Changing the substrate specificity of penicillin G acylase from *Kluyvera citrophila* through selective pressure

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Escherichia coli (*mutT*, *mutD*, *Leu*[−]) cells transformed with plasmid pYKD59 harbouring the *pac* gene encoding penicillin acylase (PA) from *Kluyvera citrophila* ATCC 21285 were exposed to environmental conditions that made expression of this enzyme essential for growth. Under these conditions, spontaneous mutants were isolated that used adipyl-L-leucine as the sole source of L-leucine. DNA sequencing of the mutant *pac* genes identified a transversion mutation of thymine to guanine at position 1163. This mutation was located in the β -subunit of the enzyme and resulted in conversion of Phe-360 to valine. The assignment of this mutation to the shift in substrate specificity was further confirmed by site-directed mutagenesis. Secondary-structure prediction of the region surrounding Phe-360 suggests that this mutation should not produce any significant structural change. The purified mutant acylase was able to hydrolyse adipyl-, glutaryl-, valeryl-, caproyl-, heptanoyl- and phenoxyacetyl-L-leucine at pH 5 with greater efficiency than the wild-type enzyme. However, the mutant enzyme was not able to hydrolyse glutaryl-7-aminocephalosporanic acid and had lost

90% and 50% of activity on penicillin G and phenylacetyl-L-leucine respectively. Nevertheless, mutant PA retained its original activity on 6-nitro-3-phenylacetamidobenzoate and *p*-nitro-phenylphenylacetate, suggesting that the binding specificity of PA by the acyl and amine moieties of the substrate are not independent phenomena. The small differences observed between the c.d. spectra of the mutant enzyme recorded at pH 5 and 8 suggest the existence of different conformational states at the two pH values, but these differences were indistinguishable from those observed in the native enzyme and cannot be correlated with the shift in substrate specificity. Our results demonstrate that it is possible to change the specificity of PA by laboratory evolution and use it to identify the amino acids involved in substrate recognition. However, the synchronous participation of the α - and β -subunits in the complex induced-fit-like mechanism of acylases suggests that, to obtain new enzymes for industrial application, the selection pressure should be specifically designed for the compound of interest.

INTRODUCTION

Penicillin acylase (penicillin amidase; EC 3.5.1.11; PA) is used industrially to hydrolyse penicillin G to phenylacetic acid and 6-aminopenicillanic acid (6-APA), the major intermediate in the production of semisynthetic penicillins [1]. Although this enzyme is produced by a wide variety of bacteria and fungi, its physiological role remains unknown. *Kluyvera citrophila* ATCC 21285 is among the micro-organisms that produce PA, and the *pac* gene encoding this enzyme has been cloned, sequenced and over-expressed in *Escherichia coli* [2,3]. This has enabled its manipulation to produce mutant proteins [4–6]. PA from *K. citrophila* is homologous with that from *E. coli* ATCC 11105 which contains two different subunits ($\alpha\beta$) derived from a single precursor protein, both of which are required for activity [3].

Although the substrate specificity [7] and kinetic mechanism [8] of PA have been extensively investigated, the molecular basis of catalysis is not known. Until its three-dimensional structure is determined [9], systematic alteration of the sequence by site-directed mutagenesis can enable study of the involvement of particular amino acids in the structure and activity of the enzyme. As these modifications are often silent or deleterious, the use of selective pressure to alter enzyme characteristics offers the advantage that only catalysts with great effectiveness are obtained and prior knowledge of enzyme structure is not needed [10]. Modification of the substrate specificity of PA might expand its

use not only in the semisynthesis of new β -lactam antibiotics but also in other synthetic and hydrolytic reactions. A variety of chemical and genetic manipulations including selective pressure [4–6,11–19] have been successfully used to direct PA evolution altering its specificity, but the amino acids involved in these changes have not been extensively investigated [20].

The aims of the present work were to study the feasibility of obtaining by selective pressure a PA able to hydrolyse new substrates and to determine the amino acids involved in the shift in substrate specificity. Selection of PA mutants with altered substrate specificity was based on the procedure developed to clone *pac* genes [2].

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli strains ES1580 (*mutT1*, *thr-1*, Δ [*gpt-proA*]62, *hisG4*, *argE3*, *thi-1*) and ES1578 (*mutD5*, *thr-1*, *leuB6*, *hisG4*, *argE3*, *thi-1*) were obtained from Barbara Bachmann (*E. coli* Genetic Stock Center). Other strains used were MC1116 (Δ [*ara-leu*]) and HB101 (*proA2*, *leuB*) [21]. *E. coli* TD21, used as the mutator strain, was obtained as follows. Bacteriophage P1 *kc* was propagated on *E. coli* MC1116 and used to transduce Δ [*ara-leu*] into *E. coli* ES1580. As co-transduction of *thr* and *ara* occurred at a frequency of 2% [13], we selected first Thr⁺ colonies on minimal M9 medium containing leucine, arginine, proline and histidine and then Leu[−]

Abbreviations used: 6-APA, 6-aminopenicillanic acid; 7-ACA, 7-aminocephalosporanic acid; NIPAB, 6-nitro-3-phenylacetamidobenzoic acid; PA, penicillin G acylase; PNPA, 4-nitrophenylphenylacetate.

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colonies by growing the cells on minimal M9 medium [21] containing arginine, proline, histidine and ampicillin. One colony with the proper phenotype (*mutT1*, Δ [*gpt-proA*], Δ [*ara-leu*], *argE3*, *hisG4*) was designated *E. coli* T67. The *MutT* phenotype was checked using the procedure described elsewhere [22]. Bacteriophage P1 *kc* was propagated on *E. coli* ES1578 and used to transduce *mutD5* into *E. coli* T67. As co-transduction of *mutD* and *proA* occurred at a frequency of 7–21% [23,24], we selected first Pro⁺ colonies on minimal M9 medium containing leucine, arginine and histidine and then colonies showing a *mutD* genotype by a previously described procedure [24]. One colony with the proper genotype (*mutT1*, *mutD5*, Δ [*ara-leu*], *argE3*, *hisG4*) was designated *E. coli* TD21 and transformed with plasmid pYKD59 [5]. This plasmid expresses the *pac* gene from *K. citrophila* under the control of its own promoter.

DNA manipulations and site-directed mutagenesis

Plasmids were prepared by the rapid alkaline method [21]. Transformation of competent cells was performed using the CIRb method [21]. Construction of the *pac* mutant by site-directed mutagenesis was carried out using the mutagenesis system from Amersham International by the procedure recommended by the suppliers. The oligonucleotide primer KLUF360V (5'-CGCCGGTGTGGTGATG-3') was synthesized by Isogen Bioscience. Restriction enzymes and T4 DNA ligase were obtained from Boehringer and Pharmacia respectively. DNA sequencing was carried out by the dideoxy-chain-termination method [25] using the kit supplied by Pharmacia.

Mutagenesis and selection of plasmids

Plasmid pYKD59 was mutagenized by passage through *E. coli* TD21. Cells transformed with pYKD59 were treated essentially as described by Forney et al. [13]. Briefly, *E. coli* TD21 (pYKD59) were grown overnight at 37 °C in Luria-Bertani medium [21] with chloramphenicol at 35 µg/ml. This strain has a defective DNA polymerase 3'-5'-exonucleolytic editing activity which results in an increased mutation frequency when the strain is grown in rich medium [23,26]. Cells were starved in 0.9% NaCl for 3 h to deplete the intracellular leucine pool and plated on selective medium. Mutants with an improved ability to hydrolyse adipyl-L-leucine or τ -(D)-aminoadipyl-L-leucine were selected in minimal M9 basal medium containing 50 µg/ml adipyl-L-leucine or τ -(D)-aminoadipyl-L-leucine as the sole source of leucine.

Plasmids from strains with mutant and wild-type PA were used to transform cells of *E. coli* HB101. The phenotype of the mutants was confirmed by plating the transformants on to minimal medium with or without acyl-L-leucine derivatives as the sole source of leucine.

C.d. spectroscopy

Measurements were made using a Jobin Yvon Dichrographe IV. Each spectrum was obtained as the difference between four average scans of the solution and four average scans of the solvent.

Purification of PA

Wild-type and mutant PA were purified as follows. Cells were

grown for 48 h, at 30 °C in 4 litres of Luria-Bertani medium containing chloramphenicol (35 µg/ml), harvested by centrifugation, resuspended in 700 ml of 33 mM Tris/HCl, pH 8.0, containing 33 mM EDTA and 20% sucrose and kept at room temperature for 30 min. Cells harvested by centrifugation were resuspended by shaking in 700 ml of cold water. Cell debris was removed by centrifugation and solid (NH₄)₂SO₄ was added to the supernatant with constant stirring to a concentration of 0.75 M. After centrifugation at 14000 *g* for 20 min, the supernatant was loaded on a phenyl-Sepharose column (32 cm × 2.5 cm) equilibrated with 50 mM Tris/HCl, pH 7.0, containing 0.75 M (NH₄)₂SO₄. Proteins were eluted with a gradient of (NH₄)₂SO₄ (0.5–0.2 M). The fractions containing PA were pooled and dialysed against 10 mM phosphate buffer, pH 6.8, and loaded on a hydroxyapatite HTP (Bio Rad) column (9 cm × 3 cm) equilibrated with the same buffer. Proteins were eluted with a phosphate gradient (10–300 mM). Fractions containing PA were pooled and stored at –20 °C.

Purity was checked by SDS/PAGE by the method of Laemmli [27] using Coomassie Blue as stain. Spectroscopic determination of protein concentration was performed using $\epsilon_{280} = 22400 \text{ M}^{-1} \cdot \text{cm}^{-1}$, a value estimated [28] from the amino acid sequence of the mature protein [3].

Kinetics of substrate hydrolysis

Hydrolysis of acyl-L-leucine derivatives was determined in reaction mixtures containing purified PA, the appropriate substrate and either 100 mM citrate buffer, pH 5, or 100 mM phosphate buffer, pH 8. The reaction mixtures were incubated at 37 °C, and released L-leucine was quantified colorimetrically [29].

Hydrolysis of β -lactam antibiotics was determined by the method of Balasingham et al. [30]. Hydrolysis of 6-nitro-3-phenylacetamidobenzoic acid (NIPAB) and 4-nitrophenylphenylacetate (PNPA) was determined colorimetrically at 405 nm [31].

Acyl-L-leucine derivatives and antibiotics were obtained from Antibióticos S.A.

Isolation of α - and β -subunits of PA

The α - and β -peptides of PA were generated and isolated as previously described by Lindsay and Pain [32]. Active PA was generated after refolding of the α - and β -subunits by dialysis.

RESULTS

Isolation of mutants that hydrolyse adipyl-L-leucine and τ -(D)-aminoadipyl-L-leucine

Mutants of *E. coli* TD21 (pYKD59) were selected by their ability to grow on minimal M9 medium containing adipyl-L-leucine or τ -(D)-aminoadipyl-L-leucine as the sole source of leucine. Several colonies appeared after 6–8 days of incubation on plates containing adipyl-L-leucine but none on plates containing τ -(D)-aminoadipyl-L-leucine. As the cells containing the PA mutants showed the highest PA activity at pH 5.5, the selection was repeated using plates buffered at this pH. Colonies growing in buffered plates containing adipyl-L-leucine appeared after 4–5 days but we were still unable to obtain colonies on the plates containing τ -(D)-aminoadipyl-L-leucine.

Plasmid DNA was isolated from each mutant and used to transform cells of *E. coli* HB101. Cells transformed with mutant plasmids were able to grow on plates containing adipyl-L-leucine as the sole source of leucine, whereas cells transformed with the wild-type plasmid did not grow. Cells with either mutant or wild-type plasmids failed to grow when adipyl-L-leucine was omitted

Figure 1 Plasmid construction

(a) Construction of plasmids encoding the chimaeric PA. (b) Construction of plasmid pADL encoding the mutant PA produced by site-directed mutagenesis. Plasmids and M13 phages are drawn in circles; the relevant elements and restriction sites are indicated. Thin line, vector plasmid; hatched box, mutant *pac* gene; open box, wild-type *pac* gene. Direction of gene transcription is indicated by arrows. Abbreviations: B, *Bam*HI; cam, chloramphenicol resistance; H, *Hind*III; S, *Sph*I; T4-ligase, DNA ligase of T4. α , α' , β , and β' represent the α - and β -subunits of wild-type and mutant PA respectively.

from the medium, whereas all grew when the medium was supplemented with L-leucine. The ability of the PA mutants to hydrolyse adipyl-L-leucine was confirmed by assaying PA activity *in vitro* (results not shown). These observations indicated that mutations within the *pac* gene were probably responsible for the new substrate specificity of PA.

DNA sequencing of mutant *pac* genes

To determine the nucleotide changes responsible for the new substrate specificity of the PA mutants, the complete DNA sequence of the *pac* genes of two mutant plasmids, named pMPA1 and pMPA2, and the wild-type *pac* gene of pYKD59 were elucidated. The wild-type *pac* gene was sequenced again as we had detected some errors in the published sequence [3], caused

by the existence of numerous GC compressions. Sequence comparison indicated that the *pac* gene from plasmid pMPA1 showed a single transversion (T/G) at position 1163 changing Phe-360 to valine, whereas the gene from plasmid pMPA2 showed the same mutation at position 1163 plus an additional transversion (T/G) at position 2155 changing Asp-690 to glutamate. These results strongly suggest that the F360V mutation was responsible for the concomitant substrate-specificity change. Surprisingly, the mutation was located in the β -subunit, a region that had not been postulated to be involved in recognition of the substrate acyl chain [11,12].

To confirm that the mutation was located in the β -subunit of PA, two chimaeric PAs were constructed by DNA manipulation of the *pac* genes according to the scheme shown in Figure 1(a). Only the *E. coli* (pM β 1) cells that express the chimaeric *pac* gene

Table 1 Hydrolytic activity of the chimaeric PA reconstructed after denaturation, isolation and renaturation of the α - and β -subunits

Wild-type and mutant controls represent the native enzymes. Wild-type and mutant enzymes were purified from *E. coli* HB101 (pYKD59) and *E. coli* HB101 (pADL) respectively. The activity on NIPAB was determined at pH 7.5 and 25 °C, and that on adipyl-L-leucine was determined at pH 5 and 37 °C. ND, not detected.

α -Subunit	β -Subunit	Activity (nmol/min per mg of protein)	
		NIPAB	Adipyl-L-leucine
Wild-type	Wild-type	3400	ND
Wild-type	Mutant	5800	455
Mutant	Wild-type	3400	ND
Mutant	Mutant	5700	685
Wild-type control		4000	ND
Mutant control		6600	1400

Table 2 Hydrolytic activities of mutant and wild-type PA

Activity was determined at 37 °C. Wild-type and mutant enzymes were purified from *E. coli* HB101 (pYKD59) and *E. coli* HB101 (pADL) respectively. Activity on NIPAB was determined at 30 °C; ND, not detected or lower than 20 nmol/min per mg of protein.

Substrate	Activity (nmol/min per mg of protein)			
	pH 5		pH 8	
	Mutant	Wild-type	Mutant	Wild-type
Adipyl-L-Leu	1496	ND	768	ND
Glutaryl-L-Leu	974	ND	25	ND
Phenylacetyl-L-Leu	11 842	22 603	15 007	28 291
Phenoxyacetyl-L-Leu	680	487	300	593
Acetyl-L-Leu	ND	ND	ND	ND
Terbutyl-L-Leu	ND	ND	ND	ND
Caproyl-L-Leu	540	191	469	335
Valeryl-L-Leu	597	ND	217	42
Heptanoyl-L-Leu	38	ND	23	ND
Octanoyl-L-Leu	ND	ND	ND	ND
Penicillin G	1009	37 050	1160	41 710
Penicillin V	160	375	72	1349
Penicillin dihydro-F	ND	535	ND	2095
Glutaryl-7-ACA	ND	ND	ND	ND
Cephalosporin C	ND	ND	ND	ND
NIPAB	8200	1600	12100	10300

Table 3 Steady-state kinetic parameters of wild-type and mutant PA

Initial-velocity measurements were made at 25 °C with NIPAB (anilide) and PNPA (ester) in 100 mM phosphate buffer, pH 7.5. Wild-type and mutant enzymes were purified from *E. coli* HB101 (pYKD59) and *E. coli* HB101 (pADL) respectively. For other details see the Materials and methods section.

Enzyme	Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
Wild-type	NIPAB	27	11	2.3
Mutant	NIPAB	28	22	1.6
Wild-type	PNPA	82	57	1.4
Mutant	PNPA	39	11	3.7

encoding the β -subunit of the mutant PA were able to hydrolyse adipyl-L-leucine (results not shown).

Construction of F360V PA mutant by site-directed mutagenesis

To confirm further that the single transversion at position 1163 was the unique cause of the change in substrate specificity of PA, this mutation was reproduced by site-directed mutagenesis (Figure 1b). The recombinant *E. coli* HB101 cells harbouring the resulting plasmid pADL were able to grow in minimal medium containing adipyl-L-leucine as the sole source of L-leucine.

To demonstrate that the α - and β -subunits of mutant and wild-type PA are interchangeable and can be folded independently, both proteins were purified and the subunits separated by f.p.l.c. on Superose 12 in the presence of 6 M guanidinium chloride (results not shown). Table 1 shows that after this treatment the mutant β -subunit retains the ability to hydrolyse adipyl-L-leucine.

The PA mutant showed an optimal pH of 5 for hydrolysis of adipyl-L-leucine and at this pH the k_{cat} and K_m values were $2.5 s^{-1}$ and 1.6 mM respectively. This result is in agreement with the previous observation that recombinant cells expressing the PA mutant grew more rapidly in minimal media buffered at pH 5.5.

The ability of mutant and wild-type PA to hydrolyse different substrates was analysed (Table 2). The mutant was able to hydrolyse glutaryl-, valeryl-, caproyl-, heptanoyl- and phenoxyacetyl-L-leucine at pH 5 with higher efficiency than the wild-type enzyme. However, the mutant enzyme shows reduced activity toward phenylacetyl-L-leucine, the best substrate of PA. Interestingly, mutant and wild-type PA showed similar kinetic parameters on NIPAB and PNPA, two alternative substrates (Tables 2 and 3). Surprisingly, the mutant enzyme was very ineffective on penicillin substrates and was not active on glutaryl-7-ACA, suggesting that the original ability of PA to hydrolyse penam and cephem derivatives with high efficiency has been partially lost. Therefore the new ability of mutant PA to hydrolyse adipyl- or glutaryl-L-leucine does not necessarily provide the enzyme with the capacity to hydrolyse other adipyl- or glutaryl-derivatives.

Mutant and wild-type PA produced identical c.d. spectra, indicating that the amino acid replacement did not produce any visible change in protein structure (Figure 2). However, both enzymes showed small but significant differences between their respective c.d. spectra at pH 5 and 8. How these conformational changes might affect the structure of the active centre increasing the catalytic efficiency of the mutant enzyme at pH 5 will have to await determination of the tertiary structure.

DISCUSSION

On the basis of the procedure developed to isolate *pac* genes [2], we have been able to select PA mutants from *K. citrophila* that hydrolyse novel substrates. Although similar procedures had been used to generate PA mutants from *E. coli* and *Proteus rettgeri* [11–14], only very limited information was available about the nature of the mutations that produce the alterations in substrate specificity [20].

As pointed out above, PA is an $\alpha\beta$ -heterodimer derived from a precursor protein and both subunits are required for enzyme activity [12,33]. The β -subunit contains a phenylmethanesulphonyl fluoride-sensitive serine residue which is directly involved in substrate hydrolysis [17,18], whereas the α -subunit has been shown to impart specificity for the penicillin side chain [12].

Early experiments demonstrated that the major specificity of

Figure 2 Far- and near-u.v. c.d. spectra of wild-type and mutant PA at pH 5 and 8

C.d. spectra of mutant (**b** and **d**) and wild-type (**a** and **c**) PA were recorded at pH 8 (—) or pH 5 (---) in the far- (**a** and **b**) and near- (**c** and **d**) u.v.

PA is towards the side chain of the penicillin molecule, as replacement of 6-APA with 7-ACA has only a minor effect on enzyme activity [34]. Moreover, PA is also able to hydrolyse other phenylacetyl esters or amides including amides of the L-isomers of amino acids [7]. Substrates with hydrophobic acyl moieties are preferred to those with ionizable (polar) functional groups. Hence amides containing acyl chains such as caproyl, malonyl or 2-thienylacetyl can also be used as substrates, whereas the enzyme is unable to hydrolyse substrates containing glutaryl, adipyl or aminoadipyl groups in the side chain [7,34].

Our approach allowed us to obtain a new PA mutant able to hydrolyse adipyl- or glutaryl-L-leucine. DNA sequencing identified the change as a single transversion. This mutation resulted in the conversion of a phenylalanine in wild-type PA to a valine in the mutant enzyme with the concomitant substrate-specificity change. Owing to the absence of crystallographic data, it is not easy to explain how this mutation produces the specificity shift. It does not produce a significant change in the c.d. spectrum of the protein and computer analysis does not predict any alteration

in the secondary structure (Figure 3a). Although we cannot rule out the possibility that this phenylalanine plays an essential role in stabilization of the tertiary structure via hydrophobic interactions, we suggest that its side chain may have an important effect on the active centre of the enzyme. It has therefore been proposed that PA follows an induced-fit-like mechanism, as interaction of the acid moiety of the substrate at the active centre induces a conformational rearrangement of the protein [4]. Hence those amino acids involved in stabilization of the first transition state as well as in the enzyme-substrate complex must play a critical role in the association equilibrium and specificity constants of the enzyme. This argument has been proposed to explain the substrate-specificity shift exhibited by the Ala-168 PA mutant [4,5]. The active site of PA seems to be maintained in a complex structure which involves hydrophobic domains recognized by interaction with the substrate, according to the induced-fit mechanism [4]. Thus it is possible that the Phe-360 residue might be involved in maintenance of one of these hydrophobic domains.

Figure 3 Secondary-structure predictions and amino-acid-sequence comparison of the region surrounding Phe-360 of PA

(a) Secondary-structure predictions near the mutation area of wild-type and mutant PA protein. α , alpha helix; β , beta sheet; t, beta turn; c, random coil. The position of the mutated residue is indicated. Predictions were carried out using the PEPTIDESTRUCTURE and PEPPLOT programs of the Wisconsin Package from Genetics Computer Group Inc. (b) Amino acid sequence comparison of the region surrounding Phe-360 of PA from *K. citrophila* with other acylases. KLU, PA from *K. citrophila* [3]; ESC, PA from *E. coli* [33]; PRO, PA from *P. rettgeri* [35]; ALC, PA from *Alcaligenes faecalis* [36]; SE83, glutaryl acylase from *Pseudomonas* sp. SE83 [37]; N176, glutaryl acylase from *Pseudomonas* sp. N176 [38]; V22, glutaryl acylase from *Pseudomonas* sp. V22 [38]; A14, glutaryl acylase from *Pseudomonas* sp. A14 [38]; GK16, glutaryl acylase from *Pseudomonas* sp. GK16 [39]. Asterisks mark the conserved amino acid residues in at least five different PA sequences.

Comparison of the amino acid sequences of several phenylacetyl and glutaryl acylases reveals that the region surrounding Phe-360 is highly conserved in these proteins (Figure 3b). This amino acid was conserved in the same position in five acylases, but, interestingly, it was replaced by a proline in the homologous PA from *Alcaligenes faecalis* [36]. The presence of a proline in this position reinforces our prediction that this region does not possess a β -sheet or α -helix structure and suggests that the replacement of Phe-360 by other amino acids does not necessarily produce a change in substrate specificity.

The observation that hydrolysis of adipyl- or glutaryl-L-leucine was carried out with higher efficiency at pH 5 agrees with the results obtained by Forney et al. [13], but there is no simple explanation. At this pH the free carboxy group of adipic or glutaric amide (pK_a around 4.7) [40] is not completely ionized and we cannot rule out it having an effect on the partial neutralization of the negative charge. On the other hand, we have shown that, at this pH, a small conformational change is produced

and, according to the induced-fit mechanism, it could modify recognition of the substrate. Therefore, at this pH, interaction of a negatively charged free carboxy group with a positively charged amino acid in the active centre might contribute to stabilization of the transition state. Kinetics and thermodynamic studies based on the pH-dependence of V/K_m for hydrolysis of penicillin G by PA from *K. citrophila* have demonstrated the existence of two pK values of 4.6 and 9.5 [41]. These studies attributed the pK_1 value to a histidine, suggesting that the high hydrophobicity postulated for the active centre of the enzyme is responsible for the low pK value of this histidine. Hence the deprotonated form of this amino acid might act as a proton acceptor which stabilizes the amide through formation of a hydrogen bond [41]. According to this observation, this histidine should be partially protonated at pH 5, a factor that might contribute to stabilization of the carboxylate form of glutaric or adipic amides. Nevertheless, we have observed that mutant PA hydrolyses substrates with non-ionizable side chains, such as phenoxyacetyl-, valeryl- or caproyl-L-leucine, more efficiently at pH 5 than at pH 8. In these cases, we cannot ascribe an effect of pH on substrate stabilization via ionic interactions.

Although the PA mutant was able to hydrolyse adipyl- and glutaryl-L-leucine, it was unable to hydrolyse other polar acyl side chain derivatives such as glutaryl-7-ACA. Interestingly, the PA mutant obtained by Forney and Wong [14], which hydrolyses D-(-)- α -aminophenylacetyl-L-leucine efficiently, was less active on cephalaxine than the wild-type enzyme. As the other PA mutants obtained by Forney et al. [13] that are able to hydrolyse glutaryl-L-leucine have not been tested on glutaryl-7-ACA, we cannot make a comparison with our results. The only PA mutant that has been tested with new penicillin derivatives is the Ala-168 mutant of PA from *K. citrophila* which was able to hydrolyse 6-bromohecanoyl-6-aminopenicillanic acid [5]. This PA mutant obtained by site-directed mutagenesis is equivalent to the BroA mutant of PA from *E. coli* [11,20]. It is noteworthy that the *E. coli* PA mutants were selected by serial passage of the strains in media containing primary amides as sole nitrogen source [11]. Apparently, the use of adipyl-L-leucine amides as a source of L-leucine instead of adipamide as a nitrogen source determines the selection of new enzymes which lose their original ability to bind penam and cephem nuclei efficiently. In this connection, it is notable that phenylacetyl amides were hydrolysed with identical or lower efficiency by our PA mutant depending on the amine moiety of the substrate.

Finally, we can conclude that selective pressure can be used to modify the substrate specificity of PA, but, owing to the complex induced-fit catalytic mechanism of this enzyme, an amino acid change that could favour binding of new acyl chains might alter the broad range of amine moieties recognized by the native enzyme. Therefore those designing experimental protocols for *in vivo* isolation of PA mutants able to hydrolyse other penicillins or cephalosporins should consider using these compounds directly to impose the selective pressure.

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