PBP-A de Thermosynechococcus elongatus, une PBP proche des ß-lactamases de classe A. Analyse structurale de l'enzyme sauvage et d'un mutant désacylant plus rapidement

Les alignements de séquence et les mutations effectuées sur PBP-A nous ont permis de mettre en évidence l'existence d'une nouvelle famille de PBPs homologues aux β-lactamases de classe A. La mutation $L_{158}E$ permet d'améliorer la vitesse de désacylation du pénicilloyl-enzyme d'environ deux ordres de grandeur. A partir des protéines sauvage et mutante purifiées et concentrées, des cristaux ont été obtenus, et la structure des protéines a été résolue (C. Evrard et J. P. Declercq).

Dans un premier temps, nous avons identifié les interactions importantes entre les résidus du site catalytique et des comparaisons ont été réalisées par rapport aux β-lactamases et PBPs dont la structure est la plus proche. Ceci nous a permis de mettre en évidence un mécanisme catalytique possible pour PBP-A. L'importance du rôle joué par l'introduction d'un glutamate en position 158 est due en majeure partie au déplacement dans le site catalytique d'une molécule d'eau similaire à celle trouvée chez les β-lactamases de classe A et essentielle pour l'étape de désacylation.

Dans un deuxième temps, l'observation de zones sensibles aux mutations chez les β-lactamases de classe A et non conservées chez PBP-A nous a permis d'élaborer une stratégie d'insertion de différentes mutations ponctuelles afin de générer une activité β-lactamase. Trois zones ont ainsi été mutées, malheureusement aucune protéine mutante n'a atteint un niveau d'activité plus élevé que celui obtenu par la seule mutation $L_{158}E$.

En étudiant la structure de PBP-A et PBP-A-$L_{158}E$, nous espérions trouver une réponse évidente à la question : pourquoi PBP-A n'est pas une β-lactamase ? Tous les résidus importants du site actif se superposent presque idéalement aux résidus de la TEM-1, et nous avons tenté de « corriger les petits défauts » observés autour du site catalytique et susceptibles d'être responsables de cette faible activité d'hydrolyse des β-lactames. Les résultats négatifs

obtenus sont assez déroutants mais témoignent des qualités d'horloge suisse des β-lactamases, suggérant d'aborder une stratégie d'évolution dirigée plutôt qu'une méthode rationnelle.

Dans le cadre de ce travail, j'ai contribué à la préparation des échantillons protéiques pour leur cristallisation (expression des protéines, purification et concentration). La partie cristallisation et résolution des structures a été réalisée par C. Evrard et J. P. Declercq. J'ai ainsi pu analyser la structure, en particulier du site actif, ce qui m'a conduit à la rédaction de ce chapitre. J'ai enfin réalisé la construction de mutants et leur analyse fonctionnelle.

PBP-A from Thermosynechococcus elongatus, a penicillin binding protein closely related to class A ß-lactamases. Structural analysis of the wild type and improved deacylation mutant.

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Abstract

Molecular evolution paths have always been matter of discussions and researchers are interested in understanding how proteins with same scaffolds can catalyse different reactions. In the superfamily of serine penicillin recognising enzymes, two main actors, DD-peptidases and β-lactamases, are phylogenetically linked, but present a high difference of reactivity towards the antibiotics penicillins. While β-lactamases hydrolyse them very fast, leading to their inactivation, penicillins act like suicide inhibitors of DD-peptidases. Recently, a new family of proteins was discovered presenting all the features of class A β-lactamases, but having a six amino-acids deletion in the conserved Ω-loop, and lacking the essential Glu_{166} known to be involved in the mechanism of deacylation. Particularly, PBP-A from *Thermosynechococcus elongatus* and the catalytically enhanced deacylation $L_{158}E$ mutant, featuring a 90 fold increase in deacylation rate but still 5 orders of magnitude less active than a typical class A β-lactamase, were characterised. In this paper, we report the crystal structure of the wild-type protein in the free and penicilloylated forms at 1.9 Å resolution and of $L_{158}E$ mutant at 1.5 Å resolution, giving insights in the catalytic mechanism of the proteins. On the basis of structural comparison with β-lactamases, several mutants were generated in order to enhance PBP-A lactamase activity, but these attempts were unsuccessful. Since all the important active site elements of PBP-A-$L_{158}E$, including an essential water molecule, are almost perfectly superimposed with those of the TEM-1 enzyme, our results emphasize how far we are from understanding the secrets of enzyme mechanisms.

Introduction

Among one million sequences from 150 genomes, there are 850 000 protein sequences clustered into 50 000 families, each of which has the same structural domain composition. 80 % of domain sequences are assigned to only 5 000 types of domain families [1]. Hence, folds must have been used several times by divergent evolution or they must have independently arisen from different protein ancestors (convergent evolution) to provide the functional range represented through the protein universe. Proteins derived from a common ancestor have evolved through duplication of a gene, substitutions, insertions and deletions giving birth to families of homologous proteins that can catalyse the same reaction with different substrate specificity or catalyse different reactions although sharing a same mechanistic attribute [2]. The heterogeneity in substrate specificity or type of reaction catalysed among a family of proteins proves the versatility of a fold, and researchers challenge this versatility by using directed evolution strategies to generate enzymes endowed with new functionalities.

For example, it is impressive that a few amino acids mutations in a D-amino acid oxidase protein are sufficient to change or broaden its substrate specificity [3; 4]. In the same way, it is possible to enhance enzymes promiscuous activities by one or two mutations [5; 6]. Even more remarkable is the ability of researchers to modulate proteins function: a hydrophenylpyruvate dioxygenase involved in tyrosine catabolism was converted by two amino acid changes into a hydroxymandelic synthase, catalysing a step in the production of glycopeptide antibiotics [7]. Playing with protein versatility seems easy but some activities are sometimes difficult to enhance though using similar protein folds, and understanding evolution pathways is often a very difficult task.

The superfamily of the serine Penicillin recognizing enzymes (PRE) [8], containing the well-known DD-peptidases and β-lactamases is a good illustration of these difficulties. DD-peptidases are bacterial cell wall enzymes involved in peptidoglycan synthesis mainly exhibiting carboxypeptidase and transpeptidase activities. β-lactam antibiotics are able to bind the catalytic serine of these enzymes and to inhibit them by forming a stable covalent adduct, hence, DD-peptidases are also called PBPs for Penicillin Binding Proteins [9]. To counteract the antibiotics, bacteria developed defence mechanisms, the main one being the expression of β-lactamases that are capable of hydrolysing penicillins rapidly. β-lactamases and DD-peptidases share a similar two steps mechanism of acylation and deacylation involving a

catalytic serine for the nucleophilic attack of the substrate, similar consensus sequences and similar fold. Though identity is very low (below 20%), PBPs and *β*-lactamases were suggested, on the basis of similarities in the catalytic site and tridimensionnal structure, to be divergent descendents of an ancestral PBP. There are three classes of serine *β*-lactamases, A, C and D, class B representing metalloenzymes, and two classes of PBPs each subdivided in three groups, and each class of *β*-lactamase is probably related to a different class of PBP [10].

Class A *β*-lactamases present three consensus sequences common to other proteins of the PRE family that are, following ambler numbering [11]: $S_{70}XXK_{73}$ containing the catalytic serine and an essential lysine, $S_{130}DN_{132}$, and $K_{234}T/SG_{236}$ whose roles in the catalytic mechanism have been reviewed [12]. Class A *β*-lactamases feature a fourth specific element called *Ω*-loop which contains a strictly conserved Glu_{166}, involved in the deacylation mechanism and whose role in acylation is still controversial [13; 14]. Though class A is the most widespread and studied class of lactamases, their catalytic mechanism remains incompletely understood, and evolutive paths leading from one PBP to a *β*-lactamase is even less clear. A PBP5-like protein from *E. coli* has recently been suggested to be a possible intermediary in evolution towards *β*-lactamases [15]. However, this hypothesis is based only on structural similarities, as kinetic parameters are very different. Finally, despite a similar organisation of the catalytic site, generating lactamase activity on a PBP scaffold is very difficult and the best achievements only allowed a 110 times increase in deacylation for penicillin substrates [16].

We have recently reported the existence of a new family of PBPs from cyanobacteria which are homologous to class A *β*-lactamases and their closer PBP parents ever known today. Of these proteins, PBP-A from *Thermosynechococcus elongatus* that shares undetectable sequence homology to DD-peptidases and in average 30 % identity with class A *β*-lactamases has been characterised and mutants were generated. PBP-A showed high activity towards thiolester substrates suggesting a DD-carboxypeptidase activity and the enzyme was sensitive to *β*-lactams with kinetic parameters typical of a PBP. The $L_{158}E$ mutation where Leu_{158} is aligned with *β*-lactamases Glu_{166}, showed a 90 times increase in deacylation rate.

In this work, we solved the crystal structure of PBP-A and $L_{158}E$ mutant. Fine analysis of the structures allowed us to compare PBP-A catalytic site to the closest members of the superfamily, clearly identifying a typical class A *β*-lactamase catalytic site and overall fold. The striking resemblance between the active sites of *β*-lactamases and $L_{158}E$ mutant is even more puzzling given the very low activity of the mutant. Our attempts in mutating some

residues susceptible to be responsible for some disturbance in the catalytic site were unsuccessful. This study is in consequence a good example of the difficulty to bypass nature using protein folds versatility.

Structure

PBP-A is a 368 amino acids protein which has been truncated of 92 N-terminal amino acids and to which an amino-terminal Met was added for cloning, giving rise to a 277 amino acids protein. Throughout this paper, residue numbering refers to the truncated protein, number one being the inserted Methionine, and number 2 being equivalent to residue 93 of the full-length protein. Residue numbering for class A β-lactamases will follow Ambler numbering scheme [11]. Four crystal structures have been determined (PBP-A, PBP-A-L_{158}E and the two penicilloylated forms) with 4 monomers in each asymmetric unit. The electron density is usually well defined except for some residues at the N-terminal and at the C-terminal parts of the polypeptides. The details are summarized in Table 16. The Ramachandran diagrams (not shown) produced by ProCheck [17] show that in all cases, 90% of the residues are in the most favoured regions. One residue (Ile_{184}) in the four independent molecules of the four structures systematically lies in a region ($\phi \sim 75°$, $\psi \sim -55°$) classified as disallowed by this programme. However, according to the new Ramachandran criteria [18], this region is now classed as allowed and corresponds to a γ-turn.

The 16 monomers are very similar, with an r.m.s. deviation of 0.427 Å between the Cα atoms of residues 11 – 274, which are observed in all of them. The largest discrepancy occurs at the level of residue 224, but this difference is induced by the packing, because in each of the four structures, only molecule C is concerned. Further similarity of the 16 molecules are their solvent accessible areas with an average of 10886 ± 77 $Å^2$ for PBP-A structures and 10940 ± 118 $Å^2$ for PBP-A-L_{158}E molecules.

Though high similarity, local structural differences are observed, that may be explained by a different exposition of monomer of one structure to the crystal packing interactions. In the same way, interactions between the monomers may be responsible for these local differences.

Overall structure.

PBP-A is composed of two domains, an all α-domain consisting of an array of five helices from residues 58 to 199, and an $\alpha\beta$-domain from residues 18 to 54, and 204 to 274, consisting of six-stranded anti-parallel β-sheet, packed on one side against C-terminal helix α-8 and on the other side on helix α-6, the active site being at the interface of the two domains. Two

hinges connecting the domains can be defined from residues 54-58 and 200-204. The N-terminal part is only observed in molecule D of each structure and consists of a completely destructurated arm (Figure 35), which is consistent with the hypothesis that the protein is membrane-anchored through its N-terminal part (page 117).

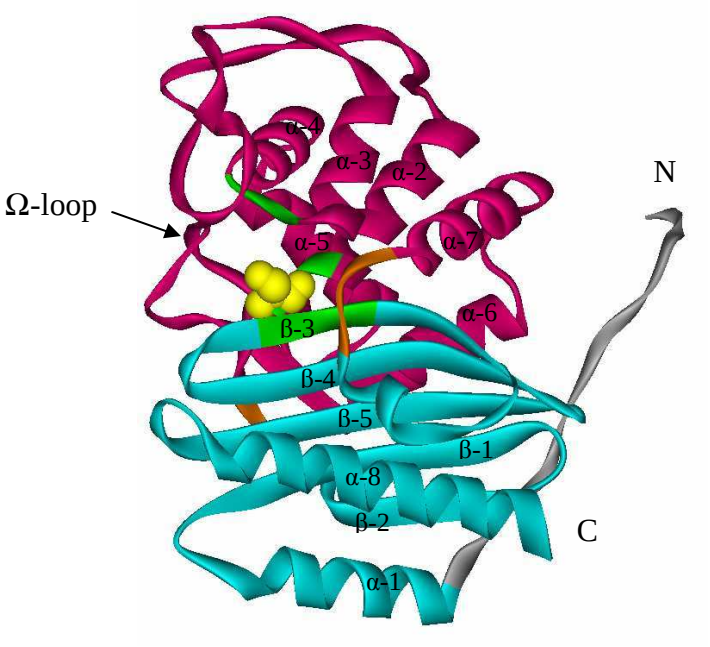


Figure 35: Ribbon diagram showing overall structure of PBP-A (molecule D). All-α domain is in magenta, αβ-domain is in cyan, the remaining portion of putative membrane anchor is grey, the hinges connecting the two domains are orange, catalytic serine is yellow and conserved motifs are indicated in green.

Amino acid sequence alignment as well as behaviour of the protein with penicillin and thiolester substrates suggested this protein to be a DD-peptidase. The overall protein structure, characteristic of the transpeptidase fold supports this idea. Moreover, when submitted to DALI server the structure matched well with Per-1 and TEM-1 and thereafter with several PBPs among which PBP-4a from *Bacillus subtilis*, *Streptomyces* K15 peptidase, PBP-5 from *Escherichia coli*, PBP-4 from *Staphylococcus aureus* and PBP-1a from *Streptococcus pneumoniae*.

Active site

The active site was located by plotting the conserved sequence motifs on the structure. The entrance to the active site is at the opposite side of the putative N-terminal membrane anchor, in an ideal position to interact with cell wall peptides. Three motifs are conserved within the ASPRE (Active Site Penicillin Recognizing Enzyme) protein family [8]: SXXK, SXN in class A *β*-lactamases or YXN in class C *β*-lactamases and KT/SG. Within PBP-A, the essential

serine Ser_{61} and lysine Lys_{64} are located at the N-terminal end of helix α-2, the turn between helices α-3 and α-4 contains Ser_{122} and Asn_{124} of the second conserved motif, and Lys_{219} , Thr_{220} and Gly_{221} are situated at the edge strand of the β-sheet. In class A β-lactamases, a fourth conserved motif located on an Ω-loop is composed of the Glu_{166}-X-X-X-Asn_{170} motif and is essential for the catalysis of acyl-enzyme deacylation [19]. Though an Ω-loop like structure is present in PBP-A, it is shorter by 6 amino acids and no residue such as Glu_{166} is present.

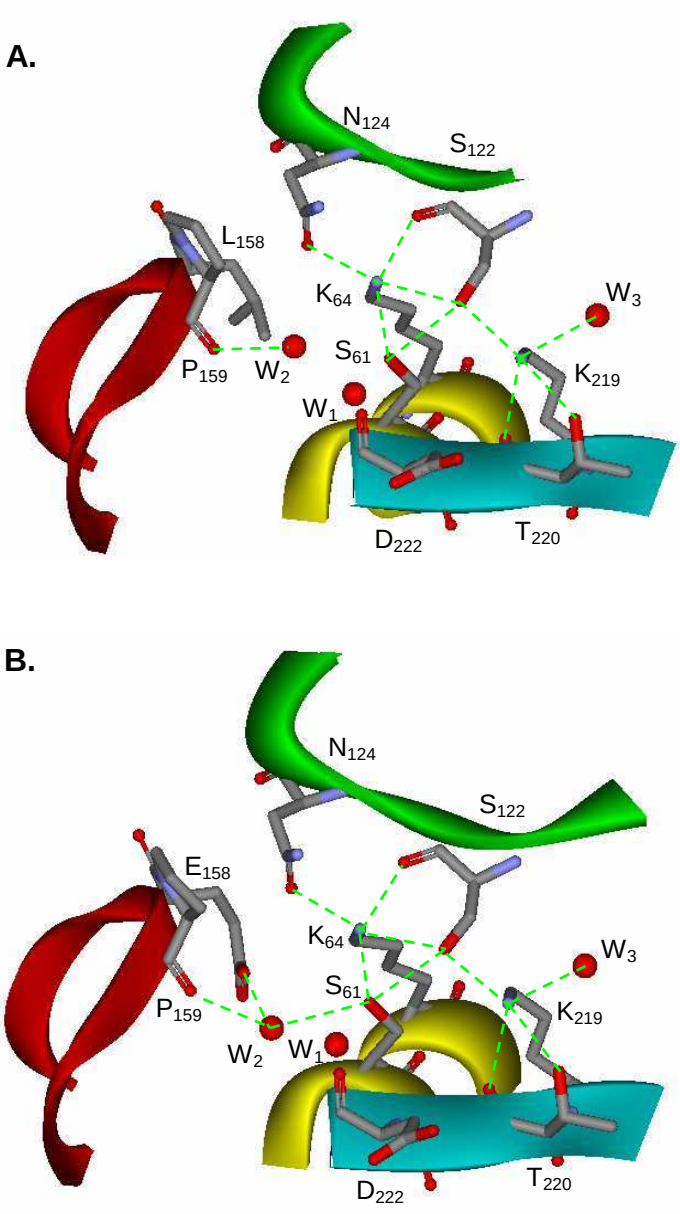


Figure 36 : hydrogen bond network in the active site of PBP-A (A) and PBP-A-L_{158}E (B). Segment of helix α-2 bearing motif SXXK is yellow, segment of loop bearing motif SDN is green, segment of Ω-loop bearing L/E_{158} is red and segment of strand β-3 bearing motif KTG is cyan. Hydrogen bonds are indicated by dashed lines. Hydrogen bonds between water molecule W1 and main chain nitrogen atoms of S_{61} and D_{222} are not indicated for better comprehension of the picture.

The active site of PRPs is highly hydrogen bonded and residues in the active site are involved in many interactions. PBP-A active site shows a dense hydrogen bonding network conserved in all 4 molecules of PBP-A and PBP-A-L_{158}E (Figure 36).

Unless specified, the distance values given below are an average of the distances measured in the 4 molecules of the crystal of PBP-A. Distances for PBP-A are quite similar to that of PBP-A-L_{158}E and only those for PBP-A are reported. In common with many other PRPs, the ζ-NH_2 group of Lys_{64} plays a pivotal role in this network, forming hydrogen bonds with the hydroxyl group of Ser_{61} (2,78 Å), the hydroxyl and backbone carbonyl groups of Ser_{122} (3,08 and 2,78 Å) and the amide carbonyl group of Asn_{124} (2,79 Å). The ζ-NH_2 group of Lys_{219} forms a hydrogen bond with hydroxyl group of Ser_{122} (2,83 Å), and is at hydrogen bond distances of Thr_{220} oxygen (2,78 Å). Finally, a hydrogen bond is formed between Ser_{61} hydroxyl group Oγ and Ser_{122} hydroxyl group Oγ (3,02 Å). This is in accordance with all PBPs and most class A β-lactamases, for these latest, distances of 3,17 Å for Per-1, 3,15 Å for TEM-1 in 1,55 Å resolution structure, (PDB code: 1zg4), but 3,55 Å in 1,8 Å resolution structure (1btl), and 2,89 Å in Toho-1 (1iyst) are found. In class A β-lactamases, Lys73 ζ-NH_2 group is hydrogen bonded to the carboxylate group of Glu_{166}, This is not the case in PBP-A-L_{158}E, between Lys_{64} and Glu_{158}.

In class A β-lactamases two molecules of water are structurally conserved [20; 21; 22], three molecules of water are conserved in PBP-A and PBP-A-L_{158}E. An important feature of serine proteases catalytic machinery is the oxyanion hole composed of a pair of backbone NH that stabilises the tetrahedral intermediate through hydrogen bonds. This oxyanion hole has been first evidenced in class A β-lactamases by *Pratt & Murphy* [23]. It is composed of main chain NH groups of Ser_{70} and of residue 237 and it contains a conserved water molecule in the ground state [20; 21; 24]. Water molecule 1 is conserved in both wild-type and mutant proteins and occupies the oxyanion hole, at 3,06 and 3,41 Å of Ser_{61} and Asp_{222} nitrogens for PBP-A and 3,05 and 3,38 Å for PBP-A-L_{158}E. The distance between W1 and Asp_{222} suggests only a weak electrostatic stabilisation of the molecule by Asp_{222} whereas it forms a real hydrogen bond with Ser_{61} nitrogen. Since the oxyanion hole is electrostatically positive, the negative charge of Asp_{222} may account for a weak destabilisation. In TEM-1, distances between main chain nitrogen atoms of Ser_{70} and Ala_{237} and the water molecule are 2,94 and 3,16 Å respectively, and in Per-1 distances are 3,2 Å between water and Ser_{70}, and 2,8 Å between water and Thr_{237}.

Class A β-lactamases active site usually contains another strictly conserved water molecule involved in penicillin hydrolysis, and found at hydrogen bond distance of hydroxyl group of

Ser_{70} and ε1-O of Glu_{166} carboxylate group. A similar water molecule W2 is found in PBP-A-L_{158}E mutant at 2,81 and 3,31 Å of Glu_{158} and Ser_{61} respectively. In β-lactamases the corresponding distances are 2,63 and 2,75 Å in TEM-1 and 2,86 and 2,98 Å in Per-1, this water molecule is also hydrogen bonded to the side chain oxygen of Asn_{170} at 3,23 Å for TEM-1 (1btl). In our case, W2 is at hydrogen bond distance of the oxygen of Pro_{159} (2,79 Å). It is worth noting that molecule D contains a water molecule W2 slightly far from the others, at 3,51 and 3,65 Å of Glu_{158} and Ser_{61} respectively, and these distances were not included in the calculation of average distances. In the wild-type protein, a nearly similar water molecule is found at 2,85 Å distance from Pro_{159} O, but the distance from W2 to Ser_{61} hydroxyl group is longer (3,59 Å) in molecules A, B and C and even longer in molecule D (5,71 Å). L_{158}E mutation seems to bring another conserved water molecule in the active site, at hydrogen bond distance of ε1-O of Glu_{158} carboxylate group and Ala_{60} oxygen (not indicated in Figure 36). A third conserved water molecule W3 is observed in the catalytic site of PBP-A, which is hydrogen bonded to the side chain of Lys_{219}. This water molecule is found in all penicilloyl serine transferases and plays a structural role in stabilising the side chain of the lysine of the KT/SG motif [25].

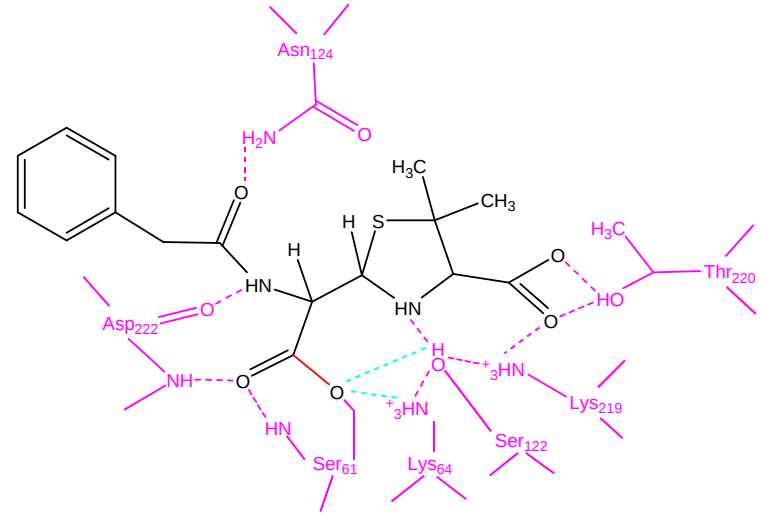
Structure of covalent adduct

In PBP-A-Pen, the acylation of Ser_{61} is clearly observed in the four molecules and the β-lactam ring has been cleaved between N_4 and C_7. The situation is much less obvious for PBP-A-L_{158}E-Pen. Some electron density appears in the catalytic pocket but the penicilloyl intermediate cannot be explicitly identified in this electron density, likely due to a low occupancy and a large agitation. Because of this problem which is probably due to the increased hydrolytic activity, this molecule will not be further discussed. The acylated form of PBP-A will be compared to the acylated form of TEM-1 E_{166}N mutant (1fqg).

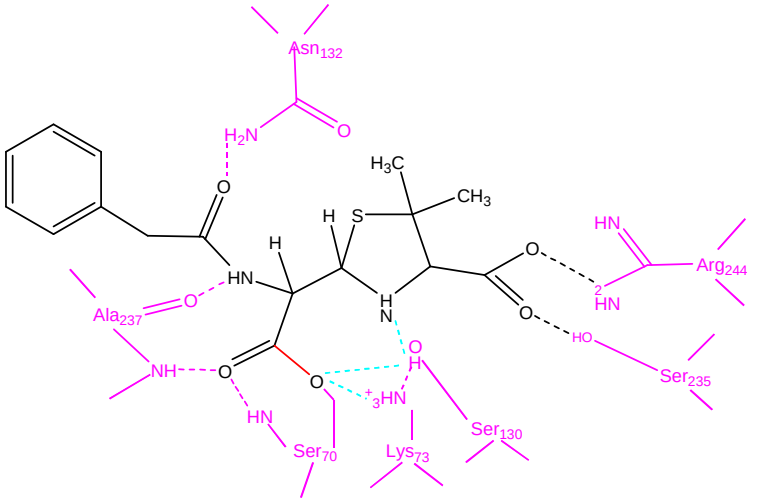
There is no striking structural difference between free-PBP-A and acyl-enzyme, as evidenced by the r.m.s (root mean square) deviation of the 8 structures: 0,425 Å for Cα and 0,828 Å for all atoms. When focusing on the active site residues (S_{61}-K_{64}, S_{122}-N_{124} and K_{219}-D_{222}), the r.m.s.d. is 0,206 Å for all atoms, indicating that binding of the substrate has no effect on the binding site cavity. It is important noting that a difference is again observed in molecule D where the orientation of the penicillin phenyl group is very different.

Besides the covalent bond between penicillin C_7 and Ser_{61} Oγ, penicillin G is stabilised by hydrogen-bonding interactions (Figure 37). The carbonyl oxygen atom O_8 of the complex is situated in the oxyanion hole and replaces the water molecule W1, forming one strong

hydrogen bond to the main chain nitrogen atom of Ser_{61} (2,8 Å) and a weaker bond to the main chain nitrogen atom of Asp_{222} (3,13 Å). N_{14} and O_{16} of penicillin G make hydrogen bonds to the main chain carbonyl oxygen of Asp_{222} (3 Å) and the amide-nitrogen of Asn_{124} (3,06 Å), respectively. Penicillin G carboxylate oxygen atom O_{12} forms a strong hydrogen bond to the hydroxyl group oxygen of Thr_{220} (2,61 Å) and a weak one to ζ-NH_2 group of Lys_{219} (3,18 Å). O_{13} is only electrostatically stabilised by the same Thr_{220} hydroxyl group (3,28 Å). Finally, the thiazolidine ring nitrogen N4 is hydrogen bonded to Ser_{122} hydroxyl group Oγ (2,99 Å) whereas the distance is longer in TEM-1 (3,22 Å).



A.



B.

Figure 37 : hydrogen bond network stabilising the acyl-enzyme complex formed with benzylpenicillin and PBP-A (A) or TEM-1 $E_{166}N$ (B). benzylpenicillin is black, residues from the enzymes are pink and pink dot lines are hydrogen bonds, and cyan dot lines are weak hydrogen bonds.

Though the catalytic site did not bear striking structural changes between the apo and acylated forms of the enzyme, the hydrogen-bonding network was slightly changed for some residues. ζ-NH_2 group of Lys_{219} hydrogen bonds with hydroxyl group of Ser_{122}, Thr_{220} oxygen, and a water molecule are maintained. The ζ-NH_2 group of Lys_{64} no longer forms a strong hydrogen bond with the hydroxyl group of Ser_{61} (3,22 Å instead of 2,78 Å), as observed in TEM-1 (3,28 Å in 1fqg and 2,9 Å in 1btl). Its hydrogen bonds to the hydroxyl and backbone carbonyl groups of Ser_{122} and the amide carbonyl group of Asn_{124} (not shown in Figure 37) are maintained, though in each case, the distance of one of the four molecules is quite different of the others. These hydrogen bonds also exist in TEM-1 (Table 12).

	PBP-Pen-A	*PBP-Pen-B*	*PBP-Pen-C*	*PBP-Pen-D*	*1fqg*
S122 Oγ	3,02	2,86	**3,14**	2,78	2,86
S122 O	2,79	2,87	2,84	**3,15**	2,91
N124 Oδ	2,82	**3,17**	2,89	3,03	2,86

Table 12 : hydrogen bond distances from ζ-NH_2 group of K_{64} in the four molecules of PBP-A crystal, and in TEM-1 $E_{166}N$ mutant in complex with benzylpenicillin (1fgq) given in Å.

Finally, the hydrogen bond between Ser_{61} and Ser_{122} hydroxyl group O*γ* is only weakly maintained (3,25 Å vs 3,02 Å), as in TEM-1 (3,25 Å). Concerning the water molecule W2 present in the free PBP-A, it still forms a strong hydrogen bond with Pro_{159} main chain carbonyl carbon (2,85 Å), but the distance to Ser_{61} hydroxyl group O*γ* has increased in all molecules (5,15 Å), whereas it is at the same place in PBP-A-$L_{158}E$ proving the importance of a charged negative side chain to maintain W2 in place for deacylation.

Comparison to ASPRE proteins: TEM-1, Per-1 and PBP-5

PBP-A general topology is similar to the conserved transpeptidase fold of the PREs. This family is composed of two main types of proteins, PBPs and *β*-lactamases.

		TEM-1	*Per-1*	*PBP5*
		1-btl	*1e25*	*1z6f*
PBP-A	*r.m.s.d (Å)*	1,83	1,58	2,27
	Number of aligned Cα	234	240	198
Per-1	*r.m.s.d (Å)*	1,75	-	ND
	Number of aligned Cα	245	-	-
PBP5	*r.m.s.d (Å)*	2,62	ND	-
	Number of aligned Cα	212	-	-

Table 13 : root mean square deviation (r.m.s.d) between PBP-A, penicillin binding domain of PBP5 and two class A *β*-lactamases. ND: Not Determined.

Comparing the overall PBP-A (molecule A) structure to other proteins, it is surprising to observe that better superpositions (Table 13) and structural alignments (Figure 38) are obtained for PBP-A with class A β-lactamases than PBPs.

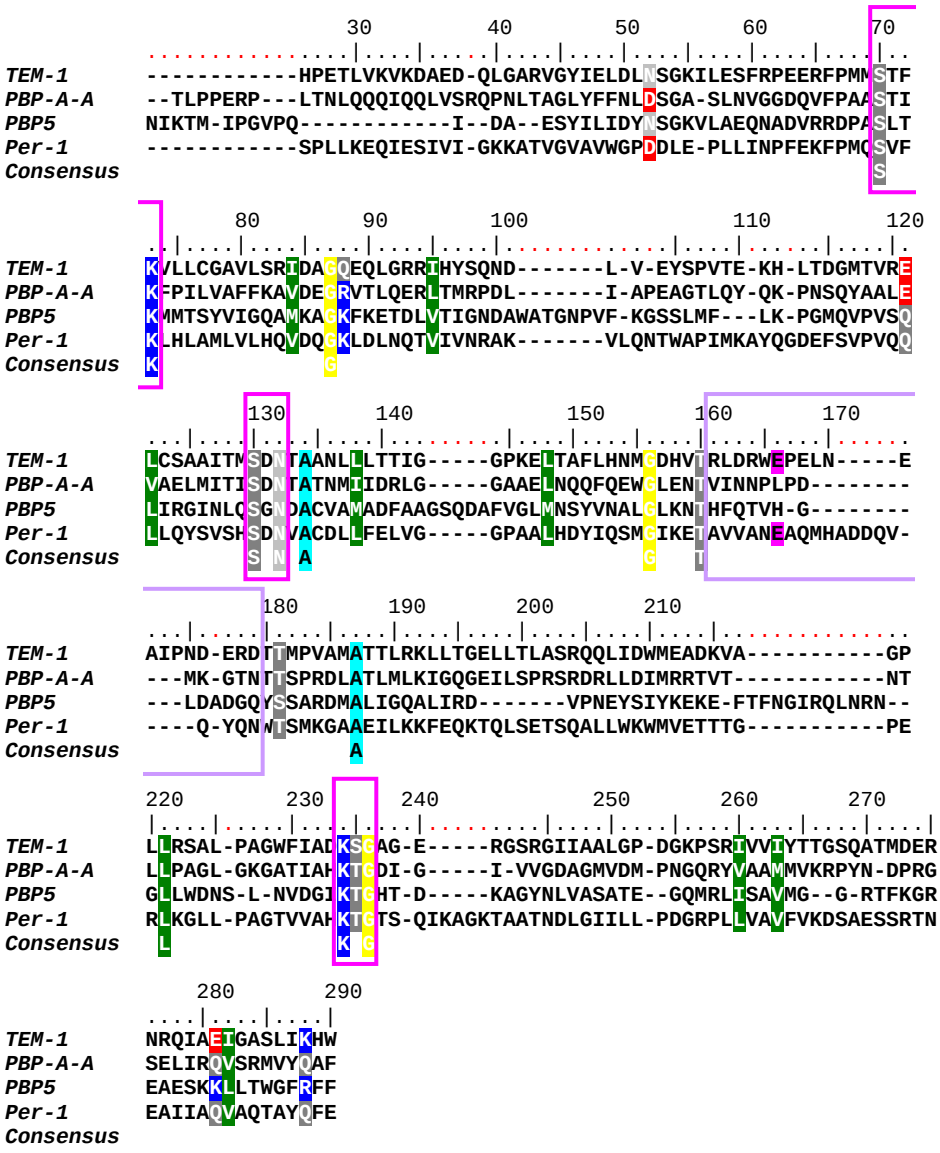


Figure 38 : structural alignment of TEM-1 (1btl), PBP-A chain A, PBP5 and Per-1. Structural alignment was achieved with Mustang, and edited using BioEdit. Residues numbering corresponds to that of TEM-1, gaps are indicates in red. Magenta boxes are repsectively SXXK, SXN and KT/SG motifs. Purple box is the Ω-loop region, and Glu_{166} is highlighted magenta.

The structural alignment of Per-1, TEM-1, PBP5 and PBP-A has been achieved with MuStAng [26]. In the same manner, the active site is remarkably well conserved at the level of the spatial arrangement of the conserved residues, and r.m.s deviation for the superposition of all atoms of active site residues S_{70}-K_{73}, S_{130}-N_{132}, K_{234}-A_{237}, E_{166} and water molecules 1 and 2 in β-lactamases and equivalent amino acids in PBP-A mutant is 0,914 Å for TEM-1 and

0,807 Å for Per-1. Hence, PBP-A overall structure is closer to that of class A β-lactamases and its structural similarity is higher than others PBPs. It is also remarkable to observe that Leu_{158}, which, on basis of sequence alignments, is aligned with Glu_{166}, is also structurally at the same spatial position and Glu_{158} side chain has the same orientation as in TEM-1 or Per-1. Though the general fold (Figure 39) and active site are well conserved, comparison of PBP-A to TEM-1 allows identification of several significant differences.

Figure 39 : superposition of PBP molecule A (green) and TEM-1 β-lactamase (red).

The most striking element that differentiates PBP-A from TEM-1 is the Ω-loop like structure from residues 155 to 165 which is six amino acids shorter than the class A β-lactamases Ω-loop, usually defined from residues 161 to 179. In class A β-lactamases, the length of the Ω-loop is strictly conserved and deletions in this region were genetically engineered to assess the impact of the mutations. It was shown that insertions at the N-terminal end of the loop are more deleterious for ampicillin hydrolysis than insertions at the C-terminal end [27]. In the same way, deletions or mutations in the Ω-loop are possible only in the region 170-178 [28; 29; 30]. This may be explained by the role played by this loop in the catalytic activity of β-lactamases. This loop presents an essential residue, Glu_{166} in the active site in a position where it allows activating the water molecule for acyl-enzyme hydrolysis, highlighting the importance of the

loop N-terminal part [20; 31; 32]. It is striking to observe that though there is no equivalent residue to Glu_{166} in PBP-A, the *Ω*-loop like structure fits better with the N-terminal end of TEM-1 *Ω*-loop (Figure 40).

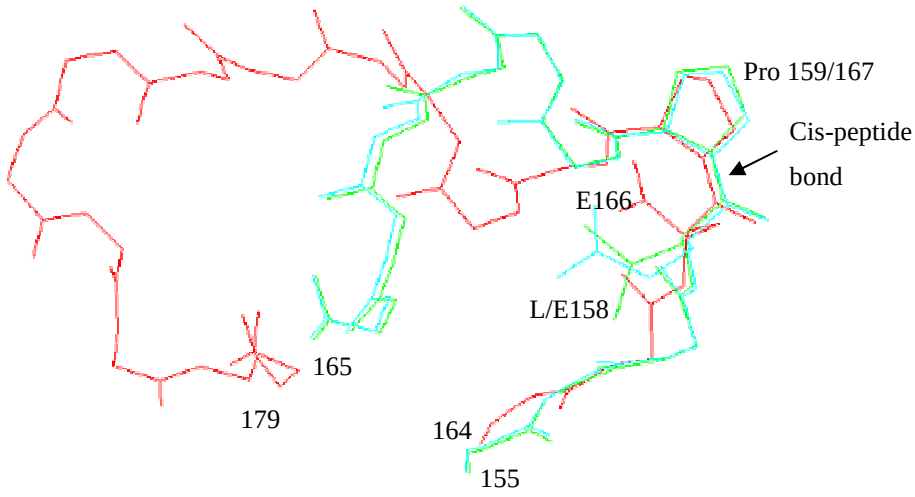


Figure 40 : superposition of PBP-A (green), PBP-A-$L_{158}E$ (cyan) and TEM-1 (red) in the region of the *Ω*-loop.

An important feature in the *Ω*-loop is the peptide bond between residues 166 and 167. Though non proline cis-peptides are energetically unfavourable and rare in proteins, a cis peptide bond between residues 166 and 167 is a typical invariant feature observed in class A *β*-lactamases except in Per-1 which displays a different *Ω*-loop fold [33]. Interestingly, a cis-proline is observed in position 159 of all PBP-A molecules. Finally, in class A *β*-lactamases, three invariable electrostatic interactions maintain the loop integrity: a hydrogen bond between the side chains of Lys_{73} and Glu_{166}, a salt bridge between Arg_{164} and Asp_{179} and hydrogen bonds between the CO and NH_2 groups of Asn_{136} side chain and the main chain NH and CO groups of Glu_{166} respectively. In PBP-A, there are many strong hydrogen bonds maintaining the loop structure but no salt bridge equivalent to Arg_{164}-Asp_{179} interaction. On the other hand, the same interactions between Asn_{136} and Glu_{166} exist in PBP-A and PBP-A-$L_{158}E$ between the equivalent residues Asn_{128} and Leu/Glu_{158}. Also, even shorter, the *Ω*-loop seems able to orientate Glu_{158} in a quite good position for water activation, but it may not be optimal.

Other difference resides in the N-terminal part of PBP-A which destructurated arm suggests that the removed N-terminal portion is a membrane anchor that is not compatible with *β*-lactamase activity as the latter are secreted or periplasmic, free enzymes.

Further element is the loop connecting helices *α*2 and *α*3 (residues 91 to 106) and overhanging the catalytic site. The conformation of this region compared to the equivalent residues 100 to 115 of the TEM enzyme is quite different. Particularly, residues 104 and 105

of TEM-1 which have been shown to play a role for β-lactamases in substrate binding and specificity [34; 35; 36] and indirectly in catalysis [37; 38] are very badly aligned in the structural alignment and there is a one amino-acid insertion. Glu_{96} and Ala_{97} seem to be the best equivalent to Glu_{104} and Tyr_{105} of TEM-1 (Figure 41, A).

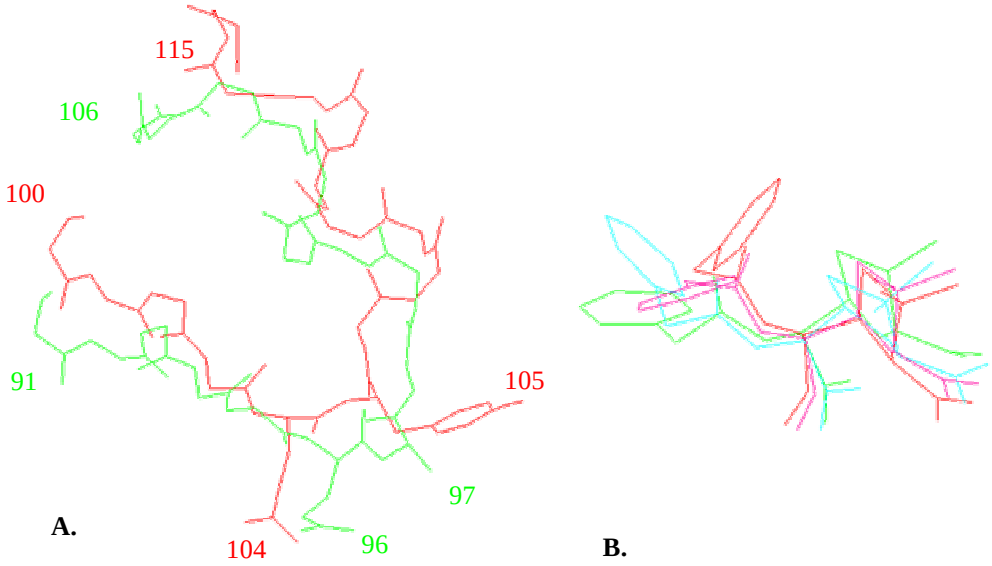


Figure 41 : A: detailed view of the conformation of the 91-106 region in PBP-A (green) and TEM-1 (red). B: orientation of benzylpenicillin in the catalytic site of TEM-1 $E_{166}N$ mutant (green), R61 DD-peptidase (cyan), PBP-A molecules A (red) and D (pink).

In class A β-lactamases amino acid 104 is hydrogen bonded to Asn_{132} side chain through the backbone carbonyl oxygen, except for Per-1 which forms this hydrogen bond with γ-OH of Thr_{104}. By stabilising Asn_{132}, Glu_{104} is indirectly involved in penicillins catalysis. In PBP-A, the carbonyl carbon oxygen of Ala_{97} is hydrogen bonded to δ-NH_2 amide group (3,2 Å) and main chain nitrogen(2,99 Å) of Asn_{124}. It is surprising to note that in molecule D of PBP-A-Pen, the distance between the carbonyl carbon oxygen of Ala_{97} and main chain nitrogen of Asn_{124} is 3,62 Å which is incompatible with a hydrogen bond. Hence, the stabilising role of this loop seems to be maintained, though the global conformation is different. The second role assigned to the loop is to form a wall in contact with the substrate and particularly Tyr_{105} in TEM-1 participates in substrate selectivity [35]. An aromatic amino acid is not necessary at this place and small residues like alanine and glycine that do not disturb the structure in the active site are allowed. In PBP-A, the amino acid in position of Tyr_{105} is an alanine and does not seem to disturb the structure of the cavity. This is less obvious for Glu_{96}: however equivalent to Glu_{104} and situated far from the substrate, its side chain has a different conformation than Glu_{104} in TEM-1 and it seems to bring a negative charge that closes the left side of the cavity and may be a problem for substrate binding (Figure 42). This hypothesis might be

strengthened by observation of penicillin conformation in the cavity of TEM-1, *Streptomyces* R61 DD-peptidase and PBP-A (Figure 41, B).

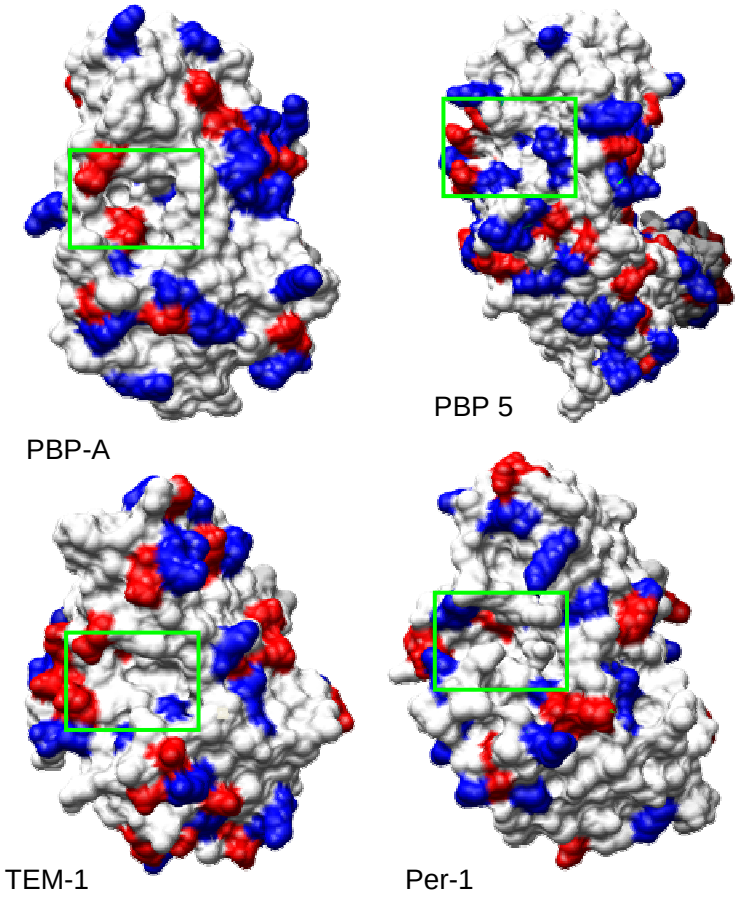


Figure 42 : repartition of charged residues in PBP-A, TEM-1, Per-1 and PBP-5. Negative residues are coloured red and positives ones are coloured blue. Active site is surrounded by green square.

Whereas benzylpenicillin side chain is directed in a same manner in R61 and TEM catalytic site, it seems more distorted in PBP-A, certainly under the influence of Glu_{96} side chain.

One deletion is observed in one of the two hinges connecting the helical domain to the $\alpha\beta$-domain β-sheet: there is no equivalent to TEM-1 Ala 217 in PBP-A. Though this region is important for domain association and thus for the good positioning of catalytic apparatus, there is no high residue conservation among class A β-lactamases in this loop and amino acid sequence depends on the environment [39]. Moreover, circularly permuted lactamases at position 228 [40], or 216 [41] retained enough activity to confer some *in vivo* ampicillin resistance. Hence, a deletion in this region may not be of great importance.

The last region that undergoes some differences when compared to TEM-1 is situated before and downstream of the KTG motif. Before the motif, Asp_{233} is a well conserved residue in

class A β-lactamases and is important for a correct active site conformation [12]. Even if the conservation is high, a histidine is found at this place in Per-1. His_{233} nitrogen atom of the imidazole ring and main chain nitrogen are hydrogen bonded to Asp_{246} side chain and Met_{211} oxygen, respectively in Per-1. His_{233} is also directly linked to Thr_{214} γ-OH by its carbonyl oxygen, and indirectly to the carbonyl oxygens of Thr_{214} and Thr_{216} through two water molecules and its imidazole ε-N. Asp_{246} is found in a β-sheet in the α/β domain, Met_{211} is situated at the end of the helix leading to one hinge connecting all α domain and α/β domain and threonines are found on the hinge. Hence, these residues are probably elements involved in hinge stabilisation for correctly adjusting one domain to another. In PBP-A, Met_{197}, Thr_{200}, Thr_{202}, His_{218} and Asp_{229} are spatially located at the same place as Met_{211}, Thr_{214}, Thr_{216}, His_{233} and Asp_{246} as in Per-1. The same interaction with Met_{197} is observed and the imidazole ring δ nitrogen is at hydrogen bond distance of Asp_{229} side chain in only two molecules (B and D, 2,71 and 2,72 Å). It is interesting that under penicillin acylation, all four molecules contain a hydrogen bond between those two residues (2,7 Å). Though interactions with threonines are different than in Per-1, Thr_{200} side chain hydroxyle is hydrogen bonded to a water molecule connected to Met_{197} trough its carbonyl oxygen, and Thr_{202} main chain carbonyl makes hydrogen bond to a water molecule connected to Asp_{229} carbonyl. Hence, it seems that in this region, a hydrogen network is in place in a comparable way as Per-1, with the probable role of maintaining a certain interaction between the all α domain and the α/β domain. In TEM-1, Asp_{246} and the two threonines are not conserved, and the only similar interaction is the hydrogen bond with Met_{211}.

Downstream the KTG motif, substitutions at residues 238-240 and 265 have been identified in TEM variants and have been shown either alone or in combination, to increase the enzyme activity against cephalosporins [36; 42], defining this region, with the 100-115 loop and the Ω-loop as the hot spots for β-lactamase specificity. In Per-1, positions 237, 238, 240 and 242 were mutated in order to assess the role of these residues in the extended spectrum activity of the protein. It was surprising to observe that none of these mutations gave significant differences in the protein activity against different cephalosporin substrates, indicating that mutating the same residues in different proteins do not have the same effect depending on their environment [38; 43]. In PBP-A, one residue is deleted after the KTG motif, however the degree of conservation in this region is so low that the precise location of the deletion is not known. Taken the differences that exist in class A β-lactamases within this region, the impact of the low conservation in PBP-A is difficult to predict.

Mutants

The structural comparison between active sites of PBP-A and class A *β*-lactamases indicates that the only significant difference is the absence of Glu_{166}, which is structurally aligned with Leu_{158} in PBP-A. Surprisingly, PBP-A-L_{158}E mutant did not lead to an important increase in activity with only a 90 fold improvement in deacylation rate when compared to wild-type protein, although the new glutamate occupies a very similar position to Glu_{166} in the active site. Hence, it seems that the catalytic site needs to be finely tuned in order to better accommodate and hydrolyse *β*-lactam substrates.

In class A *β*-lactamases, the hydrolytic water molecule is hydrogen bounded to Glu_{166} and Asn_{170}. Asn_{170} position has been shown to be important for catalysis in *Staphylococcus aureus* PC1: in N_{170}Q mutant, Gln residue allows similar polarity and functional group to be conserved, but the longer side chain occupies the space for the hydrolytic water molecule, impairing enzyme deacylation. In N_{170}M mutant, deacylation was less decreased as the mutation still allowed introduction of the water molecule, but the side chain occupied some space required for substrate binding and K_m was increased [44]. In the same manner, in the double mutant E_{166}D:N_{170}Q, Asp_{166} side chain was displaced in a position inappropriate to activate the water molecule, and Gln_{170} side chain occupied the space required for the water molecule. Hence, deacylation was completely abolished while acylation was still possible [45]. In PBP-A-L_{158}E, Glu_{158} orientation seems good for activating W2, but it may not adopt the optimal configuration, and though Pro_{159} carbonyl carbon is bound to the hydrolytic water, W2 is still 3,31 Å from hydroxyl group of Ser_{61}, which is a little far when compared to class A *β*-lactamases with 2,75 Å for TEM-1 and 2,98 Å for Per-1. Thus, introduction of a longer polar residue may push the water molecule closer to Ser_{61} and/or may influence Glu_{158} side chain conformation. Mutational modelling with SPDBViewer indicates that in the mutants D_{160}N and M_{161}N, the Asn could adopt conformations where it points towards the active site. Four mutants were generated: PBP-A-D_{160}N, PBP-A-L_{158}E-D_{160}N, PBP-A-M_{161}N and PBP-A-L_{158}E-M_{161}N, the single mutants being constructed as negative controls.

As was stated earlier, though Glu_{96} has no direct interaction with catalytic site residues or substrate, its long negative side chain may influence substrate binding. Hence, we mutated this residue into either the small non charged residue Gly or the small hydrophobic residue Val. Finally, we wanted to replace the Asp_{222} which introduces a negative charge at the bottom of the active site that has no equivalent in class A *β*-lactamases (Figure 42). Since an insertion that may influence the loop conformation between *β*-strands 3 and 4 is also found in

this region for the β-lactamases, two additional mutants were constructed where E96G or V and $M_{161}N$ mutations are combined with the replacement of D_{222} by AG: $E_{96}G$-$L_{158}E$-$M_{161}N$-$D_{222}AG$ (E96G+) and $E_{96}V$-$L_{158}E$-$M_{161}N$-$D_{222}AG$ (E96V+). The activities of the mutants were measured on both benzylpenicillin and thiolesters hydrolysis.

Regarding introduction of an asparagine close to L/E_{158}, $D_{160}N$ mutant did not have any significant effect on catalytic activity when residue 158 was a leucine. Though acylation was 5 times increased in PBP-A-$L_{158}E$-$D_{160}N$ compared to PBP-A-$L_{158}E$, deacylation was impaired to reach the level of PBP-A. This suggested that Asn influences the electrostatic field around Glu_{158} or displaces the water molecule such that it is more able to activate the serine during acylation, but is not able anymore to attack the ester bond during deacylation. $M_{161}N$ mutant showed no huge effect on catalytic efficiency in PBP-A, and a slight decrease in PBP-A-$L_{158}E$ pencillin hydrolysis (Table 14).

	k_2/Ks (M^{-1} s^{-1})	k_3 (s^{-1})	k_{cat} (s^{-1})
PBP-A	$1,07.10^4$	$1,55.10^{-4}$	$2,7.10^{-4}$
PBP-A-$L_{158}E$	$1,56.10^3$	$1,44.10^{-2}$	$1,42.10^{-2}$
PBP-A-Δ	$2,8.10^3$	$4,25.10^{-4}$	ND
$D_{160}N$	$2,9.10^4$	3.10^{-4}	$6,4.10^{-4}$
$L_{158}E$-$D_{160}N$	$7,8.10^3$	$4,9.10^{-4}$	$4,6.10^{-4}$
$M_{161}N$	$6,1.10^3$	$5,5.10^{-4}$	$3,3.10^{-4}$
$L_{158}E$-$M_{161}N$	$1,7.10^3$	8.10^{-3}	$8,1.10^{-3}$
$E_{96}G$+	$2,2.10^3$	$5,3.10^{-4}$	$3,3.10^{-4}$
$E_{96}V$+	$2,7.10^3$	$2,1.10^{-4}$	$6,2.10^{-4}$

Table 14: kinetic parameters of benzylpenicillin hydrolysis of wild-type PBP-A and mutants determined at RT. k_2/Ks and k_3 were measured using [^{14}C]-benzylpenicillin as described under Material and methods. k_{cat} was measured directly by measuring evolution of absorbance at 232 nm.

Thiolester hydrolysis was impaired in the four mutants, and the deleterious effect was additive to the effect of $L_{158}E$ mutation. These mutations also have an impact on the enzymes stereoselectivity, as three of them become able to catalyse hydrolysis of both S2d enantiomers (Table 15).

		S2a	*S2c*		*S2d*	
			X	*Y*	*X*	*Y*
PBP-A	k_{cat} (s^{-1})	≥ 7,7	≥ 6,6	≥ 0,21	≥ 52	-
	K_m (mM)	≥ 3	≥ 1,5	≥ 1,5	≥ 1,5	-
	k_{cat}/K_m ($M^{-1}.s^{-1}$)	$2,58.10^3$	$4,38.10^3$	$1,4.10^2$	$3,47.10^4$	-
PBP-A-$L_{158}E$	k_{cat} (s^{-1})	0,35	0,35	0,024	≥ 5,8	-
	K_m (mM)	2,2	1,3	1,3	≥ 1,5	-
	k_{cat}/K_m ($M^{-1}.s^{-1}$)	$1,6.10^2$	$2,68.10^2$	18,7	$3,89.10^3$	-
$D_{160}N$	k_{cat} (s^{-1})	0,62	0,99	0,073	≥ 15,45	≥ 0,8
	K_m (mM)	1,75	1,2	1,2	≥ 1,5	≥ 1,5
	k_{cat}/K_m ($M^{-1}.s^{-1}$)	$3,52.10^2$	$8,28.10^2$	60,8	$1,03.10^4$	$5,27.10^2$
$L_{158}E$-$D_{160}N$	k_{cat} (s^{-1})	0,023	0,057	-	≥ 0,9	-
	K_m (mM)	1,75	1,2	-	≥ 1,5	-
	k_{cat}/K_m ($M^{-1}.s^{-1}$)	13,1	47,8	-	61,4	-
$M_{161}N$	k_{cat} (s^{-1})	1,23	≥ 1,68	0,34	≥ 8,74	$\geq 2.10^{-4}$
	K_m (mM)	2,22	≥ 1,5	≥ 1,5	≥ 1,5	≥ 1,5
	k_{cat}/K_m ($M^{-1}.s^{-1}$)	$5,52.10^2$	$1,12.10^3$	$2,27.10^2$	$5,83.10^3$	0,16
$L_{158}E$-$M_{161}N$	k_{cat} (s^{-1})	0,057	≥ 0,02	$\geq 1.10^{-4}$	≥ 0,34	$\geq 3.10^{-4}$
	K_m (mM)	2,22	≥ 1,5	≥ 1,5	≥ 1,5	≥ 1,5
	k_{cat}/K_m ($M^{-1}.s^{-1}$)	25,7	13,3	0,07	$2,26.10^2$	0,23
E96G+	k_{cat} (s^{-1})	-	-	-	≥ 0,03	-
	K_m (mM)	-	-	-	≥ 1,5	-
	k_{cat}/K_m ($M^{-1}.s^{-1}$)	-	-	-	21,2	-
E96V+	k_{cat} (s^{-1})	-	-	-	≥ 0,007	-
	K_m (mM)	-	-	-	≥ 1,5	-
	k_{cat}/K_m ($M^{-1}.s^{-1}$)	-	-	-	5,25	-

Table 15 : kinetic parameters of thiolester hydrolysis at 37°C measured directly following absorbance at 250 nm.

Both mutants $E_{96}G$-$L_{158}E$-$M_{161}N$-$D_{222}AG$ and $E_{96}V$-$L_{158}E$-$M_{161}N$-$D_{222}AG$ showed decreased catalytic activity, here again deacylation was decreased to the wild-type enzyme level. Which of E96 or D222 mutation was responsible for the decreased deacylation was not assessed. In the quadruple mutants, only very low hydrolysis of S2d substrate was observed.

It was surprising to see that PBP-A was able to support four mutations close to the active site without loosing the activity for penicillin hydrolysis, but impairing drastically thiolesters hydrolysis.

Interaction of PBP-A with peptide substrates

Even though PBP-A is not active on peptide substrate Ac_2-L-Lys-D-Ala-D-Ala, its activity towards thiolester substrates suggests that this enzyme is a DD-carboxypeptidase (see page 119). Lower sequence and structural homologies to PBPs compared to β-lactamases are observed, and it is not obvious that structural features that contribute to peptides binding are identifiable. Actually, there are structural differences in the active site of DD-peptidases due to the fact that the composition of the interpeptidic links is variable, though the general structure of the cell wall is similar among bacteria. However, similarities do exist, the main one amongst this type of enzymes being the presence of a hydrophobic surface close to the catalytic site, that is important for binding the four methylenes of the diaminopimelate/Lys group of the natural substrate. This hydrophobic patch is absent in class A β-lactamases [46]. The region around the catalytic site of PBP-A is highly hydrophobic (Figure 43), and a hydrophobic groove leading to the catalytic serine is observed. Hence, the neighboring of the catalytic site is probably well fitted to accept a peptide substrate.

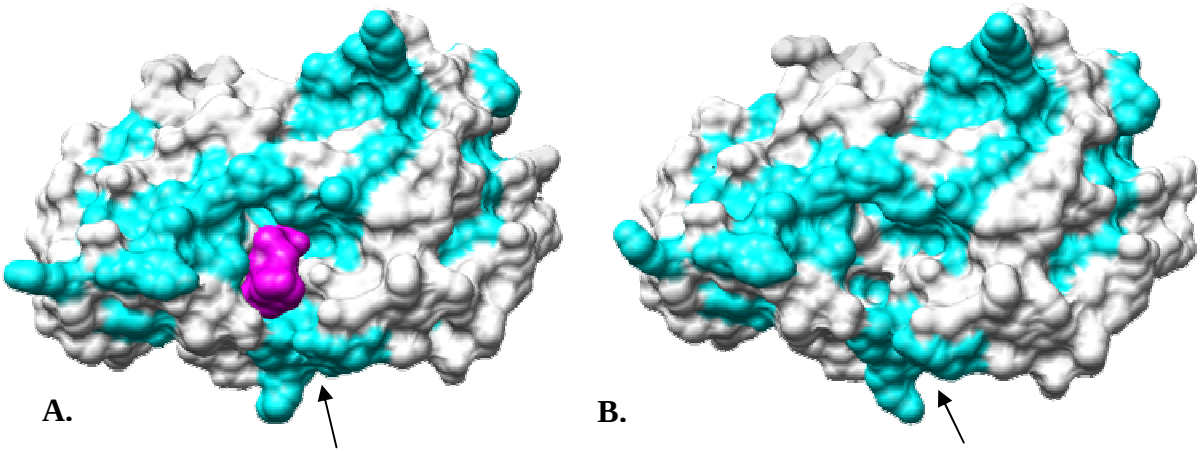


Figure 43 : PBP-A hydrophobic surface (cyan) in the apo (B) and acylated form (A) where penicillin G is in magenta. Black arrow indicates a putative hydrophobic groove for substrate binding.

Another typical characteristic of DD-peptidases is a hydrophobic pocket composed of two residues Gly-Leu with the glycine at the left side of the cavity. These residues are well conserved in PBPs and can be found in PBP5 as G_{152}-L_{153}, in *Streptomyces* K15 as G_{144}-L_{145} and in PBP3 as G_{161}-L_{162} [47; 48]. These residues are each time located on the Ω-loop like structure of these enzymes, and the absence of a side chain at the glycine position is important

to accommodate the methyl of the penultimate D-Ala of the peptidic substrate. Though structural alignment of PBP5 with PBP-A shows no equivalent residues because the Ω-loop like structures do not superimpose well, observation of the acyl-PBP-A allowed us to propose A_{97} and G_{98} as equivalent residues (Figure 44).

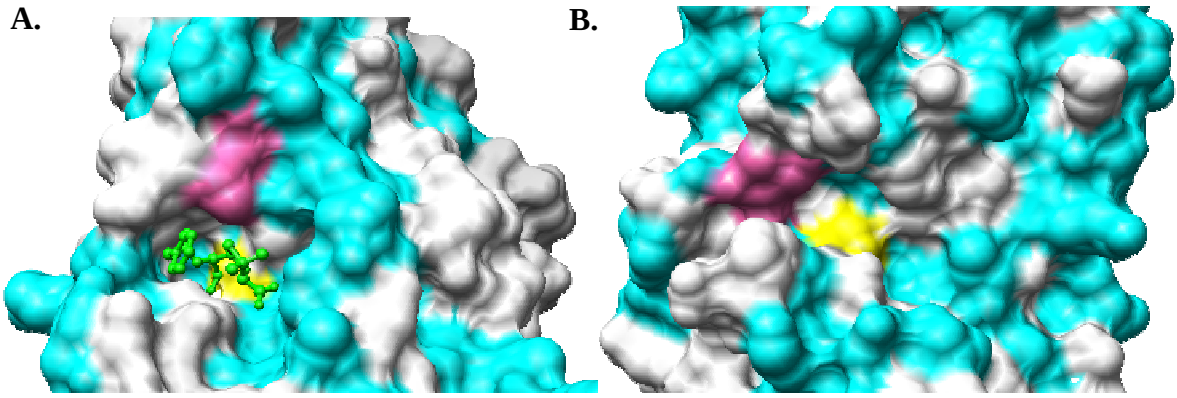


Figure 44 : solid surface view of the catalytic site of PBP-A in its Penicillin G acylated form (A) and K15 DD-peptidase (B). Catalytic serine is represented in yellow, penicillin G is represented in green, hydrophobic residues are in cyan and A_{97}-G_{98} (A) and G_{144}-L_{145} are represented in pink.

These two amino acids form a hydrophobic pocket 5 Å far from the penicillin C6, allowing space for a methyl group. However, in this case, this pocket is not placed on the left side of the catalytic site, as a consequence, peptidic substrates may not adopt a similar conformation in PBP-A compared to other known PBPs.

Finally, all the mutations around the active site affect greatly PBP-A thiolesterase activity. For each mutation on the Ω-loop that is susceptible of providing charge or electrostatic effect (L_{158}E, D_{160}N, M_{161}N), catalytic activity is decreased, indicating that electrostatic environment in the bottom of the cavity is important for protein carboxypeptidase activity.

Interaction of PBP-A with penicillins-Proposition for a catalytic mechanism

As a general rule, β-lactamases and PBPs act on their R_1-CO-X-R_2 substrates by a proton shuttle in a two steps manner. In the first step, the enzyme acylates the substrate: the nucleophilic attack of the carbonyl carbon by the activated Oγ of the catalytic serine is concomitant with the transfer of a proton from the hydroxyl group of a conserved serine to the leaving group X-R_2. In the second step, a proton from the acceptor (an amine group or a molecule of water) is abstracted, the activated acceptor attacks the carbonyle carbon of the acyl-enzyme and the abstracted proton is back-donated to Oγ of the leaving serine, hence deacylating the enzyme.

The deacylation process in class A β-lactamases has been clearly identified and involves the activation of a water molecule by Glu_{166} acting as a general base catalyst. Glu_{166} mutants of TEM-1 were generated, severely impairing deacylation [19; 49]. It was first shown by *Adachi & al.* that mutating Glu_{166} into Gln, Asn or Ala abolished penicillin hydrolysis, but mutants were still able to bind the substrates. This implied alternative mechanisms of acylation that do not involve Glu_{166}. Thus, for the acylation reaction, two residues were suggested to be involved, Glu_{166} and Lys_{73}, leading to controversial mechanisms [22; 50; 51]. Since then, many studies have been done on the acylation mechanism. NMR titration in the catalytic site of TEM-1 allowed measurement of the Lysine 73 pKa which was shown to be in a protonated state, incompatible with the mechanism proposed in which Lys_{73} is the general base for acylation [52]. The mechanism was thus puzzling as both mutants of Lys_{73} or Glu_{166} still undergo acylation [49; 53]. More recent simulations studies of acylation suggest the existence of two pathways in competition, one involving Glu_{166} and a water molecule , and another involving Lys_{73} [13; 14].

In PBPs a water molecule in a position merely equivalent to the TEM-1 deacylating water is sometimes present. However, there is no residue equivalent to Glu_{166} able to activate it, thus explaining the low deacylation rates [25; 54]. The general concerted mechanism of the nucleophilic attack of the penicillin by the catalytic serine with the transfer of a proton to the thiazoline ring nitrogen through the serine of the SXN motif can be applied to all penicilloyl serine transferases. However, it has been shown that conservation of the residues in the active site of PBPs does not necessarily implies the conservation of their role in the catalytic mechanism [55]. Hence, several mechanisms have been proposed for the acylation step, and it is most probable that different mechanisms are used from one homologous protein to another and these mechanisms could depend on the used substrate [56].

Kinetic constants of PBP-A with penicillins suggest that this enzyme has intermediate sensitivity to β-lactams. Orientation and interaction of the residues suggest that the general mechanism of acylation described for all peptidases may be a possible acylation mechanism for PBP-A. Whether another acylation path is possible may be evidenced by sequentially mutating residues in the active site.

Though a similar organization of the catalytic site, there are wide variations in penicillin acylation efficiencies among DD-peptidases, ranging from 20 $M^{-1}.s^{-1}$ for the low affinity PBP5fm to 300 000 $M^{-1}.s^{-1}$ for the best R39 PBP. These variations can be explained by the accessibility of the active site, the conservation of the essential motifs (SXC in the K15 DD-transpeptidase is less efficient than SXN), or the difference in the stabilizing elements of the antibiotic carboxylic acid [57]. PBP-A acylation efficiency is in an intermediate range, and two

reasons may explain that. First, benzylpenicillin carboxylate is stabilised by K_{219} and T_{220} side chains. Though not necessary, an guanidinium group for stabilization of this carboxylate seems to be more efficient and is present in many class A β-lactamases of which TEM-1 (Arg_{244}) [58]. It is also the case for the highly efficient R39 DD-peptidase for which an arginine is found 6 Å away from the carboxylate and in a similar place than Arg_{220} in *Streptomyces Albus G* lactamase [57]. Looking at the alignment, residues equivalent to Arg_{220} and Arg_{244} are Val_{227} and Leu_{205}. Simulations of Val_{227}Arg and Leu_{205}Arg mutations with Swiss-PdbViewer show that not Leu_{205} is in good position for supporting the mutation to Arg, and that residue would point towards the carboxylate group of benzylpenicillin. Hence, the effects of Leu_{205}Arg mutation may be interesting to investigate. Second, the active site needs to be accessible to the substrate for good acylation. In PBP-A, the accessibility does not seem to be optimal as the cavity is rather closed at the left side with Glu_{96} side chain (Figure 44). Hence, these observations may explain the inability of PBP-A to catalyse acylation more efficiently.

Improving PBP-A deacylation

As for all PBPs, inefficient deacylation of PBP-A is explained by the absence of a residue capable of activating a water molecule to attack the carbonyl of the penicilloyl enzyme. What is more unsettling is why introduction of a glutamate spatially in the same place as Glu_{166} in class A β-lactamases does not improve more than 90 times the deacylation efficiency ? In the case of PBP2x, the aromatic ring of Phe_{450} hampers the entry of a water molecule in the active site. Introduction of an aspartic acid in place of Phe_{450} increases the deacylation rate by 110 times [16]. Here, the catalytic site was less amenable to hydrolysis since no water molecule was pre-existent to the introduction of an aspartate in the catalytic site, and no water molecule was observed in the oxyanion hole [16; 59]. The catalytic site of PBP-A is very similar to that of TEM-1, and the oxyanion hole is well defined with a conserved water molecule hydrogen bonded to Ser_{70} and Asp_{222} main chain nitrogens.

Under acylation, this water molecule is replaced by the carbonyl oxygen of penicillin, and interactions with Ser_{70} and Asp_{222} are maintained. Moreover, penicilloyl group of the acyl-enzyme interacts with other residues of the catalytic site for stabilization, and except for the interaction between Lys_{64} and Glu_{158}, the H bond network is well organized for catalysis. Finally, PBP-A Ω-loop is superimposable to the TEM-1 N- terminal part which was showed to be most important, providing a good orientation of Glu_{158}. Hence, L_{158}E mutant would have been expected to reach higher levels of deacylation. To explain this low efficiency, the catalytic site was detailed and some imperfections were noted such as a slightly longer

distance between the water molecule and the acyl-enzyme carbonyl carbon. The water molecule was suggested not to be optimally positioned for efficient deacylation, and adding a long polar side chain equivalent to class A β-lactamases Asn_{170} might help optimising the position of W2. Asn mutants replacing Asp_{160} and Met_{161}, two positions where Asn was susceptible to point toward the active site, were generated. $M_{161}N$ mutation had little effect on protein activity, probably because the Asn side chain was too far to provide some effect on catalysis. $D_{160}N$ had effect in the double mutant suggesting that the electrostatic environment around Glu_{158} has changed, however in the wrong way for deacylation.

Finally, the rearrangement of two other regions of the protein was suggested by the structural analysis and the effect of synergistic mutations was assessed by constructing the quadruple mutants $E_{96}G$-$L_{158}E$-$M_{161}N$-$D_{222}AG$ and $E_{96}V$-$L_{158}E$-$M_{161}N$-$D_{222}AG$. The effect was a return of activity of $L_{158}E$ mutant to wild-type rate, indicating that the basic hydrolytic mechanism of PBP-A was not disturbed by these mutations. Although the expected increase in deacylation was not observed, it is interesting that modulating the left side of PBP-A catalytic cavity does not influence its activity, providing information on the protein high stability.

Thus, there is no clear explanation why this protein is not a β-lactamase. It may come from an unoptimised electrostatic environment or hydrophobicity of the cavity that do not provide good configuration for the important proton shuttles involved in the reaction mechanism. Modelisation studies carried on penicillin acylation in TEM-1 show that both Glu_{166} and Lys_{73} are involved, and a proton shuttle between the residues is occuring [14]. The equivalent interaction is missing between Glu_{158} and Lys_{64} and the impaired proton shuttle may have a great impact on the enzyme activity.

Phylogenetic link

Biochemical characterisation suggested that PBP-A is a DD-carboxypeptidase and sequence alignments indicated that it belongs to a new class of PBPs. In this study, the tridimensionnal structure of the protein was solved, and observations are in agreement with the previous hypotheses. On the other hand, structural superimpositions are better with class A β-lactamases. Comparison to other PBPs show less structural conservation, and though hydrophobic elements typical of PBPs seem to be present, they are not in the same place, strengthening the previous hypothesis that PBP-A like proteins are a new family of PBPs.

PBP-A is the first protein of which Ω-loop like structure fits so well with the N-terminal part of β-lactamases Ω-loop. When comparing to β-lactamases, it was shown to provide many similarities with TEM-1, but it was also sharing some similarities with the very special Per-1

β-lactamase. All these observations strengthen the theory of an ancestral PBP that would be an evolutionary intermediate between PBP-A and class A β-lactamases. Our results are a good illustration of how proteins are not as versatile as they look and that enzyme efficiencies are sometimes mysteries for rational studies.

Chemicals and antibiotics. Penicillin G was purchased from sigma. Oligonucleotides were from Eurogentec, with "up" for sense primer and "rp" for reverse primer:

D_{160}N-up 5'CCGCTGCCGAATATGAAGGGC3'

D_{160}N-rp 5'GCCCTTCATATTCGGCAGCGG3'

L_{158}E-D_{160}N-up 5'CCGGAGCCGAATATGAAGGGC3'

L_{158}E-D_{160}N-rp 5'GCCCTTCATATTCGGCTCCGG3

L_{158}E-M_{161}N-up 5'CCGCTGCCGGATAACAAGGGTACCAACACCACCAG3'

L_{158}E-M_{161}N-rp 5'CTGGTGGTGTTGGTAGGGTTGTTATCCGGCAGCGG3'

E_{96}G 5'CTCATAGCCCCCGGGGCTGGCACATTGC3'

E_{96}V 5'CTCATAGCCCCCGTGGCTGGCACATTGC3'

D_{222}AG 5'GCCCACAAAACGGGTGCAGGTATTGGTATTGTGGTCGG3'

Construction and purification of PBP-A and mutants – Cloning of wild-type PBP-A and PBP-A-L_{158}E mutant have been reported. To produce PBP-A-M_{161}N and PBP-A-D_{160}N, the double mutants L_{158}E-M_{161}N and L_{158}E-D_{160}N, and the quadruple mutants E_{96}G-L_{158}E-M_{161}N-D_{222}AG and E_{96}V-L_{158}E-M_{161}N-D_{222}AG, PBP-A or PBP-A-L_{158}E constructs were used. In these constructs PBP-A truncated for the 92 first amino acids was fused to a myc-tag and a His-tag, allowing cytoplasmic expression of the protein and further IMAC purification. The single and double mutant enzymes were prepared using the Quick Change® Site-Directed Mutagenesis kit (Stratagene), suitable construct (PBP-A or PBP-A-L_{158}E), and L_{158}E-M_{161}N-up and L_{158}E-M_{161}N-rp primers, D_{160}N-up and D_{160}N-rp primers or L_{158}E-D_{160}N-up and L_{158}E-D_{160}N-rp primers. The quadruple mutant enzymes were prepared using the Quick Change® Multi-Site-Directed Mutagenesis kit (Stratagene), PBP-A-L_{158}E construct, and M_{161}N-up, E_{96}G/V, M_{161}N and D_{222}AG primers. Mutations were confirmed by sequencing of both strands on the entire gene. PBP-A and mutants were expressed in *E. coli* and purified as described previously (page 133).

Kinetic measurements. Kinetic parameters for benzylpenicillin hydrolysis were determined as follows: k_{cat} was determined by following Penicillin G hydrolysis at 232 nm. k_2/Ks acylation constants were determined from time courses of formation of acyl-enzyme complexes of [^{14}C]-Penicillin G with the proteins, and the deacylation rate constant was derived from the first order decay rates of the [^{14}C]penicilloyl-PBP complex as previously described (page

107). Hydrolysis of thiolester substrates S2a, S2c, and S2d was performed at 37°C and monitored at 250 nm, with $\Delta\varepsilon$ = 2200 $M^{-1}.cm^{-1}$ [55; 60; 61]. Kinetic parameters were determined by fitting differential equations on the raw data and using Excel solver.

Crystallization. The concentrations of the wild type protein and of the $L_{158}E$ mutant used for crystallization experiments were about 7 $mg.mL^{-1}$, with Hepes 20 mM pH 7.4 and EDTA 1 mM. The hanging drop vapour diffusion method was used at 18°C. For the wild type protein, the reservoir was filled with 500 µL of an aqueous solution containing Hepes 0.1 M pH 7.5, proline 0.2 M, PEG-3350 10% (w/v) and NaN_3 0.02% (w/v). The crystals used for acylation were identical, but grown from a different reservoir composition: Hepes 0.1M pH 7.5, ammonium acetate 0.2M, PEG 3350 25% (w/v) and NaN_3 0.02% (w/v). For the $L_{158}E$ mutant, the reservoir solution contained Hepes 0.1 M pH 6.9, ammonium acetate 0.2 M, PEG 3350 15% (w/v) and NaN_3 0.02% (w/v). The hanging drop was composed of 1 µL of protein solution mixed with 1 µL of the reservoir. In both cases, crystals with typical dimensions 0.15 x 0.15 x 0.10 mm^3 appeared after a few days.

Data collection. Before data collection, the crystals were soaked for a few seconds in a cryo-solution similar to the mother liquor but containing 5% glycerol as cryo-protectant, and flash cooled at 100 K. In order to study the acylated form of the enzymes, some crystals were also soaked for one minute in such a solution containing penicillin G. All the diffraction data were collected using synchrotron radiation at EMBL c/o Desy (Hamburg – Germany) on beam lines BW7A or BW7B. Some statistics of data collection and processing are presented in Table 1.

Data processing, structure solution and refinement. The diffraction images of the different crystals were processed and merged with the XDS program package [62]. The crystals are orthorhombic, space group $P2_12_12_1$ and are isomorphous, with four molecules in the asymmetric unit. The structure solution was attempted by molecular replacement using the data of the non-acylated wild type enzyme. A self-rotation function computed at 3.5 Å resolution indicated the presence of a non crystallographic 4-fold axis along c, and as a consequence two additional 2-fold axes in the a,b plane, bisecting the two crystallographic axes. A native Patterson function showed a peak of pure translation at (0.00, 0.36, 0.50) occurring because the non crystallographic 4-fold axis has the same orientation as a crystallographic 2-fold screw axis. After many unsuccessful attempts, the structure was

	PBP	**PBP-pen**	**$L_{158}E$**	**$L_{158}E$-pen**
Data collection				
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions				
a (Å)	87.79	87.69	86.61	86.58
b (Å)	91.90	91.87	91.46	91.53
c (Å)	147.27	147.44	145.59	145.71
Synchrotron source	EMBL c/o DESY Hamburg			
beamline	BW7B/BW7A	BW7A	BW7B	BW7B
Detector type	MAR345/ MARCCD	MARCCD	MAR345	MAR345
Wavelength (Å)	0.8414/0.8874	1.220	0.8430	0.8430
Resolution (Å)				
Overall (ov)	39.2 – 1.9	24 – 1.9	25 – 1.5	28 – 1.7
Highest shell (hs)	1.95 - 1.90	1.95 - 1.90	1.54 - 1.50	1.74 – 1.70
Measured reflexions	483112	304600	613414	414638
Unique reflexions	94191	91292	181250	126734
Completeness (%) ov/hs	99.8/99.7	96.7/88.2	98.1/99.2	99.3/100.0
R_{sym}	0.079	0.053	0.036	0.040
$I/\sigma(I)$ ov/hs	13.9/3.8	17.3/2.6	17.6/2.3	16.8/2.6
Refinement				
R factor (Rfree) ov	0.174 (0.213)	0.190 (0.245)	0.179 (0.213)	0.180 (0.218)
hs	0.220 (0.298)	0.273 (0.346)	0.245 (0.273)	0.242 (0.285)
Estimated overall coordinate error (Å) based on				
Rfree	0.132	0.156	0.078	0.104
maximum likelihood	0.097	0.118	0.057	0.079
Limits (residue numbers) of observed electron density				
chain A	10 – 274	10 – 274	11 – 274	11 – 274
chain B	11 – 275	10 – 274	10 – 275	10 – 274
chain C	6 – 275	11 – 274	10 – 274	11 – 274
chain D	1 - 274	2 - 274	2 - 276	2 - 275
r.m.s. Deviation from ideality				
Bonds (Å)	0.015	0.016	0.017	0.015
Angles (°)	1.51	1.58	1.646	1.555
Number of solvent molecules	870	823	1527	905
of gycerol molecules			3	1
Mean B values (Å)				
Main chain	26.8	25.5	23.4	28.0
Side chain	29.1	27.7	26.2	31.0
Solvent	35.4	35.6	40.5	38.5

Table 16. Statistics of data collection and refinement

solved using the program PHASER [63] which applies the maximum likelihood to molecular replacement. A set of pre-aligned class A β-lactamases was used as a model. Their Protein Data Bank codes and the percentage of homology with *Thermosynechcoccus elongates* PBP are as follows: 1mfo – 32%, 1e25 – 31%, 1bsg – 26%,1erm – 30%, 1i2s – 29%. PHASER succeeded to orient and locate in the asymmetric unit three molecules consistent with the non crystallographic 4-fold axis. A fourth molecule was discovered by the programme FFFEAR [64] of the CCP4 suite (1994) [65]. Electron density maps were interpreted using O [66] and Coot [67]. After applying the NCS-phased refinement procedure available in the CCP4 suite, the refinement was pursued with REFMAC5 [68]. Since all the structures are isomorphous, it was possible to start immediately the refinement of the other structures, after some minor adjustments. Solvent molecules were introduced by ARP-wARP [69]. Ordered glycerol molecules were observed in PBP-A-L_{158}E and PBP-A-L_{158}E-Pen. During the final steps, the hydrogen atoms were incorporated in riding positions and the mean-square displacements of rigid bodies were refined, each polypeptide chain being defined as a different TLS group. At the end of the refinement, the TLS contribution was included in anisotropic temperature factors. Some refinement statistics are given in Table 16.

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