Site-directed Mutants of a Soluble Form of Penicillin-binding Protein 5 from Escherichia coli and Their Catalytic Properties*

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Soluble, truncated mutant and wild-type forms of penicillin-binding protein 5 (sPBP 5) from Escherichia coli were produced in large amounts by placing the dacA gene that encodes PBP 5 under the control of the trp-lac fusion promoter. The 3' end of the dacA gene used in this study contains a stop codon that results in the deletion of 15 amino acids from the carboxyl terminus and the production of a soluble protein. Using oligonucleotide-directed mutagenesis, the role of cysteine 115 in the mechanism of sPBP 5 was investigated. Alkylation of cysteine 115 with sulfhydryl reagents has previously been shown to inhibit severely the d-alanine carboxypeptidase activity of PBP 5. Alkylation also inhibits the hydrolysis of bound penicillin G, with only a slight effect on its binding. Cysteine 115 in sPBP 5 was changed to either a serine (sPBP 5Ser) or an alanine (sPBP 5Ala) residue. The wild-type and mutant sPBPs were purified in milligram amounts from induced cultures by ampicillin affinity chromatography. The mutant PBPs showed only a 2-fold increase in the half-life of the penicilloyl-PBP complex, and had a binding affinity for penicillin G identical to wild-type PBP 5. The $K_m$ for the release of d-alanine from the peptide L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Ala was 5.0, 3.5, and 7.8 mM for PBP 5, PBP 5Ser, and PBP 5Ala, respectively, while the values for $V_{max}$ were 2.5, 3.3, and 5.1 μmol/min/mg. From these data it was concluded that the cysteine residue does not directly participate in the enzymatic mechanism.

Nascent peptidoglycan in Escherichia coli consists of alternating β 1,4-linked N-acetylglicosamine and N-acetylmuramic acid residues, in which the N-acetylmuramic acid residues are substituted with the pentapeptide L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Ala (1). Cell wall synthesizing enzymes, which are located in the cytoplasmic membrane, react with the pentapeptide to form an acyl-enzyme intermediate, with the concomitant release of the carboxyl-terminal d-alanine residue. This acyl-enzyme intermediate can react with either an amino group from another peptide chain to form a cross-link (transpeptidation), or it can react with enzymes, which are located in the cytoplasmic membrane, to form a stable, inactive covalent complex.

The cell wall synthesizing enzymes that form a covalent bond with penicillin, termed penicillin-binding proteins (PBPs), are detected by incubating cytoplasmic membranes with [14C]penicillin G, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography (3, 4). In E. coli seven PBPs, designated 1A, 1B, and 2-6, have been detected and characterized. The PBPs can be divided into two distinct groups. The high molecular weight PBPs (PBPs 1A, 1B, 2, and 3) are multifunctional proteins that catalyze both the addition of the disaccharide pentapeptide to existing peptidoglycan and its subsequent cross-linking to other peptide chains (5-8). The low molecular weight PBPs catalyze secondary transpeptidase activity (PBPs 4, Ref. 9) or d-alanine carboxypeptidase activity (PBPs 4, 5, and 6; Refs. 10-13).

PBP 5 is known to catalyze the major d-alanine carboxypeptidase activity in vivo (14). It is encoded by the dacA gene, which has been cloned and sequenced (15). It is initially synthesized as a larger precursor containing 29 additional amino acids that are cleaved during insertion into the cytoplasmic membrane (16). The nucelophilic amino acid that forms the covalent bond with both substrate and penicillin has been identified as serine 44 (17, 18). A recent study on the interaction of PBP 5 with the cytoplasmic membrane has shown that the removal of as little as 10 amino acids from the carboxyl terminus is sufficient to result in the release of the protein into the periplasmic space (19). PBP 5 can thus be classified as an "ectoprotein," that is, a normal globular protein attached to the membrane by a short stretch of amino acids.

PBP 5 has also been shown to have the highest β-lactamase activity of characterized PBPs; at pH 7 and 30 °C, the half-life of the penicilloyl-PBP 5 complex is about 10 min (20). It had initially been reported by Tamura et al. (21) that sulfhydryl reagents severely inhibit the carboxypeptidase activity of PBP 5, but not the binding of penicillin to the enzyme. Further investigation by Curtis and Strominger (22) showed that the addtion of sulfhydryl reagents only slightly affects the binding of penicillin to PBP 5 (as well as cell wall substrate), but greatly increases the half-life of the penicilloyl-PBP 5 complex. The amino acid sequence of PBP 5, deduced from the DNA sequence of the dacA gene, has shown that only 1 cysteine residue is present in mature PBP 5 (15). The possibility has been put forth that this lone cysteine residue, located at position 115, resides within the active site of PBP 5. 

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1 The abbreviations used are: PBP, penicillin-binding protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; bp, base pair; sPBP, soluble penicillin-binding protein; IPTG, isopropyl-1-thio-β-D-galactopyranoside.
5 and catalyzes the hydrolysis of the acetyl-enzyme intermediate (17, 29). The hypothesis of an intimate involvement of the sulfhydryl moiety in the catalytic mechanism was further supported by the properties of a putative charged amino acid, which plays a role in the mechanism of the mutant, termed PBP 5'. PBP 5' displays a near-normal acylation rate with respect to [14C]penicillin G, but shows a drastic increase in the half-life of the [14C]penicilloyl complex (23). It is also devoid of any detectable D-alanine carboxypeptidase activity (14, 23). This mutant thus mimics the effect of sulfhydryl alkylation on the activity of the wild-type enzyme. The mutation represents a single base change that alters residue 105 from glycine to aspartic acid (24), and this mutation is only 10 residues distant from the location of the cysteine residue. It can be argued, however, that structural constraints in this region, either the addition of a bulky alkylating group or the replacement of a small neutral amino acid with a large negatively charged amino acid, are responsible for the properties of both PBP 5' and alkylated PBP 5. Thus, the actual importance of the cysteine residue itself in the catalytic mechanism remains to be clarified. The present study examines the role of this cysteine residue in the hydrolysis of the acetyl-enzyme intermediate using oligonucleotide-directed mutagenesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—All restriction enzymes and DNA modifying enzymes were from Boehringer Mannheim with the exception of Bal I exonuclease (New England Biolabs). [14C]Penicillin G was from American Corp. Synthetic oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer at the Harvard Microchemistry facility. The strains of E. coli used were either JM63 (ara, lac-pro, strA, thi, tdl, lacZAM15) with the PUC plasmids, JM105 (lac pro thi, strA, supE, endA, sbcB, hod+, F', traD60, proAB, lacZΔM15) containing M13 vectors, XA90 (tonA, lac-proABC, F' lacI proAB) with pKK, pKAN, or pATG plasmids, or HB101 (F', hsdR30 (r-p-, m-), recA13, ara-14, proA2, lacY1, galK2, rpsL20 (Sm'), xyl-5, mtl-1, supE44, x') with other plasmids. The plasmids pBS42-4 (19) and pDA11 (20) were kindly provided by Dr. Julie Pratt, University of Liverpool, and Dr. Michio Matsushashi, University of Tokyo, respectively.

**Construction of Truncated PBP 5**—The dacA gene from pDA11 was ligated into pUC12 as a 1860-base pair (bp) BamHI-EcoRI fragment and transferred to M13mp9 using the polylinker and Pst-I-EcoRI fragment (pRN1). The 187-bp BglII-HindIII fragment from pBS42-4, which contains a termination codon inserted within the coding sequence of the dacA gene, was used to replace the BglII-HindIII fragment from pRN1. The resulting tetracycline-resistant plasmid, pRN20.4, was constructed by replacing the EcoRI-BstEII fragment from pRN1. This plasmid was digested with SalI and the 400-bp fragment was cloned into pUC19, and the resulting plasmid was designated pRN20.4. The entire gene was then excised by digestion with EcoRI and HindIII and ligated into the polylinker of pKK223-3 (Pharmacia LKB Biotechnology Inc.). This plasmid contains the inducible trp-fusion promoter, followed by a polylinker and transcription signal. The plasmid pKK-plasmid construction pKK was isolated by the oam method as described in "Materials." Following rescreening of positive phage, propacase, was allowed to substitute the subation of the BacEI-BglII fragment from pRN20.4 with the corresponding fragment containing the wild-type and mutant sequences (see Fig. 1).

**Removal of the Endogenous Promoter of PBP 5**—To put the dacA gene under the control of an inducible promoter, the endogenous PBP 5 promoter was deleted using Bal I exonuclease. pSAL220 (57 μg) was linearized with PstI and digested with Bal I, followed by the generation of blunt-ended fragments by T4 DNA polymerase. The DNA was then digested with SalI, and the Bal I deletion products were ligated into the Smal-Sall sites of pUC9. Clones of appropriate length were selected, ligated into M13mp6, and sequenced. The clone pUC9-15 was used for the initiation codon.

To abate the EcoRI site to the initiation codon, loop-out mutagenesis was performed. The oligonucleotide, 5'-ACGGCCAGTTAATT-CATGAAATACATT-3', contains 15 nucleotides complementary to mp9 to the 5' end of and including the EcoRI site and 16 nucleotides of the coding sequence of the pre-PBP 5' gene by inserting the BstEII-HindIII fragment into pBAL10, resulting in pBAL20.4. The entire gene was then excised by digestion with EcoRI and HindIII and ligated into the polylinker of pKK223-3 (Pharmacia LKB Biotechnology Inc.). This plasmid contains the inducible trp fusion promoter, followed by a polylinker and transcription signal. The plasmid pKK-plasmid construction pKK was isolated by the oam method as described in "Materials." Following rescreening of positive phage, propacase, was allowed to substitute the subation of the BacEI-BglII fragment from pRN20.4 with the corresponding fragment containing the wild-type and mutant sequences (see Fig. 1).

**Construction of dacA Genes Under the Transcriptional Control of an Inducible Promoter**—pRN20.4 was used to construct the PBP 5' gene by inserting the BstEII-HindIII fragment into pBAL10, resulting in pBAI20.4. The entire gene was then excised by digestion with EcoRI and HindIII and ligated into the polylinker of pKK223-3 (Pharmacia LKB Biotechnology Inc.). This plasmid contains the inducible trp fusion promoter, followed by a polylinker and transcription signal. The plasmid pKK-plasmid construction pKK was isolated by the oam method as described in "Materials." Following rescreening of positive phage, propacase, was allowed to substitute the subation of the BacEI-BglII fragment from pRN20.4 with the corresponding fragment containing the wild-type and mutant sequences (see Fig. 1).

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2.53 described by Frere et al. (33) characterized the reactions containing between 0.6 and 10 mM concentration of peptide varied between 1.6 and 10 mM. Duplicate aliquots were removed at various times and diluted into 10 pl of 50 mM sodium phosphate, pH 6.5, to stop the reaction. The radioactivity covalently attached to the PBP was determined from a Lineweaver-Burk plot.

Determination of the Rate of Hydrolysis of [14C]Penicillin G by sPBP 5 Variants—In general, between 7 and 15 mg of protein was diluted to a final volume of 30 pl with 50 mM sodium phosphate, pH 7.0. [14C]penicillin G was added to a final concentration of 47 [mu]g/ml (1.5 [mu]l of a 1 mg/ml solution), and the sample incubated at 37 °C for 10 min. Nonradioactive penicillin G was then added to a final concentration of 4.5 mg/ml (1.5 [mu]l of 100 mg/ml penicillin G), and 5-[mu]l aliquots were removed at various times and diluted into 10 pl of 50 mM sodium phosphate, 1% sodium dodecyl sulfate, pH 6.5, to stop the reaction. The radioactivity covalently attached to the PBP was determined by injection onto a Waters HPLC system equipped with a 214-nm wavelength detector and a Waters Protein column 1 x 80, and eluted with 50 mM sodium phosphate, 0.1% SDS, pH 6.5, at 1 ml/min. The radioactivity of the protein peak was determined by liquid scintillation counting and the values were normalized to the integrated area of the initial time point (i.e., 0 min). In cases where a gel was run, samples were adjusted with one-half volume of 3 x Laemmli sample buffer, boiled for 3 min, and submitted to electrophoresis on a 12% SDS-PAGE gel. The gel was treated with ENHANCE (Du Pont-New England Nuclear) before drying and exposure to Kodak X-AR5 film.

Carboxypeptidase Assays of PBP 5 Proteins—The peptide L-Ala-D-Glu-L-Lys-D-Ala-D-Ala (Sigma) was used as the substrate for the wild-type and mutant sPBPs. The reactions contained between 0.6 and 0.9 [mu]g of sPBP in 50 pl Tris-HCl, pH 8.5, and the final concentration of peptide varied between 1.6 and 10 mM. Duplicate samples were incubated at 37 °C for 20 min and then boiled to stop the reaction. The amount of [14C]-alanine released was determined as described by Pratt et al. (33). The kinetic parameters $K_m$ and $V_{max}$ were determined from a Lineweaver-Burk plot.

RESULTS

Cloning and Purification of Soluble PBP 5'—Pratt et al. (19) have shown that the deletion of as few as 10 amino acids from the carboxyl terminus of PBP 5 results in the production of a soluble form of the protein. Since the ability to generate a soluble PBP 5 greatly simplifies its purification and manipulation, the truncated form of PBP 5 was used in these studies. pBS42-4, a low copy number plasmid that produces a stable, truncated form of PBP 5 missing its last 15 amino acids, was used to construct a plasmid containing the truncated mutant dacA gene encoding soluble PBP 5' (sPBP 5'). This plasmid (pRN20.4) was a derivative of pBR322 containing the mutant dacA gene in a 1430-bp PstI-HindIII fragment. Osmotic shock of E. coli HB101 harboring pRN20.4 resulted in the release of a significant amount of sPBP 5', when assayed by SDS-PAGE and fluorography (Fig. 2). sPBP 5' was isolated in yields approaching 1 mg of protein/liter of bacterial culture.

Production of Mutant and Wild-type PBP 5 Under the Transcriptional Control of an Inducible Promoter—The overproduction of wild-type PBP 5 is lethal to the cell and thus the dacA gene cannot be cloned into a high copy number plasmid such as pBR322 (34). When the low copy number plasmid pBS42-4, which encodes soluble, truncated wild-type PBP 5, is transformed into E. coli, sPBP 5 is produced at only 3-5 times the level of endogenous PBP 5 (19). This amount was deemed unsatisfactory for large scale purification of wild-type and mutant proteins, and therefore the dacA gene was put under the transcriptional control of an inducible promoter. The construction of this plasmid was done using the previously described mutant dacA gene. Once the level of sPBP 5' was maximized, the BatEII-BglII DNA fragment from the wild-type dacA gene (or the analogous fragment containing the Cys to Ser or Cys to Ala mutation) was used to replace the corresponding fragment from the mutant dacA gene to yield the final plasmids. The construction of these plasmids is shown in Fig. 1 and explained here briefly. Bal31 deletion of a plasmid encompassing the dacA promoter and a portion of the 5' coding region resulted in the removal of all but 15 bp of endogenous DNA 5' to the ATG initiation codon (plBal10). The mutant dacA gene, with its promoter region deleted, was then reconstructed (pBal20.4) and placed under the transcriptional control of the inducible promoter Fc (a fusion of the rpm and lac promoters), resulting in pKK20.4. It was necessary to inactivate the $\beta$-lactamase gene of pKK20.4 in order to facilitate both the identification of sPBP 5' in the

FIG. 1. Construction of plasmid pATG20.4 and its derivatives for the overexpression of soluble PBP 5 variants. Plasmid pBal10, generated by Bal31 deletion of the dacA promoter region, contains 15 bp (arrow) 5' of the initiation codon to the SacI site of the dacA gene. The BatEII-BglII fragment from pRN20.4, which contains both a previously described mutation that changes Gly-105 to Asp-105 (C) and an insertion of a stop codon that results in the removal of 15 residues from the carboxyl terminus, is then ligated into pBal10 to yield pBal20.4. The promoterless dacA gene was excised from pBal20.4 and placed under the transcriptional control of Fc, a fusion of the rpm and the lac operon, resulting in pKK20.4. The ampicillin resistance gene was inactivated by digesting pKK20.4 with SacI and inserting the gene that encodes kanamycin resistance. pATG20.4 was constructed by replacing the EcoRI-BatEII fragment of pKAN20.4 with the EcoRI-BatEII fragment from pATG4, which resulted in the removal of the 15 bp of endogenous DNA 5' to the ATG codon. To yield the final plasmids discussed in the text, the BatEII-BglII fragments from Fig. 3 containing the wild-type sequence, the Cys to Ser mutation, and the Cys to Ala mutation were used to replace the corresponding fragment from pATG20.4, which at the same time changed the mutant Asp-105 residue back to the wild-type Gly-105. The symbols $\uparrow$ and $\downarrow$ designate the position of the Cys to Ser and Cys to Ala mutations, respectively. E, EcoRI; Bs, BatEII; S, SacI; H, HindIII; Sc, ScaI; Bg, BglII.
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Osmotic fluid and the preparation of pure protein. The β-lactamase resistance gene was therefore inactivated by inserting the gene that confers kanamycin resistance, which resulted in pKAN20.4. Fig. 2 shows the result from the induction of sPBP 5' by IPTG from XA90 cells harboring pKAN20.4. It can be seen that only a small amount of protein was being secreted into the periplasmic space and subsequently released by osmotic shock (lanes 3–6, compare with lane 2). A possibility for the low level of translation was that the endogenous Shine-Dalgarno sequence (35) was deleted and thus the plasmid-derived sequence in P5555 was too distant from the AUG codon to result in efficient translation (see “Discussion”). This possibility was tested by employing loop-out mutagenesis to remove the 15 bp 5' to the initiation codon.

Single-stranded DNA from M13mp9 containing the non-coding strand of the 630-bp E. coli-SalI fragment from pKAN20.4 was used as template. An oligonucleotide complementary to 15 bp of the M13mp9 vector (ending at the EcoRI site) and identical to the first 15 bp of the dacA gene was used as primer. Mutant phage were identified by hybridization of the 32P end-labeled oligonucleotide to nitrocellulose filter lifts of the M13 bacteriophage plaques. The yield of phage containing the 15-bp deletion was ~5%. Positive phage were sequenced by the chain termination method (30), and double-stranded DNA was isolated and subcloned (pTAG4). The EcoRI-BstEII fragment from pATG4 was then used to replace the corresponding fragment of pKAN20.4. This new construction was designated pATG20.4 (Fig. 1). The level of sPBP 5' in the osmotic shock fluid from XA90/pATG20.4 was compared to that from XA90/pKAN20.4 and HB101/pRN20.4. As seen in Fig. 2, the levels produced by pATG20.4 upon induction with IPTG were much higher than pKAN20.4 and comparable to that obtained with pRN20.4.

Construction of Wild-type and Mutant dacA Genes and Purification of the Protein Products—In order to assess the role of cysteine 115 in the hydrolysis of the acyl-enzyme intermediate, the codon for cysteine was mutated to a codon specifying either serine or alanine by using oligonucleotide-directed mutagenesis (26). Single-stranded DNA from bacteriophage M13mp9 containing the noncoding strand of the 400-bp BstEII-SalI fragment from the wild-type dacA gene was used as template. Mismatched oligonucleotides, identical to the coding sequence except for either one (TGT to TCT, Cys to Ser) or two (TGT to GCT, Cys to Ala) bases, were used as primers (Fig. 3). Positive phage were identified, and the presence of the mutations were verified by sequencing. Double-stranded DNA was isolated, and the SacI-HindIII fragment from the 3' end of the gene was added. The BstEI-BglII fragment was then excised and inserted into the mutant dacA plasmid pATG20.4 to give either pATG C-S or pATG C-A, which, at the same time, replaces Asp-105 in the previously described mutant gene with Gly-105 in the wild-type gene (Fig. 3). The truncated wild-type dacA gene was constructed in a similar matter (pATG WT). Induction of XA90 carrying each of these three plasmids with IPTG resulted in the same level of [14C]penicilloyl protein in the osmotic shock fluid as was obtained with HB101/pRN20.4 or XA90/pATG20.4, i.e. ~1 mg/liter of culture (data not shown). The three proteins were designated sPBP 5, sPBP 5C-S, and sPBP 5C-A, corresponding to the soluble forms of the wild-type, cysteine to serine mutant, and cysteine to alanine mutant proteins, respectively.

The large-scale production of the soluble wild-type and mutant proteins (except sPBP 5') was achieved by IPTG induction for 4 h of a culture of XA90 carrying the appropriate plasmid. sPBP 5' was synthesized by an overnight culture of HB101 carrying pRN20.4. The osmotic shock fluids were prepared and the sPBPs were isolated by conventional ampicillin affinity chromatography. This simple purification procedure was possible due to the absence of any other PBPs in the osmotic shock fluid. The purities of the sPBP preparations, as assessed by SDS-PAGE (Fig. 4), were greater than 95%. The protein concentrations were determined, and the stoichiometry of penicillin binding was calculated from the specific activity of the [14C]penicilloyl protein. Surprisingly, sPBP 5 showed a much lower stoichiometry of penicillin binding (0.65 mol/mol protein) than did sPBP 5C-S or sPBP

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\begin{align*}
\text{WT} & \quad \text{Asp} \quad \text{Ala} \\
\text{C-S} & \quad \text{Ser} \\
\text{C-A} & \quad \text{Cys} \quad \text{Asp} \\
\text{Asp} & \quad \text{Asp} \quad \text{Ala} \quad \text{Asp} \\
\end{align*}
\]

Fig. 2. Induction of sPBP 5' with IPTG in XA90 cells harboring either pKAN20.4 or pATG20.4. Cells harboring either pKAN20.4 or pATG20.4 were grown in L broth in the presence of 50 μg/ml kanamycin sulfate to an A660 = 1, IPTG was added (t = 0), and at the indicated times 2 ml of culture was removed and subjected to osmotic shock as described under “Experimental Procedures.” A portion of the shock fluid (20 μg) was incubated at a final concentration of 40 μg/ml [14C]penicillin G for 20 min at 37 °C, submitted to electrophoresis on a 12% sodium dodecyl sulfate-polyacrylamide gel, and the bands visualized by fluorography. Osmotic shock fluid (20 μg) from an overnight culture of HB101/pRN20.4 and purified sPBP 5' (1 μg) were also treated as above.

Fig. 3. Relationship between DNA and protein of pATG WT dacA gene and the position of substitutions produced by oligonucleotide-directed mutagenesis. Point mutations are shown in boldface with the corresponding amino acid changes shown above. Only the coding strand is shown. Amino acids -29 to -1 represent the cleaved leader sequence and Ser-44 is the nucleophile that forms the acyl-enzyme complex with penicillin and substrate. The change from Gly-105 to Asp-105 is present only in sPBP 5'.
acrylamide gel electrophoresis. The proteins were purified as described under “Experimental Procedures” and run on a 12% SDS-polyacrylamide gel. Lanes 1 and 6, 1.5 μg each of mass standards (values in kilodaltons); lanes 2–5, 2 μg of sPBP 5, sPBP 5C-A, and sPBP 5C-A, respectively.

**FIG. 5.** Quantitation of the rate of hydrolysis of [14C]penicillin G by sPBP 5 variants. Purified sPBP 5 variants (7–15 μg of protein) were incubated with 47 μg/ml of [14C]penicillin G in 50 mM phosphate buffer, pH 7.0, for 10 min at 30°C. A 100-fold excess of nonradioactive penicillin G was added (t = 0), and aliquots were removed at the indicated times and denatured in buffer containing 2% SDS. The radioactivity remaining covalently attached to protein was determined by gel filtration on high performance liquid chromatography.

5C-A (~1 mol/mol protein). The reason for this discrepancy is not understood. By normalizing the concentration to the amount of active protein (i.e. sPBP 5 that bound [14C]penicillin G), however, this problem was minimized.

**Activities of the Mutant Proteins**—To look at the effect of the cysteine mutations, a [14C]penicillin G hydrolysis assay was performed. The PBP5s were incubated with a saturating amount of [14C]penicillin G for 10 min at 30°C. A 100-fold excess of nonradioactive penicillin G was added (t = 0), and at various times aliquots of the sPBPs were removed and denatured in SDS. The amount of radioactivity covalently attached to protein was measured by gel filtration on high performance liquid chromatography. The first-order rate constants and half-lives for the enzymatically catalyzed hydrolysis of penicillin were calculated from a semi-log plot of percent radioactivity remaining versus time of incubation (Fig. 5; Table I). It is obvious that mutation of the cysteine residue to either a serine or alanine residue had a minimal affect (~2-fold higher half-life) upon the rate of hydrolysis of the acyl-enzyme intermediate. The t1/2 value for sPBP 5 was identical to the value of the detergent-soluble native form (18), which strongly supports the notion that the removal of the last 15 amino acids does not affect the enzymatic activity. As reported previously for PBP 5', the half-life of the sPBP 5' complex was extremely long (230 min) when compared to either PBP 5 or sPBP 5 (~10 min). The values of sPBP 5' and sPBP 5 are somewhat larger than the values previously reported for PBP 5' and PBP 5 (60 and 5 min, respectively; Ref. 21).

The effect of the mutations on the d-alanine carboxypeptidase activity was also investigated. Using the substrate L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Ala, the kinetic parameters of the release of d-alanine were determined (Table I). Curiously, PBP 5C-A showed a reproducibly lower K₅0 than PBP 5, while PBP 5C-A displayed a higher Kₑ. The values for Vₐ₅ for PBP 5' and PBP 5 are somewhat larger than the values previously reported for PBP 5' and PBP 5 (60 and 5 min, respectively; Ref. 21).

**DISCUSSION**

The recent report on the production of soluble PBP 5 by removing between 10 and 25 residues from the carboxyl terminus (19) presented an easy way to purify the PBP 5 variants. Since there are no detectable PBPs in the soluble fraction of an E. coli lysate, the purification of plasmid-derived sPBP 5 is simplified, without worries of contamination from other PBPs. The truncated forms of wild-type and mutant PBP 5 were therefore used in these studies. It is known that the overproduction of wild-type PBP 5 is lethal; presumably, the excess carboxypeptidase activity is sufficient to prevent the normal cross-linking of the peptidoglycan. Initial attempts were made to clone the Cys to Ser and Cys to Ala mutants.
into pBR322, as the previously described mutant had been cloned (pRN20.4). Surprisingly, these attempts consistently failed, which suggested that the mutants may be active in vivo and thus be lethal to the cell when present in large amounts. Overproduction of sPBP 5' is not lethal, as it displays no β-alanine carboxypeptidase activity in vivo. Since the level of sPBP 5 from *E. coli* carrying pBS42-4 was too low for large-scale purification, plasmids were constructed in which the *dacA* wild-type and mutant genes were placed behind the control of the inducible promoter F*<sub>16</sub>*.

The initial constructs (pKAN plasmids) contained 15 bp of endogenous DNA 5' to the ATG codon of the *dacA* gene. Upon induction with IPTG, very low amounts of protein were synthesized. Inspection of the DNA sequence 5' to the ATG codon revealed only one possible region with homology to the 3' sequence of the 16 S RNA (35), at -22 to -13 bp. This sequence contains 6 out of 10 bp that are complementary to the Shine-Dalgarno sequence (AGGA). These bases are situated at -19 to -17 from the initiation codon. The majority of the endogenous consensus sequence is therefore missing in the pKAN constructs, and thus the plasmid-derived sequence must be used. This sequence is 25 bp 5' to the initiation codon, and this distance presumably was a major factor in limiting the expression of the protein. The distance of the Shine-Dalgarno sequence from the initiation codon has been shown to dramatically affect the efficiency of translation (36). When loop-out mutagenesis was performed, the resulting construct placed the plasmid-derived Shine-Dalgarno sequence at -12 to -8 bp from the AUG, and this resulted in a large increase in the expression of the sPBP 5 proteins. The distance of the presumed endogenous Shine-Dalgarno sequence from the initiation codon may also be a way the bacterium regulates the translation of a gene product that is lethal in large amounts.

The codon for cysteine 115 of wild-type sPBP 5 was mutated to a codon specifying either serine or alanine. The choice of amino acids for replacement was straightforward; if the —SH moiety of cysteine was directly involved in the mechanism, then its substitution with the —OH group of serine might be expected to result in a mutant with a lower but measurable activity, or possibly even result in the exchange of the acyl group from the active site serine to the serine from the mutation. It has been shown that the replacement of the active site serine in RTSM-β-lactamase with cysteine results in a catalytically active protein, but with an 10-fold decrease in K<sub>m</sub> (37). One might also expect the reverse to be true. The replacement of the —SH group of cysteine with the hydrogen of alanine should completely prevent any active participation of this residue.

When the half-lives of the PBP-acyl-enzyme complexes were determined (Fig. 4; Table I), both mutant proteins displayed an approximately 2-fold increase in the half-life of the acyl-enzyme intermediate when compared to wild-type. This relatively small difference could be due to small perturbations in the overall structure of the active site, but it is interesting that both the serine and alanine mutations have the same effect. The similarity of the half-lives of the two mutant proteins argues against the importance of hydrogen bonding of the sulfhydryl group of cysteine in the catalytic mechanism of penicillin hydrolysis, even in an indirect role. Since the binding of penicillin to the mutant proteins (except sPBP 5') was identical to wild-type sPBP 5, it is also clear that this cysteine has no role in the recognition of or reaction with β-lactam antibiotics. This is consistent with the fact that sulfhydryl reagents still allow penicillin to bind to PBP 5.

In order to assess the effect of the mutations on the *in vivo* reaction of PBP 5, β-alanine carboxypeptidase assays were performed. The results, summarized in Table I, show that the mutations had very little affect on the carboxypeptidase activity. This result is to be expected, taking into account the results of the hydrolysis assays. The most reliable parameter, K<sub>m</sub>, due to its being independent of the enzyme concentration, varies only by a factor of 2 in the three different sPBPs. It is noteworthy that sPBP 5'-8 displayed an ~2-fold decrease in the K<sub>m</sub> for the reaction when compared to sPBP 5, but yet showed a 2-fold increase in the half-life of the penicilloyl complex. This suggests that there may be subtle differences in the mechanism of hydrolysis of substrate *versus* penicillin. The effectiveness of the two mutants *in vivo* is seen by their apparent lethality when cloned into pBR322 (see above).

It can thus be concluded that reaction of the cysteine residue in sPBP 5 with sulfhydryl reagents inhibits the hydrolysis of both substrate and penicillin by either altering the three-dimensional conformation of the active site or by sterically preventing water from entering the active site. The properties of PBP 5' give much support to the first conclusion. Since this mutation affects the activity of PBP 5 in a manner identical to sulfhydryl alkylation, it seems reasonable to conclude that the bulky alkylating reagents themselves are not preventing water from entering the active site. Rather, the effect upon the activity may be due to the steric perturbation of an active site amino acid that is involved either in positioning or activating the water molecule that reacts with the acyl-enzyme intermediate. It should be noted that the inhibitory effects of sulfhydryl reagents that have been proposed to inactive by reacting with an essential cysteine residue has recently been challenged by several groups doing site-directed mutagenesis. For example, both the lac permease (38) and the glycyl-aminoc acid synthetase (40) of *E. coli* have cysteine residues that upon reaction with N-ethylmaleimide results in a pseudo first-order loss of activity. When the cysteine residue whose alkylation results in the inhibition of the enzymatic activity was replaced with serine (38, 39) or alanine (39), the mutant proteins in each case displayed normal or near-normal activity. It is therefore apparent that the proposed essential cysteine residue in each protein is not directly involved in the molecular mechanism of its enzyme.

It should be emphasized that, unlike the serine proteases, the mechanism of PBP 5 is not symmetrical. It is clear that acylation of PBP 5' can occur at a rate very similar to wild-type PBP 5 (23), but the hydrolysis of the acyl-enzyme intermediate is drastically altered. It should be very illuminating to work out the detailed mechanism of this carboxypeptidase and see how much similarity is present between these two different classes of serine enzymes. Along these lines, crystals of sPBP 5' that diffract to greater than 3 Å resolution have recently been obtained. Analysis of these crystals should elucidate the three-dimensional structure and molecular mechanism of this enzyme.

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**REFERENCES**


2. R. A. Nicholas, B. Samareou, M. Matsushishi, and J. L. Strominger, manuscript in preparation.
Properties of Soluble, Mutant PBP 5s from E. coli