Site-directed mutagenesis of proposed active-site residues of penicillin-binding protein 5 from *Escherichia coli*

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Alignment of the amino acid sequence of penicillin-binding protein 5 (PBP5) with the sequences of other members of the family of active-site-serine penicillin-interacting enzymes predicted the residues playing a role in the catalytic mechanism of PBP5. Apart from the active-site (Ser⁴⁴), Lys⁴⁷, Ser¹¹⁰-Gly-Asn, Asp¹⁷⁵ and Lys²¹³-Thr-Gly were identified as the residues making up the conserved boxes of this protein family. To determine the role of these residues, they were replaced using site-directed mutagenesis. The mutant proteins were assayed for their penicillin-binding capacity and DD-carboxypeptidase activity. The Ser⁴⁴Cys and the Ser⁴⁴Gly mutants showed a complete loss of both penicillin-binding capacity and DD-carboxypeptidase activity. The Lys⁴⁷Arg mutant also lost its DD-carboxypeptidase activity but was able to bind and hydrolyse penicillin, albeit at a considerably reduced rate. Mutants in the Ser¹¹⁰-Gly-Asn

fingerprint were affected in both acylation and deacylation upon reaction with penicillin and lost their DD-carboxypeptidase activity with the exception of Asn¹¹²Ser and Asn¹¹²Thr. The Asp¹⁷⁵Asn mutant showed wild-type penicillin-binding but a complete loss of DD-carboxypeptidase activity. Mutants of Lys²¹³ lost both penicillin-binding and DD-carboxypeptidase activity except for Lys²¹³His, which still bound penicillin with a k_{+2}/K' of 0.2% of the wild-type value. Mutation of His²¹⁶ and Thr²¹⁷ also had a strong effect on DD-carboxypeptidase activity. Thr²¹⁷Ser and Thr²¹⁷Ala showed augmented hydrolysis rates for the penicillin acyl-enzyme. This study reveals the residues in the conserved fingerprints to be very important for both DD-carboxypeptidase activity and penicillin-binding, and confirms them to play crucial roles in catalysis.

INTRODUCTION

The penicillin-binding proteins (PBPs) of *Escherichia coli* are located in the periplasmic space and perform a key role in peptidoglycan synthesis. With the exception of PBP4 (Mottl and Keck, 1991), these proteins are membrane-associated and can generally be divided into two groups. The high-molecular-mass PBPs, PBP1A, 1B, 2 and 3 fulfil essential functions in peptidoglycan synthesis during growth and division. These enzymes are the lethal targets of β -lactam antibiotics. The low- M_r PBPs are monofunctional enzymes with DD-endopeptidase and/or DD-carboxypeptidase activities. Their enzymic action is also inhibited by penicillin and they are believed to have functions in the recycling and maturation of murein (PBP4) and in the regulation of the level of cross-linkage (PBP5 and PBP6) (Goodell and Schwarz, 1983; Nicholas and Strominger, 1988). PBP5 and PBP6 are also involved in the switch from cylindrical to hemispherical peptidoglycan synthesis (Begg et al., 1990; van Heyenoort et al., 1992).

All PBPs interact in a similar way with the natural substrate, the D-alanyl-D-alanine portion of the pentapeptide side chain of murein, and with β -lactam antibiotics which are substrate analogues that inhibit enzymic action (Tipper and Strominger, 1965). To get more insight into the exact mode of interaction between PBPs and their substrates or inhibitors, these enzymes are studied using site-directed mutagenesis (Nicholas and Strominger, 1988; Malhotra and Nicholas, 1992; Adachi et al., 1992). Since it is available in a soluble form, we have chosen

PBP5, one of the low- M_r PBPs of *E. coli* for our mutagenesis studies (Ferreira et al., 1988).

PBP5 is a DD-carboxypeptidase cleaving the D-alanyl-D-alanine peptide bond in the pentapeptide side chain of the murein, to produce a tetrapeptide, by means of an acylation–deacylation mechanism. An overexpression system producing large amounts of the soluble form of PBP5 in the cytoplasmic space has been established (van der Linden et al., 1992). This cytoplasmically overexpressed soluble form of PBP5, PBP5^{s353+9} (van der Linden et al., 1993), was the basis for site-directed mutagenesis. Although a three-dimensional structure of PBP5 could not yet be obtained due to lack of isomorphism between native and heavy-atom derivative crystals, residues likely to play a role in the catalytic mechanism could be identified. Sequence alignments of PBP5 with β -lactamases of class A and C revealed several conserved residues (Joris et al., 1988). The solved three-dimensional structures of class A and C β -lactamases revealed these conserved amino acids to be located in the active site, playing key roles in the catalytic mechanism (Herzberg and Moulton, 1987; Lamotte-Brasseur et al., 1991; Knox and Moews, 1991). Since structural similarities were found between class A β -lactamases and DD-peptidases (Kelly et al., 1986; Dideberg et al., 1987), the three-dimensional structure of the class A β -lactamases can be used as a reference for all serine β -lactamases and DD-peptidases (Joris et al., 1991). In this study, the three-dimensional structure of the β -lactamase of *Streptomyces albus* G was taken as a model structure for PBP5 (Dideberg et al., 1987).

The conserved fingerprints in PBP5 were Ser⁴⁴-Leu-Thr-Lys,

Abbreviation used: PBP, penicillin-binding protein.

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Ser¹¹⁰-Gly-Asn, Asp¹⁷⁵ and Lys²¹³-Thr-Gly. The Ser⁴⁴-Leu-Thr-Lys tetrad contains the active-site serine (Glauner et al., 1984) which covalently binds either substrate or inhibitor. The Lys⁴⁷ residue is also likely to be important for catalysis. The homologous Lys⁷³ in class A β -lactamases probably plays a role in acylation (Lamotte-Brasseur et al., 1991). The conserved triads Ser-Asp(Gly)-Asn and Lys-Thr(Ser)-Gly flank the active site in the β -lactamase structure. The serine residue of the first triad maintains a functional active site in the class A β -lactamases, whereas the asparagine determines substrate specificity (Jacob et al., 1990a,b). The lysine residue in the Lys-Thr(Ser)-Gly-box seems to be important in stabilizing the transition state during both acylation and deacylation in β -lactamases (Ellerby et al., 1990; Brannigan et al., 1991), but the analogous histidine in *Streptomyces* R61 DD-peptidase seems to play a different role (Hadonou et al., 1992a). Residues downstream of the Lys-Thr-Gly-box are thought to influence the substrate specificity of β -lactamases (Sougakoff et al., 1988; Healey et al., 1989; Jacobs et al., 1992). The conserved negative charge at Asp¹⁷⁵ is supposed to correspond to Glu¹⁶⁶ in class A β -lactamases, where it plays a role in acylation and deacylation (Gibson et al., 1990; Adachi et al., 1991; Delaire et al., 1991; Escobar et al., 1991).

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli strain MC1061 (Meissner et al., 1987) was used as a host for subcloning and expression of wild-type and mutant proteins. Site-directed mutagenesis was performed using *E. coli* strains CJ236 (Bio-Rad Laboratories, Richmond, VA, U.S.A.) and JM101 and phage M13mp19 (Norrande et al., 1983). The plasmid pLF1-5 was from our own collection (Ferreira et al., 1988) and the plasmid pPROFIT-5C^s was described previously (van der Linden et al., 1992). Bacteria were grown with aeration at 30 °C in Luria-Bertani (LB) medium supplemented with kanamycin (50 μ g $\cdot$ ml⁻¹) for antibiotic-resistant strains. At an A_{500} of 0.6 the culture temperature was shifted to 42 °C to induce the λ -p_R promoter.

Enzymes and radiochemicals

Restriction enzymes, Klenow polymerase, T4-DNA ligase and T4-polynucleotide kinase were obtained from Boehringer and used as recommended by the supplier. For penicillin-binding assays, potassium 6-phenyl-[1-¹⁴C]acetamidopenicillanate (specific radioactivity 1.97 GBq $\cdot$ mmol⁻¹) was used. Deoxyadenosine 5'-[α -³⁵S]thio]triphosphate was used for DNA sequencing. Both radiochemicals were obtained from Amersham International.

Site-directed mutagenesis

The 1.7 kb *Bam*HI-*Eco*RI fragment from pLF1-5 containing the *dacA*^s gene coding for PBP5⁵³³⁻⁵⁹ was cloned into the phage M13mp19 using the *Bam*HI and *Eco*RI restriction sites. Mutations were made using the method described by Kunkel et al. (1987). The mismatch oligonucleotides used were synthesized by Eurosequence (Groningen, The Netherlands) using an Applied Biosystems Model 380B DNA synthesizer. The sequences of the oligonucleotides are listed in Table 1. Mutations were screened for by single-track DNA sequencing using the Sanger dideoxy method (Sanger et al., 1977). Positive clones were verified by four-lane DNA sequencing. Mutated genes were recombined into the vector pPROFIT-5C720 using the *Sma*I and *Eco*RI restriction sites. pPROFIT-5C720 differs from pPROFIT-5C (van der Linden

et al., 1992) only in that the *Sma*I-*Eco*RI fragment from *dacA*^s has been replaced by a 0.7 kb *Sma*I-*Eco*RI fragment of phage λ .

Purification of mutant proteins

All mutant proteins were purified to homogeneity (yield 10–100 mg) using the newly developed dye-ligand chromatography method described previously (van der Linden et al., 1992). Cytoplasmic overexpression of wild-type and mutant PBP5 was achieved by placing the respective genes under the control of a temperature-inducible λ P_R promoter. Yields of 20 mg of PBP5 per litre of culture medium were reached.

SDS/PAGE

SDS/PAGE was performed as described by Lugtenberg et al. (1975) using a 10% (w/v) running gel and a 4.5% (w/v) stacking gel. The acrylamide/bisacrylamide ratio was 30:0.5.

N-terminus determination

N-terminus determination was performed by means of Edman degradation of protein immobilized on a poly(vinylidene difluoride) filter (Matsudaira, 1987).

Determination of the kinetic parameters

Penicillin-binding assays

The acylation rate of the mutant proteins was determined by incubating 24 μ g (600 pmol) of protein with [¹⁴C]benzylpenicillin (50 μ M) at 37 °C in a total volume of 100 μ l. Samples were taken at various times and the activity was determined as described (van der Linden et al., 1992). Mutants with a very high acylation rate as well as the wild-type protein were incubated with a [¹⁴C]benzylpenicillin concentration equal to 10% of the protein concentration and samples were withdrawn every 5 s up to 1 min thereafter.

For the determination of the deacylation rate, protein (600 pmol) was saturated with [¹⁴C]benzylpenicillin (50 μ M) during incubation for 10 min to 2 h, depending on the actual acylation rate. For each mutant protein, saturation was verified by removing an aliquot (50 pmol) of protein and determining the amount of bound penicillin. Unlabelled benzylpenicillin was then added to the sample to a concentration of 5 mM and aliquots were withdrawn every minute up to 10 min. The procedure was repeated removing aliquots at longer or shorter intervals depending on the acylation rate. The quantitative determination of the bound radiolabelled penicillin was done as described (van der Linden et al., 1992).

Kinetic measurements

The DD-carboxypeptidase activity of the mutant proteins was determined by incubation of 250 pmol amounts of purified protein (100 mM Tris, pH 8.0, 37 °C) with various concentrations (6–25 mM) of the artificial substrate bisacetyl-L-Lys-D-Ala-D-Ala (Sigma). After 10 min (60 min for slower mutants), the amount of released D-alanine was determined using D-amino acid oxidase and *o*-dianisidine chloride as described (Frère et al. (1976).

Kinetic background of the wild-type enzyme

Acylation and deacylation can be studied separately for β -lactams. This yields the values of k_{+2}/K' ($K' = (k_{+2} + k_{-1}/k_{+1})$) for acylation and k_{+3} for deacylation. With the peptide substrate, k_{cat} and K_m were determined.

Figure 1 Purified mutant forms of PBP5

The Figure shows a Coomassie-Blue-stained SDS/polyacrylamide gel of the different mutant forms of PBP5 ($\sim 1/\mu\text{g}$). The proteins were purified using the immobilized dye Procion Rubine MX-B. LMW, low-molecular-mass markers.

Other techniques

The concentration of proteins was routinely determined by the method of Bradford (1976), which was calibrated by a PBP5 standard subjected to an amino acid determination on h.p.l.c. after hydrolysis.

RESULTS AND DISCUSSION

Expression and purification of mutant proteins

The mutated proteins were overexpressed in the cytoplasm of *E. coli* MC1061 cells to a level comparable with that of the overexpressed wild-type PBP5 (van der Linden et al., 1992). All mutated proteins were purified in 10–100 mg amounts using the immobilized dye Procion Rubine MX-B (Figure 1). The dye chromatography showed to be independent of a functional active site as was found before (van der Linden et al., 1992). The purified mutant protein PBP5^{S353+g}Ser⁴⁴Cys showed a degradation product of 30 kDa, which had an N-terminal deletion of 87 amino acids (van der Linden et al., 1992). A degradation product of similar molecular mass was found for the Ser⁴⁴Gly and Lys⁴⁷Arg mutant proteins and, to a lesser extent, for the Ser¹¹⁰Ala, Ser¹¹⁰Glu and Ser¹¹⁰Thr mutant proteins. The Ser-Xaa-Xaa-Lys box is proposed to be located at the interface of the two domains of the protein, as was found for the class A β -lactamases. The homologous serine residue (Ser¹³⁰; numbering according to Ambler et al., 1991) in the Ser-Asp-Asn box of the β -lactamase of *S. albus* G forms a hydrogen bond to the α/β domain (Jacob et al., 1990b; Lamotte-Brasseur et al., 1991). Apparently mutations in these two conserved boxes open up the active-site cavity and make the protein more accessible to proteases. The cleavage point was found to be located in a region corresponding to an ill-defined loop in the *S. albus* G β -lactamase structure.

Kinetic properties of the wild-type enzyme

Since all mutant proteins were derived from the cytoplasmically overexpressed soluble form of PBP5, PBP5^{S353+g}, the kinetic properties found for this protein were taken as reference. Kinetic properties of the native membrane-bound PBP5 (PBP5^{nc}) remain difficult to obtain but are supposed to be very similar to those of PBP5^{sc} (Ferreira et al., 1988; van der Linden et al., 1992, 1993). In contrast with other PBP species, PBP5 is capable of releasing bound penicillin (Spratt, 1977; Nicholas and Strominger, 1988; van der Linden et al., 1992). PBP5 thus functions as a weak β -lactamase.

Kinetic characterization of the mutant enzymes

Ser-Xaa-Xaa-Lys fingerprint

The active-site Ser⁴⁴ (Blauner et al., 1984) was altered to a cysteine residue. This rendered the protein completely inactive towards both the peptide substrate bisacetyl-L-Lys-D-Ala-D-Ala and benzylpenicillin (Tables 1 and 2). Similar mutations in β -lactamases resulted in enzymes with reduced activities (Sigal et al., 1982; Dalbadie-McFarland et al., 1982; Jacob et al., 1991). For the thiol-PBP3 of *E. coli*, considerable residual penicillin binding was observed (Houba-Hérin et al., 1985). In contrast, the Ser⁶²Cys-mutant DD-peptidase of *Streptomyces* R61 was completely inactive (Hadonou et al., 1992b). Apparently structural requirements on the active-site serine are more strict in PBPs than in β -lactamases. Replacement of the active-site serine by glycine abolished the activity of the protein, which was expected, since the mutant protein lacks the functional group for acylation of both substrate and penicillin.

The lysine residue three residues downstream of the active-site serine is highly conserved among the members of the family of active-site-serine penicillin-interacting enzymes. In β -lactamases,

Table 1 Penicillin-binding characteristics of mutants

The rate constants for acylation and deacylation of the different mutant proteins were determined as described in the Materials and methods section. k_{+2}/K' values were determined at a single concentration of penicillin. The kinetic pathway for the reaction of a α -carboxypeptidase (E) with a substrate or an inhibitor (C) is:

where EC is the Michaelis–Menten complex and EC^* is the acyl-enzyme. D-Alanine is only released with the peptide substrate. P_H is the hydrolysed product (bisacetyl-L-Lys-D-Ala for the peptide substrate or penicilloic acid for penicillin). ND, not determined; WT, wild-type, –, not defined.

Mutant	k_{+2}/K'		$10^3 \times k_{+3}$	
	$M^{-1} \cdot s^{-1}$	% of WT	(s^{-1})	% of WT
PBP5 ^{sc} (WT)	12100 ± 300	100	3.3 ± 0.13	100
Ser ⁴⁴ Cys	0	–	–	–
Ser ⁴⁴ Gly	0	–	–	–
Lys ⁴⁷ Arg	20 ± 1	0.2	0.21 ± 0.06	6
Ser ¹¹⁰ Ala	148 ± 1	1.2	0.44 ± 0.09	13
Ser ¹¹⁰ Glu	44 ± 1	0.4	0.21 ± 0.02	6
Ser ¹¹⁰ Thr	115 ± 1	1	0.10 ± 0.02	3
Asn ¹¹² Thr	2580 ± 100	21	0.61 ± 0.08	18
Asn ¹¹² Ser	228 ± 1	2	2.92 ± 0.16	88
Asp ¹⁷⁵ Asn	200 ± 1	2	2.97 ± 0.19	90
Lys ²¹³ Glu	0	–	–	–
Lys ²¹³ Gln	0	–	–	–
Lys ²¹³ His	25 ± 1	0.2	ND	–
His ²¹⁶ Ser	10100 ± 200	83	2.78 ± 0.22	84
Thr ²¹⁷ Ser	6630 ± 170	55	11.15 ± 0.93	340
Thr ²¹⁷ Ala	5440 ± 200	45	5.33 ± 0.30	160
Thr ²¹⁷ Pro	30 ± 15	0.2	0.16 ± 0.05	5

a basic amino acid is necessary at this position which is believed to play a role in hydrolysis of the bound β -lactam (Gibson et al., 1990; Lamotte-Brasseur et al., 1991). Mutation of the lysine to an arginine resulted in enzymes with reduced activities. In the DD-peptidase of *Streptomyces* R61, the Lys⁶⁵Arg mutant had a strongly reduced activity on the peptide substrate and showed almost no penicillin-binding (Haddonou et al., 1992b). In PBP5, the replacement of Lys⁴⁷ by arginine completely abolished DD-carboxypeptidase activity; residual penicillin binding, however, was observed, acylation being more affected than deacylation.

Ser-Xaa-Asn fingerprint

Comparison of the sequences of class A β -lactamases revealed a highly conserved Ser-Asp-Asn sequence. In the β -lactamase

structure this conserved fingerprint is located in the α -domain, flanking the active-site cavity (Joris et al., 1991). In most PBPs, a corresponding conserved box was found, and in PBP5, the sequence appeared to be Ser¹¹⁰-Gly-Asn. Mutations in both the serine and the asparagine residue were made. Ser¹¹⁰ was mutated to alanine, glutamic acid and threonine. In all cases, the DD-carboxypeptidase activity was lost. Residual penicillin-binding activity was observed for the three mutants, acylation being more affected than deacylation. Mutation of the homologous serine (Ser¹³⁰) of *S. albus* G β -lactamase strongly affected the activity of the enzyme (Jacob et al., 1990b). Ser¹³⁰ forms a hydrogen bond to Lys²³⁴ of the Lys-Thr-Gly fingerprint, thus stabilizing the active-site structure. Furthermore, Ser¹³⁰ was proposed to play a role in ligand binding through interaction of the OH group with the carboxylate group on the ligand, and in hydrolysis of the bound ligand (Lamotte-Brasseur et al., 1991). From our results it is clear that Ser¹¹⁰ of PBP5 plays a role in catalysis. Omission of the OH group replacing serine with alanine had a strong effect on activity towards both substrate and penicillin. This is in agreement with the results described for Ser¹³⁰ in the β -lactamase. Even replacement by threonine, which conserves the OH-group, abolished DD-carboxypeptidase activity and had a stronger effect on deacylation than the Ser¹¹⁰Ala mutant. The more bulky side chains of threonine and glutamic acid might disturb the active-site structure.

Substitution of Asn¹¹² by threonine or serine had a strong effect on DD-carboxypeptidase activity, but did not completely abolish it. Both mutants had a much higher K_m , indicating a role for Asn¹¹² in ligand recognition. Lamotte-Brasseur et al. (1991) described a role for the corresponding asparagine (Asn¹³²) in *S. albus* G β -lactamase in acylation of the ligand, asparagine forming a hydrogen bond with the ligand.

In PBP5, both acylation and deacylation of penicillin were affected for the Asn¹¹²Thr mutant. For the Asn¹¹²Ser mutant, acylation was decreased even more but deacylation remained nearly unchanged. It remains difficult to explain these phenomena.

Asn¹⁷⁵

In class A β -lactamases, Glu¹⁶⁶ was described to play an important role in catalysis (Gibson et al., 1990; Escobar et al., 1991; Delaire et al., 1991). Glu¹⁶⁶ is involved in acylation by abstracting the proton from the active-site serine. During deacylation, it activates two water molecules to perform hydrolysis of the acyl enzyme (Lamotte-Brasseur et al., 1991). Sequence alignments indicated Asp¹⁷⁵ to be the corresponding residue in PBP5. The Asp¹⁷⁵Asn mutant completely lost its DD-carboxypeptidase activity. However, it still showed interaction

Table 2 α -Carboxypeptidase activity of mutants

The α -carboxypeptidase activity of the mutant proteins was determined using the artificial substrate diacetyl-L-Lys-D-Ala-D-Ala. All other mutants show no activity towards this substrate. The kinetic pathway for the reaction of a α -carboxypeptidase is given in the legend to Table 1. WT, wild-type.

Mutant	$10^{-3} \times V_{\max}$ ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	$10^{-3} \times k_{\text{cat}}$		K_m		k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
		(s^{-1})	% of WT	(mM)	% of WT	
PBP5 ^{sc} (WT)	2200 ± 180	1460 ± 100	100	14 ± 2	100	104 000
Asn ¹¹² Thr	11 ± 1	7 ± 1	0.5	34 ± 7	240	200
Asn ¹¹² Ser	20 ± 5	13 ± 3	1	34 ± 16	240	380
His ²¹⁶ Ser	520 ± 30	350 ± 20	24	11.6 ± 1.6	80	30 000
Thr ²¹⁷ Ser	9 ± 1.5	6 ± 1	0.5	50 ± 11	360	120

with penicillin. Acylation was strongly affected but hydrolysis of the acyl enzyme was nearly unchanged. This last result is in contrast with the findings for β -lactamases, where mutations in Glu¹⁶⁶ strongly diminish deacylation rates. Mutation of the corresponding residue in *Streptomyces* R61 DD-peptidase (Asp²²⁵Glu/Asn) hardly affected enzymic activity (Hadonou et al., 1992b). In the class C β -lactamase of *Citrobacter freundii*, the corresponding aspartic acid residue (Asp²¹⁷) also plays a role different from the class A β -lactamases (Tsukamoto et al., 1990). From our results, it can be concluded that Asp¹⁷⁵ plays a role in acylation of both peptide substrate and penicillin. In contrast with the β -lactamases of class A, however, Asp¹⁷⁵ seems not to be important for deacylation.

Lys-Thr-Gly fingerprint

The Lys-Thr(Ser)-Gly fingerprint is located on a β -strand on the side of the active-site cavity that faces the Ser-Asp-Asn box. The Lys-Thr(Ser)-Gly triad is highly conserved in the β -lactamases of classes A, C and D and in the PBPs. The DD-peptidase of *Streptomyces* R61 forms an exception, having a histidine residue instead of a lysine at the first position of the triad.

The lysine residue was found to play a major role in catalysis in class A β -lactamases, the positive charge stabilizing the transition state in both acylation and deacylation (Brannigan et al., 1991; Lenfant et al., 1991). The corresponding residue in PBP5 (Lys²¹³) was mutated to glutamic acid, glutamine and histidine. All three mutants completely lost their activity on the peptide substrate and only the Lys²¹³His mutant retained residual penicillin-binding activity. Variation of the pH (pH 4–10) did not show increase of the penicillin-binding activity of PBP5Lys²¹³His below pH 6.5, as was seen for the Lys²³⁴His mutant of the β -lactamase of *S. albus* G (Brannigan et al., 1991). It is clear, however, that a positive charge at this position is essential since both the Lys²¹³Glu and the Lys²¹³Gln mutant were completely inactivated. This positive charge could interact with the carboxylate group on penicillin or the C-terminal carboxylate on the peptide substrate, similar to the pattern found for class A β -lactamases (Ellerby et al., 1990; Lenfant et al., 1991; Brannigan et al., 1991). Using site-saturation mutagenesis on Lys²¹³ of PBP5, Malhotra and Nicholas (1992) found that only the Lys²¹³Arg mutant was still able to bind penicillin. In *Streptomyces* R61 DD-peptidase, His²⁹⁸ appears to play a role in acylation of both peptide substrate and penicillin. Its role in deacylation, however, is limited to the transpeptidase reaction (Hadonou et al., 1992a).

The residues just downstream of the Lys-Thr-Gly fingerprint were found to be involved in ligand binding (Healey et al., 1989; Jacobs et al., 1992). In PBP5 we have mutated His²¹⁶ to serine and Thr²¹⁷ to serine, alanine and proline.

The His²¹⁶Ser mutation still had a considerable amount of DD-carboxypeptidase activity. The k_{cat} was decreased to 24 % of the wild-type value but the K_m was lowered. A Thr³⁰¹Ser mutation in the *Streptomyces* R61 DD-peptidase also showed a decrease in k_{cat} . The K_m , however, was similar to the wild-type value (J. M. Wilkin, personal communication). In the β -lactamase of *S. albus* G, the backbone carbonyl and NH group of the corresponding residue (Ala²³⁷) are involved in hydrogen bonding to the ligand and polarization of Ser⁷⁰ respectively (Lamotte-Brasseur et al., 1991). Alterations in the side chain of this residue should not cause too large effects. Even so, alterations of substrate specificity were found in a class A and a class C β -lactamase carrying mutations in the corresponding residues (Healey et al., 1989; Jacobs et al., 1992). It is not clear from our results whether the positively charged histidine side chain plays a role in the

catalytic mechanism. At least the interaction with penicillin is not affected very much upon removal of the positive charge.

Thr²¹⁷ was mutated to serine, alanine and proline. Only the Thr²¹⁷Ser mutant showed residual activity with the peptide substrate. Both the Thr²¹⁷Ser and the Thr²¹⁷Ala mutant, however, showed increased hydrolysis rates with penicillin. Acylation was only moderately affected. A reduction in the size of the side chain at this position apparently makes the complex more susceptible to hydrolysis, probably by making the active site more accessible to water. It is interesting to note that in most β -lactamases of classes A and C, a glycine is found at the corresponding position, whereas more bulky or polar residues are found in the PBPs. The Thr²¹⁷Pro mutant almost completely lost its penicillin-binding activity, which can be understood in terms of the structural deformation caused by the internal ring of this residue.

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