. [14C]Penicillin G was chosen for this experiment since its 50-fold higher specific activity compared with [14C]penicillin G should have allowed us to observe even low level binding. As shown in Fig. 3A, however, only the Arg mutant and the "revertant" wild-type sPBP 5 proteins were detected under these conditions. To test whether adequate levels of sPBP 5 mutant proteins were present in the samples that did not show binding, immunoblot analysis was performed on each sample (Fig. 3B). The immunoblot revealed that 18 (out of 19 total) variants were stable and expressed at levels equal to or exceeding those of sPBP 5. To ensure that the gene encoding sPBP 5-K213R had not reverted back to the wild-type sequence, the plasmid was isolated and submitted to double-stranded sequencing, which confirmed the presence of an arginine codon (CGT) at the site-saturation position that was substituted with 18 other amino acids. Serine 44 is the active-site nucleophile that forms the covalent bond with β-lactam antibiotics. Kb, kilobase pairs; aa, amino acids.

![Fig. 2. Expression vector for overproduction of sPBP 5 mutants.](image-url)

**FIG. 1. Box VII sequences of selected PBPs and β-lactamases.** The sequences encompassing the Box VII region of PBPs and β-lactamases are compared with one another; and the KTG, KSG, or HTG conserved triad is boxed. The positions of the sequences in the primary structure of each enzyme are noted, and the lysine residue that was selected for mutation in PBP 5 is indicated in boldface type.
Site-saturation Mutagenesis of Soluble PBP 5 from E. coli

The purified protein was incubated with a saturating amount of [14C]penicillin G that results in 50% binding for each protein is indicated versus time of incubation (Fig. 5 and Table I). The identical half-lives of the penicilloyl-sPBP complexes for both sPBP 5 (t_{1/2} = 9.1 min) and sPBP 5-K213R (t_{1/2} = 9.1 min) clearly indicate that the arginine mutation has no effect upon the rate of hydrolysis of the acyl-enzyme complex. As a comparison, the hydrolysis rate of sPBP 5' is also shown. sPBP 5' contains a point mutation (G105D) that results in a drastically reduced deacylation rate of the penicilloyl-sPBP 5' complex while still allowing near-normal acylation with penicillin G (33, 34). As reported previously (22), the half-life of the penicilloyl-sPBP 5' complex with penicillin G compared with sPBP 5 (affinity = 5.2 μg/ml).

The effect of the arginine mutation on the interactions of sPBP 5 with penicillin G was further assessed by determining the rate of deacylation of the [14C]penicilloyl-sPBP complex.

| sPBP     | IC_{50} for affinity of [14C]penicillin G | Hydrolysis of [14C]penicillin G | Carboxypeptidase activity |
|----------|------------------------------------------|---------------------------------|---------------------------|
| sPBP 5   | 5.1                                      | 9.2 0.00125                     | 7.98 ± 0.45 1.41 ± 0.18    |
| sPBP 5-K213R | 4.4                                  | 9.1 0.00126                     | ND                         |
| sPBP 5'-G105D | 14                                 | 244 4.7 × 10^{-5}              | ND                         |

* Values were calculated from the data in Fig. 4.

** Values were calculated from the data in Fig. 5.

* Values were determined from double-reciprocal plots of initial rates.

** ND, not detected.

**Fig. 3. Analysis of sPBP 5 mutants by [3H]penicillin G binding and immunoblotting.** A, osmotic shock fluids (20 μg) were incubated with [3H]penicillin G at a final concentration of 40 μg/ml for 20 min at 30 °C, following which the samples were denatured in SDS and subjected to electrophoresis on a 12% SDS-polyacrylamide gel. The bands were visualized by fluorography. The amino acid substitution at position 213 is indicated above each lane. B, osmotic shock fluids (20 μg) were subjected to electrophoresis as described above and transferred to nitrocellulose, and the bands were visualized by immunoblotting with antisera against sPBP 5'. In both cases, only the relevant part of the fluorograph or immunoblot is shown.

**Fig. 4. Affinity of sPBP 5 and sPBP 5-K213R for [14C]penicillin G.** Aliquots of purified sPBP 5 or sPBP 5-K213R were incubated for 20 min at 30 °C with increasing concentrations of [14C]penicillin G. Following this incubation, the samples were submitted to SDS-polyacrylamide gel electrophoresis on a 12% gel and fluorographed. The amount of radioactivity bound was determined by a densitometric scan of the bands following fluorography, and the percent of [14C]penicillin G bound at each concentration (calculated from the value at 80 μg/ml) was graphed on a semilog plot versus the concentration of [14C]penicillin G. The concentration of [14C]penicillin G that results in 50% binding for each protein is indicated (---).

**Fig. 5. Quantitation of rate of hydrolysis of [14C]penicillin G by sPBP 5, sPBP 5', and sPBP 5-K213R.** Purified sPBP 5 proteins (6 μg) were incubated with 40 μg/ml [14C]penicillin G in 25 mM Tris-HCl, pH 7.3, for 20 min at 30 °C. A 100-fold excess of nonradioactive penicillin G was then added (t = 0); and aliquots were removed at the indicated times, denatured, and submitted to electrophoresis on a 12% SDS-polyacrylamide gel. Following fluorography, the radioactivity remaining covalently attached to protein was determined by densitometric scanning.
was extremely long (244 min) when compared with that of sPBP 5-K213R or sPBP 5 (9 min). The half-life determined for sPBP 5 differed slightly from the value determined previously (10.5 min) and may be a result of the higher pH (7.3 versus 7.0) used in these experiments.

Because sPBP 5-K213R showed essentially no difference in its interaction with penicillin G, we reasoned that it would also catalyze D-alanine carboxypeptidase activity. To conduct D-alanine carboxypeptidase assays, it was necessary to purify both sPBP 5 and sPBP 5-K213R to homogeneity in large yields. Utilizing covalent ampicillin affinity chromatography, sPBP 5 (1.0 mg) and sPBP 5-K213R (2.6 mg) were purified from osmotic shock fluids and were used to assess D-alanine carboxypeptidase activity (22). The kinetic parameters of the release of D-alanine were determined from the purified proteins and the substrate L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Ala (Table I). Surprisingly, whereas sPBP 5 displayed D-alanine carboxypeptidase activity, no activity could be detected with sPBP 5-K213R. The purified protein was reasayed for penicillin binding at the same time to determine whether the protein had lost its binding activity, but it showed the identical activities that were determined in osmotic shock fluid.

A competition experiment in which L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Ala was preincubated at pH 8.5 with sPBP 5-K213R for various times before the addition of [14C]penicillin G showed that sPBP 5-K213R did not appear to accumulate an acyl-enzyme complex (data not shown). Our sPBP 5 showed a reproducibly higher $K_m$ (8.0 ± 0.5 mM) than the sPBP 5 (5.0 ± 0.8 mM) reported previously (22), but it is not clear why these values are different from one another.

**DISCUSSION**

The proposed role of the histidine residue from the Box VII region in the catalytic mechanism of the Streptomyces R61 DD-peptidase was based upon the molecular coordinates of the histidine residue within the active-site cavity determined from the high-resolution crystal structure. The histidine side chain appears to be in position to contribute to the initial binding of substrate and β-lactam antibiotics, presumably by providing a positive charge that can interact with the carboxylate moiety of these ligands (16). We were interested in this hypothesis and sought to confirm the role of this amino acid in another low molecular weight PBP (PBP 5). The role of Lys$^{213}$ from the Box VII region of PBP 5 in the catalytic mechanism of this D-alanine carboxypeptidase enzyme was assessed by the technique of site-saturation mutagenesis. We reasoned that if the protonated amino group on Lys is responsible only for forming an electostatic interaction with the penicillin carboxylate, then one might expect that either arginine or histidine could substitute for lysine in the active site of sPBP 5. If Lys$^{213}$ directly participates in the acid-base reactions that occur during the acylation and deacylation of penicillin G with PBP 5 (as might the histidine residue), then only the protein with a histidine at this position should be able to bind [1H]penicillin G. Finally, if Lys$^{213}$ is not important in the binding of penicillin G, then at least some of the mutations should show significant binding activity.

Using site-saturation mutagenesis, we substituted lysine 213 of the Box VII region of sPBP 5 with 18 other amino acids and tested the ability of these mutant proteins to bind [1H]penicillin G. Only one of these proteins (sPBP 5-K213R) was capable of forming a stable covalent complex with penicillin. It is very illuminating that only arginine was capable of substituting for lysine at this position; since the high $pK_a$ of the guanidinium group of arginine makes it unsuitable for participating in acid-base catalysis, the results strongly suggest that it is simply the positive charge contributed by these 2 residues that is required for penicillin binding. The inability of histidine to substitute for lysine was unexpected since histidine is found at the same position in the Box VII region of Streptomyces R61 DD-peptidase. Since histidine is capable of contributing its positive charge in the Streptomyces enzyme, there are apparently other subtle conformational differences within the active site of sPBP 5 that prevent the productive interaction of the histidinium ion with the penicillin carboxylate moiety.

The activity of sPBP 5-K213R was further investigated, and the mutant was found to exhibit wild-type values of binding affinity and hydrolysis of [14C]penicillin G. The equivalence of these properties in sPBP 5 and sPBP 5-K213R suggests that the arginine residue has virtually no effect on the structure of the active-site cavity in respect to penicillin binding. Interestingly, when D-alanine carboxypeptidase activity was assayed, sPBP 5-K213R consistently showed no activity, even though its interaction with penicillin was normal. This suggests that the arginine residue, although situated in a suitable geometry for interacting with penicillin, is unable to form a productive electrostatic interaction with the substrate carboxylate. This in part may be due to a different positioning of the substrate carboxylate in the active site as compared with the penicillin carboxylate, which is supported by modeling studies of L-lysyl-D-Ala-D-Ala and cephalosporin C in the active-site cavity of the Streptomyces R61 DD-peptidase (16). Another reason for the loss of D-alanine carboxypeptidase activity in sPBP 5-K213R may be the different geometry of the arginine side chain as compared with the lysine side chain. This difference includes both a 1-Å increase in the length of the arginine side chain and the planar geometry of the guanidinium moiety. These structural differences may influence the binding of substrate such that arginine is not capable of interacting with the substrate carboxylate. Although we cannot be sure of the structural perturbations that have occurred in the active site when Lys$^{213}$ is replaced by Arg, it does appear that the positioning of the substrate carboxylate in the initial Michaelis complex is different from the penicillin carboxylate and that the arginine residue is capable of discriminating between the two compounds.

Other reports have detailed experiments aimed at the role of lysine in the Box VII region of β-lactamases. The role of Lys$^{244}$ (from the KTG triad) in the catalytic mechanism of B. licheniformis β-lactamase was addressed by site-directed mutagenesis, in which it was mutated to either an alanine or glutamate residue (35). These changes resulted in alterations (by 1–3 orders of magnitude) of both $K_m$ and $k_{cat}$, and this led to the conclusion that Lys$^{244}$ is involved in both ground-state and transition-state binding. The primary sequence of the class C β-lactamase PSE-4 has been recently shown to exhibit a notable variation in the conserved triad, in which the Lys residue is replaced with Arg (36). This was the first report of any native penicillin-interactive protein that contains Arg at this position and supports the view that this residue provides a positively charged electrostatic environment for the binding of the substrate carboxylate. Recently, a report appeared in which Lys$^{213}$ from the KSG triad of TEM β-lactamase was mutated to an arginine residue (37). The arginine-substituted β-lactamase displayed near wild-type levels of activity with cephalosporins, but showed a 10-fold decrease in $K_m$ with no effect on $k_{cat}$ for several penicillins, including penicillin G. These results also suggest that arginine is capable of replacing lysine in the KSG triad and maintaining near normal activity (at least for some substrates). Our results indicate that the
mutation of Lys$^{213}$ to Arg in sPBP 5 does not affect any of the parameters of penicillin G binding.

In contrast to its interaction with penicillin G, sPBP 5-K213R does not catalyze any detectable D-alanine carboxy-peptidase activity. We do not know which of the three parameters in the kinetic scheme of D-alanine carboxypeptidation (binding, acylation, or deacylation) were altered in sPBP 5-K213R. Of these three, we favor the alteration of the binding affinity and/or acylation rate as the parameter(s) most likely altered in the mutant. We base this choice on two factors: 1) the deacylation rate displayed by sPBP 5-K213R for [3H]D-alanyl-enzyme complex with the peptide substrate could be correlated with an alteration in k₃, the rate of hydrolysis of penicillin G.

It is clear that we shall not truly understand the structural changes that have occurred in the mutation of Lys-213 to Arg until the crystal structure of sPBP 5 has been elucidated. Work on solving the three-dimensional structure of sPBP 5' is in progress, and once this work comes to fruition, it will be very informative to determine what structural perturbations have occurred within the active site of sPBP 5-K213R. We have shown that this mutant can be isolated in high yield and purity, and it should be well suited for growing crystals.

Comparison of the crystal structures of sPBP 5, sPBP 5', and sPBP 5-K213R should allow us to further define the roles of the active-site residues that participate in binding, acylation, and deacylation of substrate and penicillin G.

Acknowledgments—We thank Derk Schultz for many helpful discussions and suggestions and for critical reading of the manuscript. We thank James Knox for helpful discussion concerning the crystal structure of the Streptomyces R61 dd-peptidase. We also acknowledge David Lamson for technical support and oligonucleotide synthesis.

REFERENCES

1. Tipper, D. J., and Strominger, J. L. (1965) Proc. Natl. Acad. Sci. U.S.A. 52, 1133-1141
2. Spratt, B. G. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2999-3003
3. Nakagawa, J., Tamaki, S., and Matsushashi, M. (1979) Agric. Biol. Chem. 43, 1379-1380
4. Ishino, F., Mitani, K., Tamaki, S., and Matsushashi, M. (1980) Biochem. Biophys. Res. Commun. 97, 287-293
5. Ishino, F., and Matsushashi, M. (1981) Biochem. Biophys. Res. Commun. 101, 905-911
6. Nakagawa, J., Tamaki, S., Tomioka, S., and Matsushashi, M. (1984) J. Biol. Chem. 259, 13937-13946
7. dePedro, M. A., and Schwarz, U. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 5865-5869
8. Tamura, T., Imae, Y., and Strominger, J. L. (1976) J. Biol. Chem. 251, 414-423
9. Matsushashi, M., Murayama, I. N., Takagaki, Y., Tamaki, S.,
   Nishimura, Y., and Hirota, Y. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 2631-2635
10. Spratt, B. G., and Strominger, J. L. (1976) J. Bacteriol. 127, 660-663
11. Medeiros, A. A. (1984) Br. Med. Bull. 40, 18-27
12. Kelly, J. A., Dideberg, O., Charlier, P., Wery, J. P., Libert, M., Moews, P. C., Knox, J. R., Duez, C., Fraipont, C., Joris, B., Dusart, J., Feire, J.-M., and Ghysen, J.-M. (1986) Science 231, 1429-1431
13. Samzou, B., Sutton, B. J., Todd, R. J., Artyomouk, P. J., Waley, S. G., and Phillips, D. C. (1986) Nature 329, 378-380
14. Kelly, J. A., Knox, J. R., Moews, P. C., Hite, G. J., Bartolone, J. B., Zhao, H., Joris, B., Freire, J.-M., and Ghysen, J.-M. (1985) J. Biol. Chem. 260, 16, 59-58
15. Joris, B., Ghysen, J.-M., Dure, G., Renard, A., Dideberg, O., Charlier, P., Frére, J.-M., Kelly, J. A., Boyington, J. C., Moews, F. C., and Knox, J. R. (1988) Biochem. J. 250, 313-324
16. Amanuma, H., and Strominger, J. L. (1980) J. Bioi. Chem. 255, 11173-11180
17. Amanuma, H., and Strominger, J. L. (1988) J. Biol. Chem. 263, 2034-2040
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488-492
20. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467
21. Sharp, P. M., and Li, W. H. (1987) Nucleic Acids Res. 15, 1291-1295
22. Kelly, J. A., Knox, J. R., Hao, Z., Freire, J.-M., and Ghysen, J.-M. (1988) J. Mol. Biol. 209, 281-295
23. Broome-Smith, J. K., Edelman, A., and Spratt, B. G. (1983) in The Target of Penicillin (Hakenbeck, R., Holtje, J.-V., and Labischinschi, H., eds) pp. 403-408, Walter de Gruyter & Co., Berlin
24. Matsuhashi, M., Tamaki, S., Curtis, S. J., and Strominger, J. L. (1983) J. Biol. Chem. 257, 644-647
25. Pratt, J. M., Holland, I. B., and Spratt, B. G. (1981) Nature 293, 307-309
26. Pratt, J. M., Jackson, M. E., and Holland, I. B. (1986) EMBO J. 5, 2399-2405
27. Amanuma, H., and Strominger, J. L. (1980) J. Biol. Chem. 255, 11173-11180
28. Nicholas, R. A., and Strominger, J. L. (1988) J. Biol. Chem. 263, 2034-2040
29. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
30. Ghysen, J.-M., Frére, J.-M., Leib-Bouille, M., Nguyen-Disteche, M., and Cayette, J. (1986) Biochem. J. 235, 159-165
31. Frére, J.-M., Mélinà, L.-B., Ghysen, J.-M., Niel, M., and Perkins, H. R. (1977) Methods Enzymol. 45, 610-636
32. Broome-Smith, J. K., and Spratt, B. G. (1989) FEBS Lett. 265, 185-189
33. Amanuma, H., and Strominger, J. L. (1984) J. Biol. Chem. 259, 1294-1298
34. Ellory, J. M., Escobar, W. A., Fink, A. L., Mitchinson, C., and Wells, I. A. (1990) Biochemistry 29, 5797-5806
35. Levesque, R. C., and Boisvert, M. (1990) J. Biol. Chem. 265, 1225-1230
36. Lezert, F., Labia, R., and Masson, J. M. (1991) J. Biol. Chem. 266, 17187-17194
37. Dues, C., Piron-Fraipont, C., Joris, B., Dusart, J., Urdea, M. S., and Cojan, C. (1990) Biochemistry 29, 5797-5806
38. Amanuma, H., and Strominger, J. L. (1984) J. Biol. Chem. 259, 1394-1398