Organic & Biomolecular Chemistry

www.rsc.org/obc

Volume 10 | Number 17 | 7 May 2012 | Pages 3345–3564

ISSN 1477-0520

RSC Publishing

FULL PAPER

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1477-0520(2012)10:17;1-D

Organic & Biomolecular Chemistry

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Cite this: *Org. Biomol. Chem.*, 2012, **10**, 3388

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PAPER

Promiscuous enantioselective (−)-γ-lactamase activity in the *Pseudomonas fluorescens* esterase I

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Received 9th November 2011, Accepted 9th January 2012
DOI: 10.1039/c2ob06887g

A promiscuous but very enantioselective (−)-γ-lactamase activity in the kinetic resolution of the Vince lactam (2-azabicyclo[2.2.1]hept-5-en-3-one) was detected in the *Pseudomonas fluorescens* esterase I (PFEI). The lactamase activity was increased 200-fold by the introduction of a point mutation and resulted as enantioselective as the *Microbacterium* sp. enzyme used industrially in this resolution. The structural and mechanistic determinants for the catalytic promiscuity and enantioselectivity were identified by molecular modeling, setting a ground stone to engineer further amidase-related activities from this esterase.

Introduction

The stereoisomers of the industrially relevant Vince lactam are precursors to pharmaceutically active compounds. They were initially used for the synthesis of carbocyclic nucleosides intended for antiviral (*e.g.* anti-HIV) therapies,[1,2] but the scope of applications has widened significantly in the past years[3] and due to the growing relevance of such syntheses, efficient processes for the kinetic resolution of this substrate are needed.

To that extent, in 1993, the company Chirotech (now Dr Reddy's, Slough, UK) discovered lactamase activity in *Aureobacterium* sp. (now classified as *Microbacterium*). Line *et al.* isolated and overexpressed the corresponding enzyme, purified it and solved its three-dimensional structure.[4] The enzyme exhibits a typical α/β-hydrolase fold and high homology with cofactor free haloperoxidases; indeed, bromoperoxidase activity was observed at pH 4. The active site is comprised of residues Ser98–Asp230–His258; the backbone nitrogen atoms of Tyr32 and Met99 form the oxyanion hole. The enzyme's natural function has not yet been determined and the name was assigned due to the reaction it catalyzes. Other enzymes described to act on the racemic Vince lactam have been identified in *Pseudomonas fluorescens*,[5] *Burkholderia cepacia*,[6] *Comamonas acidovorans*[2] and *Sulfolobus sulfataricus*[7] which show (+)-γ-lactamase activity, while the described enzyme isolated from *Microbacterium*,[4] *and the Pseudomonas fluorescens* esterase I (PFEI) described in this manuscript exhibit (−)-γ-lactamase activity.

Promiscuity has been defined as the ability of enzymes to catalyze more than one reaction per active site. Whether the promiscuous reaction is natural or induced, involves different reaction conditions, a new substrate or a new mechanism has given rise to different types of enzyme promiscuity.[8,9] From an industrial or applied angle, promiscuous enzymes provide a good starting point for protein engineering. One particular case of catalytic promiscuity that has been continuously addressed over the past 20 years is the promiscuous amidase activity in esterases/lipases, however with scattered results and a small number of theories to explain the phenomenon.[10–12] Certain members of both types of enzymes share the same catalytic triad (Ser–His–Asp) and a similar reaction mechanism and yet, there are very few examples of esterases/lipases hydrolyzing amides,[12–15] while amidases and proteases (such as subtilisin) catalyze ester hydrolysis. Although the ester and amide function may seem similar at first sight, the resonant amide bond is more stable than the ester bond and thus, it is logical to assume that the mechanism, although very similar, cannot be completely identical. To support such a hypothesis, extra hydrogen bonding of the transition state either with itself or with the enzyme has been recently proposed as a determinant of amidase activity that is present in amidases/proteases but missing in esterases.[12]

In this paper we describe the promiscuous and enantioselective (−)-γ-lactamase activity on the Vince lactam (2-azabicyclo [2.2.1]hept-5-en-3-one, Scheme 1) of a wild-type esterase from *Pseudomonas fluorescens* with none of the structural determinants for amidase activity described above and how the introduction of such determinants improved reaction turnover without affecting enantioselectivity.

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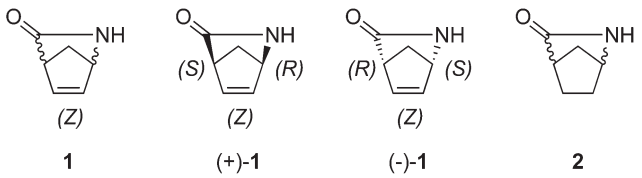

Scheme 1 **1**, 2-Azabicyclo[2.2.1]hept-5-en-3-one (Vince Lactam); **2**, 2-azabicyclo[2.2.1]heptan-3-one.

Results and discussion

Structural alignment of PFEI

A search for structural homologues of PFEI (PDB: 1VA4) having amidase activity was conducted using the protein structure comparison service Fold at European Bioinformatics Institute, authored by E. Krissinel and K. Henrick[16] (http://www.ebi.ac.uk/msd-srv/ssm). The highest-ranking amidase was the (−)-γ-lactamase from *Microbacterium* sp. (PDB: 1HKH) that aligned with 254 residues to PFEI, of which 39% were identical. The alignment was further supported by a RMSD of 0.98 Å, which allows a close superposition of both the highly conserved α/β-hydrolase fold domain and the more variable cap domain.

Substrate scope of PFEI as an amidase

The substrate scope of PFEI acting as amidase was investigated, by setting up reactions with pure enzyme and the substrates and conditions detailed in the Experimental section. In addition, the scope of the Leu29Pro mutant of PFEI was also investigated, as it has been described to have lactonase activity with cyclic substrates that partially resemble the Vince lactam.[17]

Both enantiomers of the Vince lactam could be hydrolyzed by either the wild-type or the Leu29Pro mutant. Furthermore, whereas PFEI exhibited lactamase activity, in turn, the *Microbacterium* sp. γ-lactamase (MGL) did not exhibit esterase activity with standard PFEI substrates, such as *p*-nitrophenyl acetate or butyrate. However, the MGL has been reported to hydrolyze ester-like bonds, as in cyclic ethylene carbonate.[4]

Lactams bearing resemblance to **1**, such as **2**, δ-valerolactam, 2-pyrrolidinone and other amides detailed in the Experimental section could not be hydrolyzed by MGL, PFEI or Leu29Pro.

Characterization of the lactamase activity of PFEI

The promiscuous lactamase activity of PFEI was characterized in terms of activity and enantioselectivity. First of all, the pH optimum was determined, as the native esterase activity of PFEI has been reported to be active over a broad range of pH values.

As detailed in Fig. 1, a slightly larger conversion was observed for PFEI between pH 6 and 10 in comparison with the MGL, dropping abruptly after pH 10.

Due to the differential behavior of MGL and PFEI with pH, enantioselectivity and total activity were calculated both at pH 7.5 and pH 9. As shown in Table 1, the three enzymes tested were enantioselective with preference towards **(−)-1** in the kinetic resolution of the racemic mixture at both pH values.

Additional insight on the selectivity of the reaction was obtained by steady-state kinetics (Table 2). Hydrolysis of the favored enantiomer exhibited in all cases a faster turnover and lower K_m. The introduction of the Leu29Pro mutation, did slightly alter K_m, but drastically increased turnover, regardless of the enantiomer hydrolyzed.

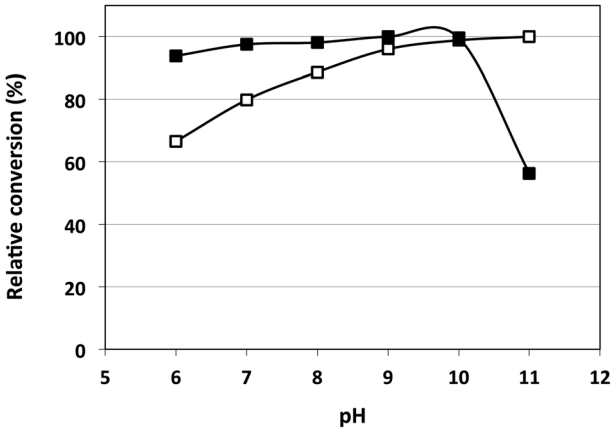

Fig. 1 pH dependency of the hydrolysis of (rac)-**1** by MGL (empty squares) and wild-type PFEI (full squares). Values at 100% correspond to 1.4 U (PFE) and 7.7 mU (MGL) and 13.2 μg protein in both cases.

Table 1 Enantioselectivity of PFEI variants and *Microbacterium* γ-lactamase in the kinetic resolution of (rac)-**1**

	E^a	
Protein	pH 7.5	pH 9
MGL	>100 (43%)	>100 (46%)
PFEI	>100 (17%)	>100 (17%)
L29P	>100 (43%)	>100 (48%)

[a] *E* values were calculated at the conversion indicated in parentheses.

Table 2 Kinetic parameters for the hydrolysis of each enantiomer of **1** catalyzed by wild-type PFEI or its Leu29Pro variant

	(−)-**1**			(+)-**1**		
Protein	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
PFEI	13.5	9.9×10^{-2}	7.3×10^{-3}	21.8	5.3×10^{-4}	2.4×10^{-5}
Leu29Pro	6.4	12.1	1.9	33.1	0.16	4.8×10^{-3}

Molecular modeling of the lactamase activity

In order to gain more insight on the causes of the regio- and enantioselectivity of the promiscuous reaction, and the effects caused by the introduction of the Leu29Pro mutation, both enantiomers of the Vince lactam were docked into the structure of the wild-type PFEI and the Leu29Pro variant. Fig. 2 shows that both enantiomers bind in the active site, but in the case of the (−)-enantiomer, the oxygen atom in the tetrahedral intermediate is somewhat closer to the oxyanion hole, which may contribute to the higher efficiency in the hydrolysis of the favored enantiomer.

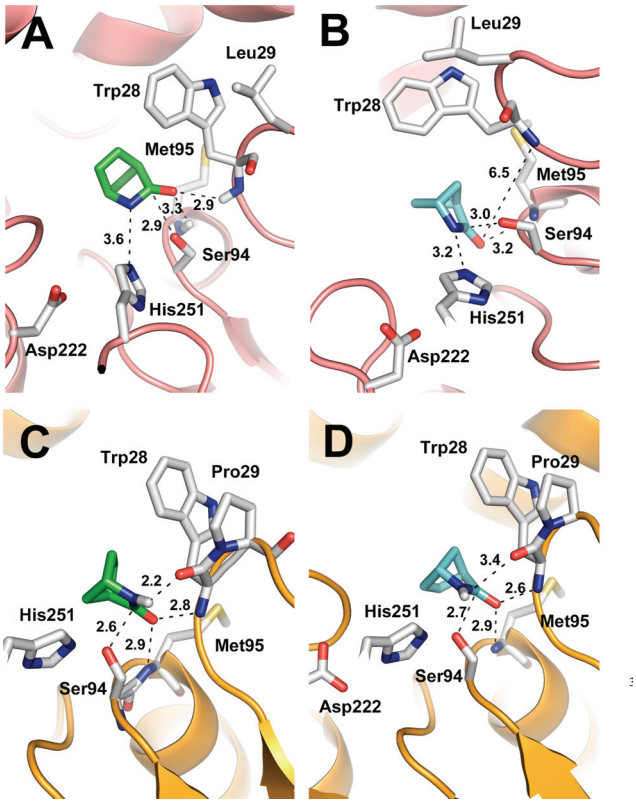

Fig. 2 Docking solutions of (−)-**1** (A) and (+)-**1** (B) in wild-type PFEI and (−)-**1** (C) and (+)-**1** (D) in the L29P variant. The amide nitrogen has been made explicit and part of the cartoon has been hidden for clarity. Distances are indicated in Angstroms.

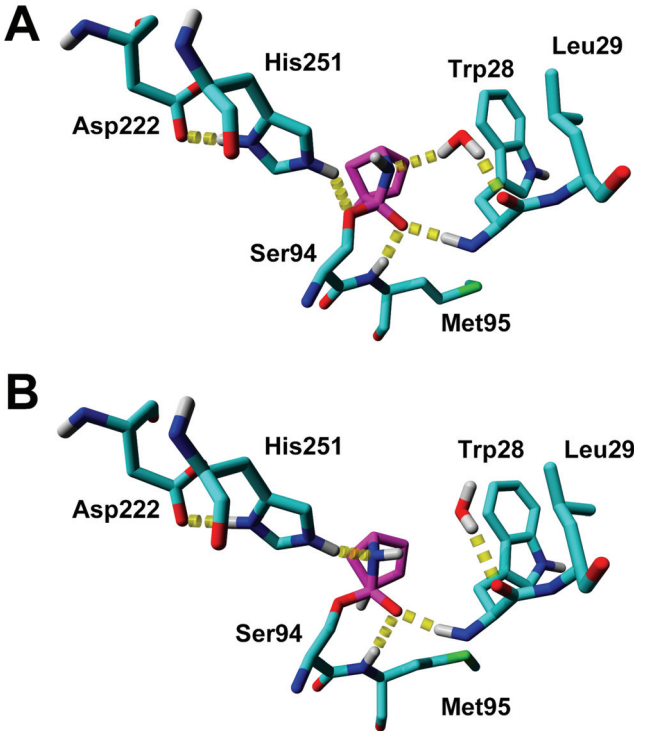

Fig. 3 Snapshots of the MD simulations of wild-type PFEI with the tetrahedral intermediate originated by attack on the *si* face of (−)-**1** (in magenta). A) before and B) after nitrogen inversion. Simulations were carried out as detailed in the Experimental section. The hydrogen bonding network necessary for catalysis is shown only with the strictly necessary H atoms for clarity.

However, affinity itself does not fully explain the large increase in efficiency originated by the Leu29Pro mutation. For that reason, to further explore the enzyme mechanism, molecular dynamics simulations were carried out on the modeled tetrahedral intermediates of (−)-**1** in wild-type PFEI and variant Leu29Pro using the Yamber3 force field (Fig. 3). As previously described by Syren and Hult, a pyramidal inversion of the nitrogen atom is a requisite for amide hydrolysis.[12] Such inversion was described to be favored by a hydrogen bond acceptor that is only found in amidases (either on the enzyme or on the substrate itself) and not in esterases. In the case of Leu29Pro, the pyramidal inversion of the amide nitrogen occurred spontaneously on average 500 times faster than in the wild-type PFEI (on average 1.2 *vs.* 200 ps), with the leaving amine hydrogen atom directed towards Trp29, and the lone pair oriented towards the protonated catalytic His251. Furthermore, the N atom inversion was favored by the Trp28 carbonyl group, which has been described to be displaced towards the substrate, as a result of the introduction of the proline residue in position 29.[18]

Nitrogen inversion in wild-type PFEI was not mediated by any atom on the protein or on the substrate and it took place at a slower rate. In fact, inversion could be hindered by an adequately located water molecule in the alcohol pocket of the active site, which was observed during the simulation acting as a hydrogen-bond donor to the lone pair of electrons on the nitrogen atom (Fig. 3). This water molecule is absent in the case of Leu29Pro owing to the space occupied by the proline side chain. The introduction of a proline residue has been shown to reduce the alcohol binding site in comparison to the wild-type PFEI.[17] Since the requirement for lactamase activity seems to be the distance between the carbonyl group in Trp28 and the scissile nitrogen, it may be hypothesized that replacements in the adjacent position 29 may yield different distances and thus broaden the narrow substrate scope observed. Furthermore, carbonyl groups may be introduced elsewhere in the active site as long as they are congruent with a particular amide substrate, in order to attempt creating new amidase activity.

Experimental

All reagents used were of analytical grade, purchased from Sigma-Aldrich (St. Louis, MO, USA). Solvents were HPLC grade (Merck). Restriction enzymes, polymerases and primers were obtained from Fermentas (Vilnius, Lithuania), EurX (Gdansk, Poland) and Sigma-Aldrich (St. Louis, MO, USA) respectively. Sequence analysis was carried out at Secugen, S.A. (Madrid, Spain).

Cloning of *Microbacterium* γ-lactamase

A construct harboring the gene coding for the *Microbacterium* γ-lactamase was kindly provided by Dr Reddy's (Slough, UK) and amplified by PCR to introduce NdeI and BamHI restriction sites at the 5′ and 3′ ends, respectively and to remove the stop

codon. The PCR product was digested with NdeI and BamHI and ligated with the vector pGASTON, predigested with the same enzymes, allowing the translation of a C-terminal His-tagged product.

Expression and purification of enzymes

The ampicillin-selectable vector pGASTON[19] with a rhamnose-inducible promoter was used for the expression of all enzymes in *E. coli* DH5α at 22 °C. Induction was performed by addition of L-rhamnose to a final concentration of 0.2% (v/v) at OD_{600} 0.5. Twenty hours later, cells were harvested by centrifugation (15 min, 4 °C, 3939 × g), the pellet was washed with phosphate buffer (50 mM, pH 7.5), resuspended in 10 ml of the same buffer and disrupted by sonication on ice (10 min, 50% pulse, 50% power). After another centrifugation step (15 min, 4 °C, 3939 × g), the supernatant containing the soluble protein fraction was subjected to IMAC chromatography using a Talon Cell-through resin (Clontech, Saint-Germain-en-Laye, France), diafiltrated and concentrated with Amicon UltraCel 10 kDa centrifugal devices (Millipore, Carrigtwohill, Ireland) and finally supplemented with glycerol to a final concentration of 50% (v/v) to be stored at −20 °C until further use. Soluble expression was confirmed by SDS PAGE (data not shown). Typical enzyme yields were in the range of 75–100 mg protein l^{-1} culture.

Qualitative determination of substrate scope

Samples of reactions with 10 mM Vince lactam, 2-pyrrolidinone, 5-methyl-2-pyrrolidinone or δ-valerolactam in 50 mM phosphate buffer pH 7 were applied to a silica-coated TLC plate (Merck, Darmstadt, Germany) and placed in a TLC cuvette containing *n*-butanol : acetic acid : water 9 : 2 : 1 as mobile phase. Following separation, the plate was sprayed with ninhydrin solution (1.5% ninhydrin in *n*-butanol, 3% acetic acid) and developed by heating. Suitable product standards were generated by hydrolyzing the substrate with 1 M HCl and subsequent neutralization with sodium carbonate.

Hydrolysis of penicillins G, V and dihydro F was carried out by incubation of 20 mM of each substrate in 10 mM sodium phosphate buffer pH 7.5 with 100 μg purified protein for up to 48 h at 37 °C. Samples were withdrawn at regular intervals, and filtered through 10 kDa filters to remove the protein. The amount of β-lactam produced was quantified by derivatization with fluorescamine.[20] Suitable autohydrolysis controls without enzyme and controls of interference in the determination assay with enzyme but without substrate were carried out. All measurements were carried out in duplicate.

Hydrolysis of NIPAB (6-nitro-3-phenylacetamide benzoic acid) was carried out by incubating 20 mM NIPAB in 10 mM sodium phosphate buffer pH 7.5 with 100 μg purified protein for up to 48 h at 37 °C. Suitable autohydrolysis controls without enzyme and controls of interference in the determination assay with enzyme but without substrate were carried out. All measurements were carried out in duplicate.

Hydrolysis of *p*-nitrophenyl butyrate (*p*-NPB) and *p*-nitrophenyl butyramide (*p*-NPBA) was carried out by incubation of the purified enzyme with 1 mM of each substrate in 50 mM phosphate buffer pH 7.5 at 37 °C and determination of A_{410}. Suitable autohydrolysis controls without enzyme were carried out. All measurements were carried out in duplicate.

Steady-state kinetics of lactam hydrolysis

Enzymatic reactions were set up in 0.5 ml 10 mM phosphate buffer pH 7.5 containing 100 mM NaCl, 0.5–20 mM of either enantiomer and 2.5–10 μg pure enzyme. The reactions were incubated at 37 °C and 800 rpm shaking and samples were withdrawn at different time intervals. The reaction was stopped by shock freezing at −72 °C. The amount of amino acid released was quantified by derivatization with fluorescamine at pH 9.0.[20] Suitable autohydrolysis controls without enzyme and controls of interference in the determination assay with enzyme but without substrate were carried out. All measurements were carried out in quadruplicate. Kinetic parameters were calculated by non-linear regression to the Michaelis–Menten equation using GraphPad Prism. R^2 values were larger than 0.99 in all cases.

Determination of enantioselectivity by HPLC

Enzymatic reactions were set up in 1 ml of either 10 mM phosphate buffer pH 7.5 of containing 8 mM substrate, 50 μg ml^{-1} of *p*-aminobenzoic acid as internal standard for quantitation and an adequate amount of pure enzyme. The reactions were incubated at 37 °C and 800 rpm shaking and samples were withdrawn at different time intervals and subjected to enzyme inactivation by incubation at 95 °C for 20 min.

The stereoisomers of the Vince lactam were separated on a Chiralcel OD-RH column (150 × 4.6 mm ID, cellulose tris (3,5-dimethylphenylcarbamate) coated on 5 μm silica-gel, Chiral Technologies Europe, France) with a mobile phase of 95% ultrapure water (MQ) containing 0.1% trifluoroacetic acid (TFA) and 5% acetonitrile (ACN) at a flow of 0.4 ml min^{-1}, with UV monitoring at 225 nm, in an HPLC system (ELITE LaChrom, VWR). As the products could not be resolved, *p*-aminobenzoic acid was added to the samples as an internal standard and the ratio of peak areas of lactam and standard was used to calculate the conversion. The retention times were: (−)-Vince Lactam, 8.3 min; (+)-Vince Lactam, 9.2 min; internal standards, 18.9 min. Average RSD between repeated experiments was 10%.

The stereoisomers of the saturated lactam **2** were separated on a Chiralcel OD-RH column as well (150 × 4.6 mm ID, cellulose tris (3,5-dimethylphenylcarbamate) coated on 5 μm silica-gel, Chiral Technologies Europe, France) with a mobile phase of 100% MQ containing 0.1% TFA for the first 18 min, ramping to 95% MQ, 0.1% TFA:5% ACN up to 30 min, and ramping again to 100% MQ with 0.1% TFA for 5 additional min, with UV monitoring at 225 nm, in a HPLC system (ELITE LaChrom, VWR). The retention times for the substrates were 14.6 and 15.7 respectively and 36.5 for the internal standard.

Enantioselectivity was calculated as the *E* value using the Chen equation[21] with ee_S and the conversion as input.

Docking studies

The three-dimensional structure of (+)**-1** was obtained from the Cambridge Structural Database (http://www.ccdc.cam.ac.uk/) with reference code Ruvzen. The three-dimensional structures of (−)**-1** was modeled using the Dundee PRODRG2 Server.[22]

Stereochemistry of ligands was checked with Mercury program.[23] Molecular docking of both stereoisomers of the Vince lactam in the wild-type PFEI and the three mutants described above was carried out using GOLD (Genetic Optimization for Ligand Docking) software,[24] that uses the Genetic Algorithm (GA). This method allows a partial flexibility of protein and full flexibility of the ligand. The cavity was defined from to 7 Å around Ser94. For each of the 25 independent GA runs, a maximum number of 100 000 GA operations were performed on a set of five groups with a population size of 100 individuals. Default cutoff values of 2.5 Å (dH–X) for hydrogen bonds and 4.0 Å for van der Waals distance were employed. When the top three solutions attained RMSD values within 1.5 Å, GA docking was terminated. The RMSD values for the docking calculations are based on the RMSD matrix of the ranked solutions. We observed that the bestranked solutions were always among the first 10 GA runs, and the conformation of molecules based on the best fitness score was further analyzed. Images were created with Pymol 0.99 (Delano Scientific).

All positions given throughout the paper are numbered according to the PFEI structure (PDB ID: 1VA4), which is missing the initial methionine.

Molecular dynamics simulations

Tetrahedral intermediates were prepared in YASARA Structure by covalently bonding the docked (−)-lactam to the catalytic serine of wild-type PFEI and the L29P variant (PDB ID: 3HI4) through the *si* face of the carbonyl group,[4] and minimizing the resulting molecule using the Yamber3 force field with a cutoff for van der Waals forces of 7.86 Å.[25] Molecular dynamics simulations (MD) were run on the minimized tetrahedral intermediate for 1.5 ns at 298 K using the same force field, and setting the lone electron pair on the lactam N antiperiplanar to the attacking oxygen atom. During the course of the MD simulation, snapshots were generated at suitable time intervals, that were subsequently analyzed for hydrogen bond formation between the substrate and the catalytic His, correct placement and interaction of the intermediate charged oxygen with the oxyanion hole formed by N atoms belonging to the main chain of Trp28 and Met95, as well as for the presence of water molecules in the active site cavity adjacent to the substrate, and their hydrogen bonds. Simulations were repeated 3 times for consistency. Images were created with Yasara Structure (Yasara Biosciences).

Conclusions

A promiscuous, enantioselective (−)-lactamase activity was found in an esterase and improved by the introduction of the Leu29Pro mutation to reach same magnitude than the one observed in an industrially relevant enzyme. This mutation increased the turnover in comparison to the wild type enzyme, and its effect was successfully explained through docking and molecular dynamics simulations. This leads us to think that further replacements may be introduced in addition to Leu29Pro in order to improve lactam binding and hydrolysis and eventually create a specifically tailored amidase activity in this esterase.

Acknowledgements

A. H. and J. B. acknowledge funding from the Spanish Ministry of Science (grants BIO2008-01481, RyC2006-02441), an institutional grant from Fundación Ramón Areces to CBMSO and a generous allocation of computing time at the Scientific Computation Center of the UAM (CCC-UAM). A. H. and J. B. are thankful to M. J. de Soto and E. Sánchez for their assistance. J. A. H. acknowledges funding from the Spanish Ministry of Science (grant BFU 2008-01711). N. S.-M. is a fellow of the Fundayacucho Foundation (Venezuela). A. S., M. S. and U. T. B. acknowledge funding from the Deutsche Bundesstiftung Umwelt (Osnabrück, Germany) grant no. AZ13159. We thank Dr Richard Lloyd (Dr Reddy's, Cambridge, UK) for providing the gene coding for the *Microbacterium* sp. γ-lactamase, Prof. R. Kazlauskas for the gene encoding the L29P variant of PFEI, and Dr J. A. Gámez for fruitful discussion.

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