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Structural and kinetic studies on ligand binding in wild-type and active-site mutants of penicillin acylase

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Penicillin acylase catalyses the condensation of Cα-substituted phenylacetic acids with β-lactam nucleophiles, producing semi-synthetic β-lactam antibiotics. For efficient synthesis a low affinity for phenylacetic acid and a high affinity for Cα-substituted phenylacetic acid derivatives is desirable. We made three active site mutants, αF146Y, βF24A and αF146Y/βF24A, which all had a 2- to 10-fold higher affinity for Cα-substituted compounds than wild-type enzyme. In addition, βF24A had a 20-fold reduced affinity for phenylacetic acid. The molecular basis of the improved properties was investigated by X-ray crystallography. These studies showed that the higher affinity of αF146Y for (*R*)-α-methylphenylacetic acid can be explained by van der Waals interactions between αY146:OH and the Cα-substituent. The βF24A mutation causes an opening of the phenylacetic acid binding site. Only (*R*)-α-methylphenylacetic acid, but not phenylacetic acid, induces a conformation with the ligand tightly bound, explaining the weak binding of phenylacetic acid. A comparison of the βF24A structure with other open conformations of penicillin acylase showed that βF24 has a fixed position, whereas αF146 acts as a flexible lid on the binding site and reorients its position to achieve optimal substrate binding.

Keywords: 3D structure/kinetics/ligand binding/mutants/penicillin acylase

Introduction

Penicillin acylase (PA) from *Escherichia coli* ATCC 11105 is a heterodimeric periplasmic protein consisting of a small α-subunit of 23 kDa and a large β-subunit of 62 kDa. The enzyme catalyses the hydrolysis of penicillin G to yield phenylacetic acid (PAA) and 6-aminopenicillanic acid, a building block of semi-synthetic β-lactam antibiotics (Bruggink *et al*., 1998). PA is of biotechnological interest because it catalyses the condensation of 6-aminopenicillanic acid and a synthetically produced acyl donor, to produce semi-synthetic β-lactam antibiotics (Arroyo *et al*., 2003). The proposed catalytic cycle starts with a nucleophilic attack by the hydroxyl group of the active-site serine, βS1, on the carbonyl carbon atom of the amide or ester bond of the substrate (the amino acid residues are labelled to indicate the polypeptide chain, α or β, and the residue number in this chain) (Duggleby *et al*., 1995). Via a tetrahedral oxyanion intermediate an acyl-enzyme is formed concomitant with the departure of the leaving group. The acyl-enzyme can subsequently be deacylated by a β-lactam nucleophile yielding a semi-synthetic β-lactam antibiotic, or by H_2O, resulting in the hydrolysis product.

The synthetic acyl donors that are used in the synthesis of semi-synthetic β-lactam antibiotics are in general activated PAA derivatives that carry substituents on the Cα position or on the phenyl ring. For instance, for the synthesis of ampicillin and amoxicillin, amides or esters of α-aminophenylacetic acid (phenylglycine) and *p*-hydroxy-α-aminophenylacetic acid (*p*-hydroxyphenylglycine) are used, respectively. The production of cefamandole requires the use of activated α-hydroxyphenylacetic acid (mandelic acid). Unfortunately, the K_m values of PA for the conversion of these derivatives are in the order of 10–100 mM, which is 100- to 1000-fold higher than the K_m values for substrates with phenylacetic acid as the acyl group (Margolin *et al*., 1980; Svedas *et al*., 1996; Alkema *et al*., 1999; Lummer *et al*., 1999). A higher affinity of PA for synthetic acyl donors would therefore be desirable for maintaining a high activity at low substrate concentrations. A second requirement for high enzyme activity is a low affinity for PAA, which is a strong inhibitor of penicillin acylase and may be present in preparations of 6-aminopenicillanic acid derived from penicillin G.

The 3D structure of the enzyme with bound PAA revealed that the acyl binding site is a hydrophobic pocket formed by the side chains of αM142, αF146, βF24, βV56 and βI177 (Duggleby *et al*., 1995). One oxygen atom of the carboxylate group of PAA interacts with the oxyanion-hole residues βN241 and βA69. Binding of *para*- and *meta*-substituted phenylacetic acid derivatives causes the enzyme to switch from a closed to an open active site conformation (Done *et al*., 1998; Alkema *et al*., 2000; McVey *et al*., 2001). In the open conformation, αR145 and αF146 have moved away from the active site, creating more space in the acyl binding site. Kinetic studies with Cα-substituted phenylacetic acid derivatives have shown that the substrate binding site can accommodate small hydrophobic substituents, but that large and polar substituents bind only with low affinity (Svedas *et al*., 1996). However, no structural information is available on the binding of these compounds.

To identify the factors that play a role in the binding of synthetic acyl donors, we carried out kinetic and crystallographic studies of wild-type enzyme and the active-site mutants βF24A and αF146Y, which were found to have an increased affinity for PAA derivatives with an NH_2 group at the Cα position. The results show that the kinetic properties of the βF24A mutant can be explained by the enzyme adopting two distinct stabilizing conformations upon binding of PAA and (*R*)-α-methylphenylacetic acid. The αF146Y mutation introduces van der Waals interactions that stabilize the binding of (*R*)-α-methylphenylacetic acid.

Materials and methods

Crystallization of wild-type and mutant penicillin acylases

Site-directed mutants were constructed as described (Alkema *et al*., 2002a). The double mutant αF146Y/βF24A was made by isolating the *Eco*RV–*Cla*I fragment of the penicillin acylase gene containing the αF146Y mutation and subsequent cloning of this fragment in the plasmid carrying the βF24A mutation. Purified wild-type and mutant enzymes were obtained as described (Alkema *et al*., 2000).

Crystals of wild-type, αF146Y and βF24A PA were grown at 4°C using the hanging-drop vapour diffusion set-up combined with streak seeding. Drops of 6 µl were prepared by mixing equal volumes of protein [4–10 mg/ml protein in 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer, pH 7.2] and reservoir solution [50 mM MOPS buffer, pH 7.2, 12–20% (w/v) polyethylene glycol monomethylether 2000 (PEG MME 2k) (Sigma)]. Crystals appeared in 1–2 days and reached typical sizes of $0.2 \times 0.15 \times 0.1$ mm. Crystals of wild-type and mutant enzymes complexed with PAA and (*R*)-α-methylphenylacetic acid (MPAA) were obtained by co-crystallization in the presence of 2.5 mM PAA and MPAA, respectively. Prior to data collection, the crystals were transferred to a solution of 20% (w/v) PEG MME 2k, 50 mM MOPS, pH 7.2, and 5% (v/v) glycerol. The crystals were flash frozen using 35% (w/v) PEG MME 2k and 7.5% (v/v) glycerol as cryoprotectant. For the ligand binding studies, these solutions contained 2.5 mM PAA or MPAA.

Data collection and refinement

All data were collected using a MacScience DIP2020 image-plate detector equipped with a CAD4 κ-goniostat, with mirror monochromatized Cu Kα radiation from a Nonius FR591 rotating anode X-ray generator. Data were integrated and reduced with the programs DENZO and SCALEPACK (Otwinowski and Minor, 1997). The intensities were converted to structure factor amplitudes and brought to absolute scale with TRUNCATE from the CCP4 package (CCP4, 1994). The structures were refined using CNS (Brünger *et al*., 1998) alternated with minor manual model building using O (Jones *et al*., 1991) and Quanta (Accelrys). Bulk solvent correction and overall anisotropic *B*-factor scaling were applied. Ligands were identified and modelled in the active site. For PAA in the βF24A enzyme, the density suggested multiple conformations, of which the most prominent one was modelled. Final rounds of refinement were performed with REFMAC (Murshudov *et al*., 1999), alternated with ARP/wARP (Lamzin *et al*., 1999) to analyse water molecules. The quality of the structures was validated using PROCHECK (Laskowski *et al*., 1993) and programs from the CCP4 suite (CCP4, 1994). The final models consist of residues α3–α208, β1–β557, one calcium ion, a variable number of water molecules and ligands where appropriate. Data collection and refinement statistics are summarized in Table I.

Determination of binding constants

The binding constants for the inhibitors and substrates were determined by measuring K_{mapp} for the hydrolysis of the chromogenic substrate D-2-nitro-5-[(phenylglycyl)amino]benzoic acid (NIPGB) in the presence of varying substrate and inhibitor concentrations. Inhibition by PAA and phenylacetamide was shown to be competitive and it was assumed that inhibition by the other PAA derivatives was also competitive, because of the similar binding of these compounds in the PAA binding site (Svedas *et al*., 1996). The rate of hydrolysis (V) of a coloured

Table I. Data processing and refinement statistics: all enzymes crystallized in space group P1

	WT-MPAA	βF24A	βF24A-PAA	βF24A-MPAA	αF146Y-MPAA
Resolution (Å)	30–2.15	30–2.28	19–2.34	30–2.43	30–2.30
Highest resolution shell (Å)	2.19–2.14	2.28–2.36	2.38–2.34	2.47–2.43	2.34–2.30
No. of observations	64473	193740	55158	58181	67386
No. of unique reflections	36555	30372	28382	26632	30886
R_{sym} (%)	6.8 (22.9)	6.8 (16.3)	4.3 (9.8)	3.7 (6.6)	8.2 (17.3)
Completeness (%)	96.7 (87.5)	95.1 (93.6)	92.9 (77.5)	96.2 (95.7)	95.4 (92.7)
$< I/\sigma(I) >$	10.1 (2.8)	9.6 (3.9)	15.9 (5.5)	21.1 (10.9)	10.2 (4.6)
R-factor (%)	18.4	16.2	16.3	17.3	15.1
R_{free}-factor (%)	21.6	21.5	21.8	23.3	20.7
No. of atoms					
Protein	6072	6068	6068	6068	6074
Water	319	433	358	360	475
Average *B*-factor (Å^2)					
All atoms	24.07	14.8	17.9	18.1	15.7
Solvent	28.61	16.3	18.0	22.5	17.9
Ligand	19.29	–	68.3	21.7	13.4
r.m.s.d. from ideality					
Bond lengths	0.016	0.013	0.020	0.009	0.010
Bond angles	1.589°	1.384°	1.251°	1.131°	1.267°
Dihedral angles	18.9°	15.783°	16.076°	16.787°	17.128°
Estimated coordinate error from σ_A[a]	0.21	0.24	0.27	0.21	0.18
PDB accession number	1k7d	1xj9	1k5q	1k5s	1kec

[a]As described in Read (1986).

substrate (S) is given by

$$V = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

where K_m is the Michaelis–Menten constant for S and V_{max} the rate of conversion at saturating substrate concentration. The rate (V_i) in the presence of a competitive inhibitor I is given by

$$V_i = \frac{V_{max}[S]}{K_m\left(1 + \frac{[I]}{K_i}\right) + [S]} \quad (2)$$

where K_i is the binding constant of the inhibitor to the enzyme. The ratio V/V_i is given by

$$\frac{V}{V_i} = \frac{K_m\left(1 + \frac{[I]}{K_i}\right) + [S]}{K_m + [S]} \quad (3)$$

from which K_i can be calculated as

$$K_i = \frac{K_m[I]}{(K_m + [S])\left(\frac{V}{V_i} - 1\right)} \quad (4)$$

Inhibition experiments were carried out with $[S] \ll K_m$ so that the determination of K_i is independent on the precise knowledge of K_m and [S], reducing the experimental error in the measurements. If I is an inhibitor, K_i is equal to the binding constant for binding of the inhibitor to the enzyme. If I is a substrate, K_i is equal to K_m of that substrate. For phenylglycinamide and phenylacetamide, the K_m values are equal to the dissociation constants K_s, since the step leading to the formation of the covalent intermediate is strongly rate limiting (Alkema *et al*., 2003). All experiments were carried out at 30°C, pH 7.0, in 50 mM phosphate buffer.

Chemicals

α-Methylphenylacetic acid, α-ethylphenylacetic acid and α-hydroxyphenylacetic acid were obtained from Aldrich. The enantiomers of α-methylphenylacetic acid and α-ethylphenylacetic acid were prepared by classical resolution of their racemates by crystallization with (−)-α-phenylethylamine as described (Aaron *et al*., 1967). Enantiomeric purities of the resulting acids were established with chiral HPLC [Chiralcel OD, hexane–2-propanol–acetic acid (98:2:1), flow-rate 0.5 ml/min] and found to be >95%. Phenylglycinamide was obtained from DSM-Gist (Delft, The Netherlands). NIPGB was obtained from Syncom (Groningen, The Netherlands). Phenylacetamide was obtained as described (Alkema *et al*., 2003).

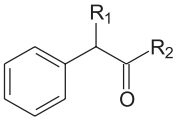
Results and discussion

Binding constants for PAA derivatives

The objective of this study was to obtain insight into the factors that play a role in the binding of Cα-substituted compounds to penicillin acylase (PA). To this end we determined the binding constants of various PAA derivatives with distinct substituents at the Cα position, varying in size, polarity and ability to accept or donate hydrogen bonds. For these experiments wild-type and the two active site mutants αF146Y and βF24A were used, in addition to the double mutant αF146Y/βF24A. In the wild-type enzyme, αF146 and βF24 line the acyl binding pocket in the active site (Duggleby *et al*., 1995; Done *et al*., 1998; McVey *et al*., 2001; Alkema *et al*., 2002a; Morillas *et al*., 2003). They play a role in substrate binding through hydrophobic interactions with the phenyl ring of the substrate. Previous studies have shown that the βF24A and αF146Y mutants have increased affinities for phenylglycine methyl ester and phenylglycinamide, which are used for the production of ampicillin (Alkema *et al*., 2002a).

The wild-type enzyme had a K_i for PAA of 0.06 mM and showed a decreasing affinity with increasing size and polarity of the Cα substituent (Table II). A methyl group on the Cα position led to a 10-fold reduced affinity, whereas an OH group caused a more than 200-fold reduced affinity. The zwitterionic phenylglycine did not inhibit the enzyme, indicating that a

Table II. Inhibition constants of wild-type penicillin acylase and the mutants αF146Y, βF24A and αF146Y/βF24A for Cα-substituted phenylacetic acid derivatives



Ligand	R_1	R_2	WT		βF24A		αF146Y		αF146Y/βF24A	
			K_i (mM)	K_{iR}/K_{iS}	K_i (mM)	K_{iR}/K_{iS}	K_i (mM)	K_{iR}/K_{iS}	K_i (mM)	K_{iR}/K_{iS}
Phenylacetic acid	H	OH	0.06	–	1.1	–	0.03	–	1	–
(*R*)-α-Methylphenylacetic acid	CH_3	OH	0.47	0.42	0.124	0.083	0.2	0.25	0.076	0.211
(*S*)-α-Methylphenylacetic acid	CH_3	OH	1.1		1.5		0.5		0.36	
(*R*)-α-Ethylphenylacetic acid	CH_2CH_3	OH	5.7	2.7	0.4	0.36	3.3	3.3	0.276	0.745
(*S*)-α-Ethylphenylacetic acid	CH_2CH_3	OH	2.1		1.1		1.0		0.37	
(*R*)-α-Hydroxyphenylacetic acid	OH	OH	31	3.1	3.5	0.61	13	2.9	6.2	0.76
(*S*)-α-Hydroxyphenylacetic acid	OH	OH	10		5.7		4.05		8.2	
(*R*)-Phenylglycine	NH_2	OH	ni[a]		ni[a]		ni		ni	
(*R*)-Phenylglycinamide	NH_2	NH_2	40	–	25	–	4		15	
Phenylacetamide	H	NH_2	0.2	–	2	–	0.05	–	1.5	–

The values reported are the average of five independent measurements carried out with concentrations [I] of the inhibitor ranging from $0.5K_i$ to $2K_i$. The standard deviation was in all cases <10% of the average.
[a]No inhibition was observed at an inhibitor concentration of 100 mM.

positive charge on the Cα position prevents the ligand from binding in the apolar active site. Increasing the size of the Cα-substituent from a methyl to an ethyl group led to a more than 10-fold reduced affinity. Interestingly, whereas the enzyme was enantioselective (determined as K_{iR}/K_{iS}) for the *R*-enantiomer of α-methylphenylacetic acid, it had an opposite enantioselectivity for the low affinity inhibitors α-ethyl- and α-hydroxyphenylacetic acid. Overall, these results show that substrate specificity and enantioselectivity of the wild-type enzyme are determined both by the size and by the polarity of the substituent at the Cα position, in agreement with results that were obtained by Svedas *et al*. (1996).

The K_i values of the βF24A mutant for the various compounds differed significantly from those of the wild-type. The most striking difference was that the βF24A mutant had a 20-fold increased K_i value for PAA, whereas K_i for PAA derivatives with a substituent at the Cα position ranged from values equal to wild-type values to values up to 10-fold lower compared with the wild-type enzyme. The substrate with a Cα-amino group, phenylglycinamide, was also bound with higher affinity to the mutant enzyme, in contrast to the 10-fold decreased affinity for phenylacetamide, which contains no substituent at the Cα position. These findings suggest that a substituent at the Cα position of PAA is necessary for tight binding of the ligand to the βF24A active site. Furthermore, the mutant enzyme had, compared with the wild-type, a higher enantioselectivity for the *R*-enantiomers of all ligands, indicating that the side chain of the residue at position β24 strongly influences the enantioselectivity of PA.

The αF146Y mutant enzyme showed an increased affinity for all compounds tested. The largest effect was observed with phenylglycinamide, for which a 10-fold increased affinity was found. The increased affinity of the αF146Y mutant for PAA derivatives did not depend on the type of Cα-substituent and the enantioselectivity for all compounds was similar to that of the wild-type enzyme.

In the double mutant αF146Y/βF24A, approximate additivity of the effects of the single mutations was observed. The affinity for α-methylphenylacetic acid and α-ethylphenylacetic acid was higher than the affinity of both single mutants. For the other Cα-substituted ligands, the K_i values averaged those of the single mutants with the exception of PAA, for which the αF146Y/βF24A mutant showed the same low affinity as the single βF24A mutant. As a result, the double mutant had a 44-fold increased preference for phenylglycinamide over PAA (K_{iPGA}/K_{iPAA} = 0.07), compared with the wild-type (K_{iPGA}/K_{iPAA} = 0.0015).

Structure of wild-type enzyme co-crystallized with MPAA

The kinetic data for the wild-type enzyme showed that substituents at the Cα atom of PAA result in a decreased affinity and that the stereoselectivity of the enzyme is dependent on the type of substituent at the Cα position. To obtain a structural explanation for these observations, we determined the crystal structure of wild-type penicillin acylase in a complex with (*R*)-α-methylphenylacetic acid (MPAA). This structure revealed that MPAA is bound in the acyl binding site of the enzyme, where it is stabilized by hydrophobic interactions with the side chains of αM142, βF146, βF24 and βI177, and by hydrogen bonds with βA69:N and βN241:Nδ2 (Figure 1).

The mode of MPAA binding is similar to that reported previously for PAA (Duggleby *et al*., 1995), but small shifts of the active site residues and the ligand are necessary to accommodate the methyl substituent at the Cα position. For instance, the Cα carbon atom of MPAA shifts by 1.1 Å towards the phenyl ring of αF146, and the phenyl rings of αF146 and βF24, on both sides of the Cα atom, move by 0.5 Å. In this way the methyl group of MPAA comes at van der Waals distance from βF24:Cδ1 (3.6 Å) and αF146:Cε2 (3.5 Å) and is also close to βS1:Oγ (3.2 Å) and βQ23:O (3.0 Å) (Figure 1).

Since the MPAA Cα–CH_3 bond is directed towards βF24:Cα, with the methyl carbon atom at 4.1 Å from βF24:Cα, accommodation of a larger ethyl group is not possible without conformational changes of the protein or a different binding mode of the ligand. Thus, (*R*)-α-ethylphenylacetic acid cannot bind in the active site in a similar way to MPAA. Likewise, the ethyl substituent of (*S*)-α-ethylphenylacetic would come too close to αF146 to be accommodated without conformational changes. The 10-fold lower affinity for α-ethylphenylacetic acid compared with α-methylphenylacetic acid and the reversal of enantioselectivity from 2.7 for the wild-type to 0.36 for the βF24A mutant confirm that an ethyl substituent is not easily accommodated in the active site. The structure suggests that residues αF146 and βF24 limit the allowed size of the Cα-substituent. The apolar nature of these residues, together with the overall hydrophobic character of the acyl-binding pocket, also explains the enzyme's low affinity for substrates with polar substituents (Table II).

Structure of βF24A

The affinity of PA for (*R*)-methyl- and (*R*)-ethyl-PAA can be increased by removing the aromatic ring of residue βF24 (see Table II). To ascertain the structural basis of this increased affinity, we determined the crystal structures of βF24A PA and its complexes with PAA and MPAA (Figure 2). In these structures, the active-site and oxyanion-hole residues βS1, βA69 and βN241 occupy almost the same position as in the wild-type enzyme, and also the loop-containing residue βF24 has hardly undergone positional rearrangements. In contrast, residues αN144–αF146 and βY31 show considerable differences. The α-helical conformation of αN144–αF146 is distorted. As a result, residues αR145 and αF146 have moved away, creating a more open and accessible active site. Residue βY31 has moved towards the acyl binding pocket and occupies some of the space created by the βF24A mutation. The more open active site of the βF24A mutant is very similar to the open conformation that was observed in the enzyme–penicillin G complex and in enzyme complexes with disubstituted phenylacetic acids (Done *et al*., 1998; Alkema *et al*., 2000).

Binding of PAA in the active site of βF24A penicillin acylase leaves the open conformation of the empty βF24A enzyme intact. Interestingly, the positions of both the phenyl ring and the carboxylate moiety of PAA are different from those in the wild-type enzyme (Figure 2B). Although the electron density indicates that PAA is present in more than one conformation, it is clear that the compound has lost the hydrogen bonds with the oxyanion-hole atoms βA69:N (at 4.2 Å distance) and βN241:Nδ2 (5.2 Å) that were observed in the wild-type enzyme. The hydrophobic interactions with the side chains of αM142 and αF146 are also not conserved. The loss of binding interactions is in agreement with the 20-fold reduced affinity of this mutant for PAA (Table II).

In contrast, the affinity of the βF24A mutant enzyme for MPAA is higher than that of wild-type PA (Table II). The

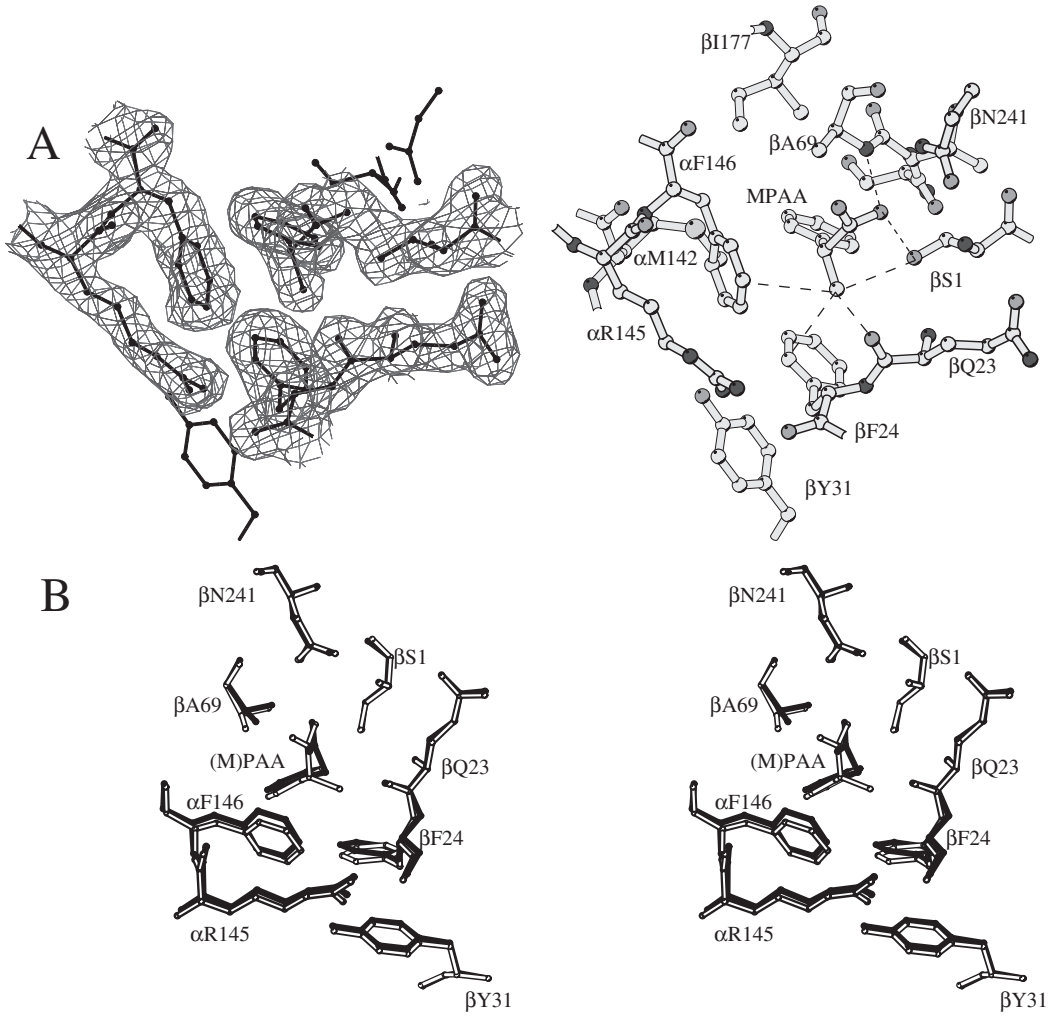


Fig. 1. Structure of wild-type penicillin acylase complexed with MPAA. (**A**) Simulated annealed omit map of the density of αR145, αF146, βS1, βF24, βQ23 and MPAA, contoured at 1 σ. The most important interactions between the enzyme and the Cα substituent are given with dashed lines. (**B**) Superposition of the structures of wild-type complexed with MPAA (black bonds) and PAA (open bonds). PAA and MPAA bind in essentially the same way to the wild-type enzyme, but a small positional shift of MPAA, αF146 and βF24 is necessary to accommodate the extra methyl group in MPAA in the active site. All figures were prepared using Molscript (Kraulis, 1991) and Bobscript (Esnouf, 1997).

crystal structure of this PA mutant with MPAA bound in the active site reveals that the open conformation of the βF24A and βF24A–PAA structures is not present in the βF24A–MPAA complex. Instead, MPAA is bound in a very similar way to that in the wild-type enzyme (Figure 2C) with αF146 moved in to cover the acyl binding site. MPAA has moved about 0.6 Å towards βA24:CB, occupying part of the space created by the βF24A mutation. Although βY31 also takes some of the space caused by the mutation, sufficient space remains to accommodate an ethyl group or an even larger substituent. This suggests that whereas α-methylphenylacetic acid and α-ethylphenylacetic acid have different binding modes in the wild-type enzyme, they bind in a similar way in the βF24A mutant. This is corroborated by the kinetic data, which show that the two substrates bind with similar affinities and enantioselectivities to the βF24A mutant.

Based on kinetic data, it was concluded that a Cα-substituent is necessary for tight binding of a ligand in the βF24A active site. The structural data show that tight binding is correlated with a switch from the open to the closed conformation. This leads to restored hydrophobic enzyme–ligand contacts with residues from the acyl-binding site and restored hydrogen bonds of the substrate carbonyl oxygen with the oxyanion-hole residues. A similar correlation between the K_i values and the presence of an open or closed conformation has been observed for the binding of *para*- and *meta*-substituted phenylacetic acids to PA (Done *et al*., 1998), supporting the functional relevance of the observed conformational changes for the enzyme's substrate preferences.

Structure of αF146Y co-crystallized with MPAA

The αF146Y mutant enzyme has an increased affinity for PAA and the Cα-substituted derivatives tested. The crystal structure of this mutant with bound MPAA shows that MPAA binds in a similar way to the enzyme as to the wild-type and the βF24A mutant. The additional OH group in the αF146Y mutant is accommodated without conformational changes of the protein or the ligand. It has van der Waals contacts with αR145:NH_2 and the Cα-substituent of the ligand, and makes a water-mediated hydrogen bond to Q23:O (Figure 3). The Cα–CH_3 bond of MPAA and the CZ–OH bond of αY146 are directed parallel to each other. This precludes hydrogen bond formation in case of an OH or NH_2 substituent at the Cα position, assuming that no conformational rearrangements take place. The

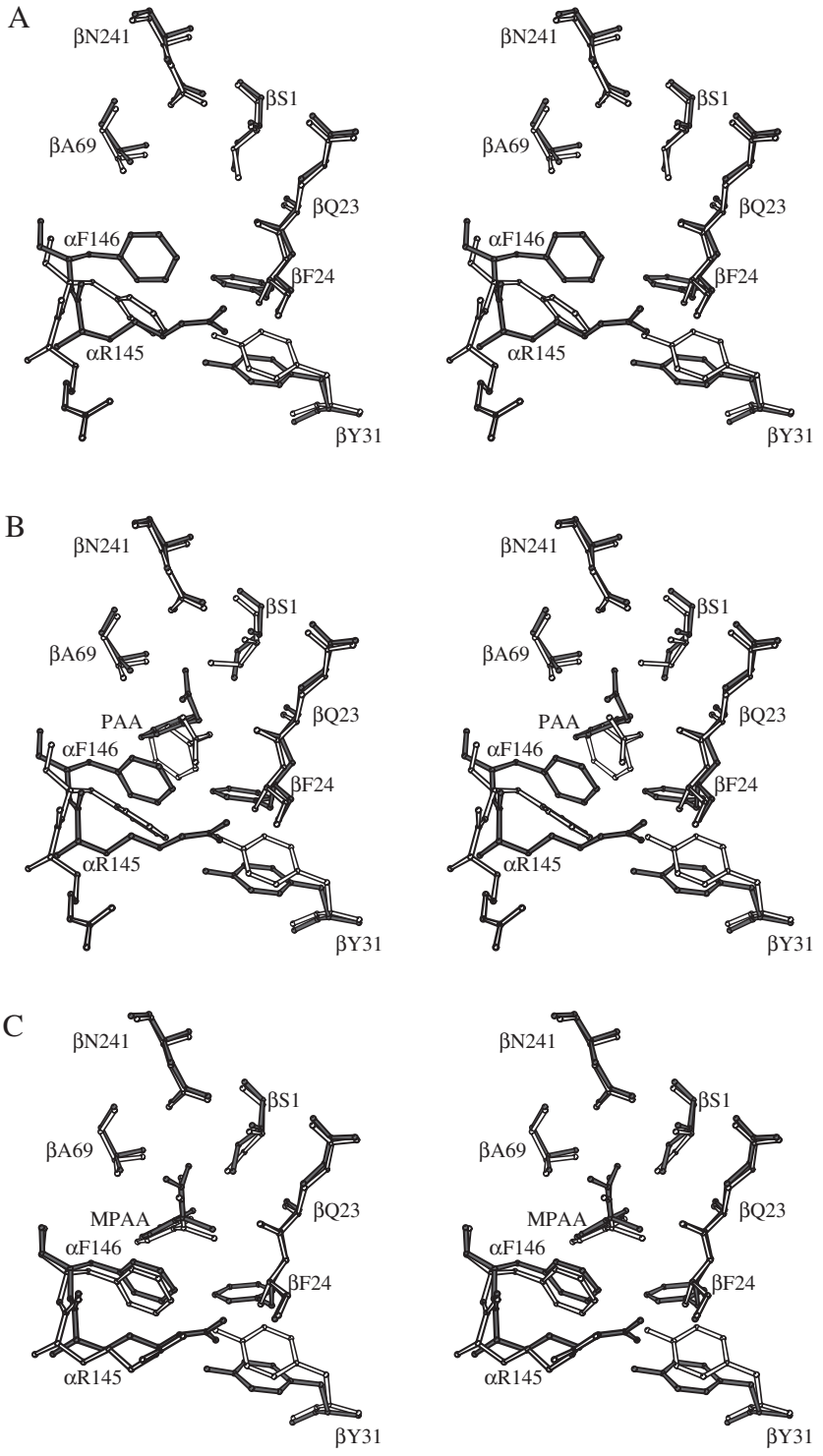


Fig. 2. Stereo view of the active-site structures of wild-type (black bonds) and βF24A (open bonds) penicillin acylase. (**A**) Superposition of the structures of wild-type and βF24A showing the open conformation of the βF24A mutant. (**B**) Superposition of WT-PAA and βF24A-PAA. (**C**) Superposition of WT-MPAA and βF24A-MPAA.

increased affinity of the αF146Y mutant for the *R*-enantiomers of the PAA derivatives may therefore be due to the added van der Waals interactions between αY146:OH and the Cα substituent.

Interaction of the αF146Y/βF24A double mutant with ligands

On the basis of the crystal structures of the single mutants and the wild-type enzyme, a structural explanation for some of the properties of the αF146Y/βF24A double mutant can be deduced. This mutant has the same low affinity for PAA and phenylacetamide as the βF24A mutant, whereas the affinity for the Cα-substituted ligands α-methylphenylacetic acid and α-ethylphenylacetic acid is 3- to 10-fold higher than the wild-type enzyme. This suggests that the same open and closed forms regulate ligand binding as observed for the βF24A mutant. For α-methylphenylacetic acid, which binds to the

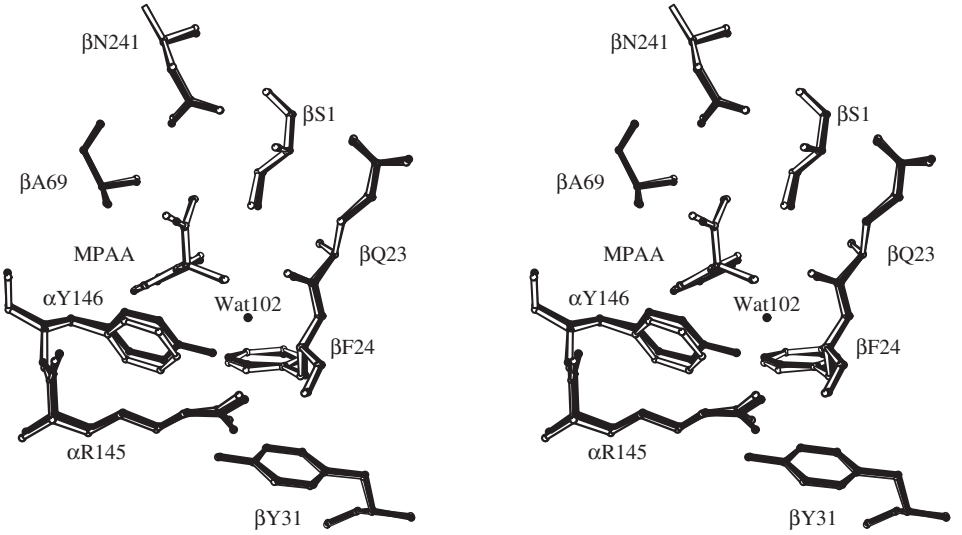


Fig. 3. Stereo view of the active site of αF146Y complexed with MPAA. The structure of the mutant, shown in black, is superimposed on the WT-MPAA structure, shown in open bonds.

closed form, the affinity of the double mutant is higher than the affinity of each of the single mutants, indicating that the effects of the single mutations are additive. The combination of the lower steric hindrance caused by the βF24A mutation and the improved enzyme–ligand interactions of the αF146Y mutant thus results in an almost 10-fold increased affinity of the αF146Y/βF24A double mutant for α-methylphenylacetic acid compared with the wild-type enzyme (Table II).

Structural comparison of open conformations

The data presented here show that ligand-induced structural rearrangements of the active site of PA play an important role in ligand binding. This is evident, for example, from the transition from an open to a closed active site that the βF24A mutant undergoes upon binding of α-methylphenylacetic acid. Similarly, binding of penicillin G and 3,4-dihydroxyphenylacetic acid to the wild-type enzyme cause the enzyme to switch from the closed to the open conformation (Done *et al*., 1998; Alkema *et al*., 2000; Morillas *et al*., 2003). Superposition of these open and closed conformations shows that in all cases the conformational change involves the movement of the same two residues, αR145 and αF146, whereas the other residues of the acyl binding site, e.g. αM142, βF24, βV56, and βS67, and the catalytic βS1 do not change position (Figure 4). In all open structures the side chain of αR145 occupies the same position, making a water-mediated hydrogen bond to the main-chain carbonyl oxygen of βF460. In the structure with penicillin G and the empty βF24A structure, an additional water-mediated H-bond to αD68:OD2 further stabilizes the side chain of this residue. The side chain of αR145 also assists in transition-state stabilization in the hydrolysis of penicillin G by the formation of a hydrogen bond between αR145:NH1 and the carboxylate group of the thiazolidine ring of the 6-APA group (Alkema *et al*., 2002b). The fact that the same position is occupied by αR145, irrespective of whether the open conformation is caused by a mutation in the enzyme, or by binding a substrate or inhibitor, indicates that this open conformation corresponds to a distinct low-energy conformation of the enzyme, in line with suggestions made by Done *et al*. (1998).

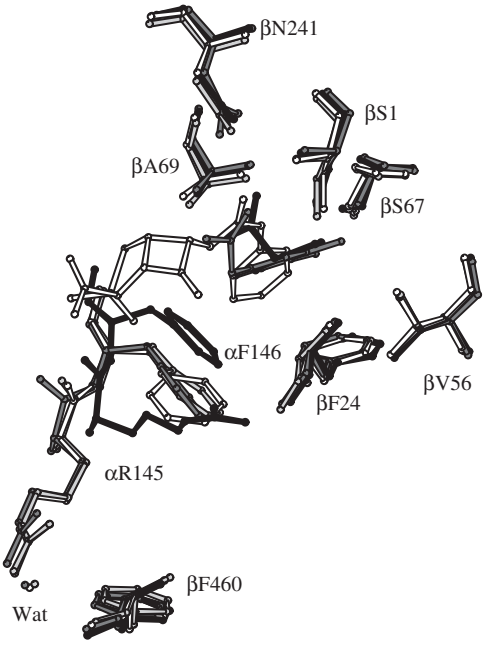


Fig. 4. Comparison of structures of PA in the open and closed conformation. The open conformations are induced by binding of the substrate penicillin G (white), binding of the inhibitor 3,4-dihydroxyphenylacetic acid (dark grey) and the βF24A mutation (light grey). The closed conformation of the wild-type enzyme complexed with PAA is shown in black. In all open conformations αR145 occupies the same position, in which the side chain is stabilized by hydrogen bonding to βF460:O, mediated by a water molecule (Wat). The side chain of αF146 can adopt multiple conformations dependent on the type of ligand that is bound in the active site. The positions of the residues in the catalytic centre, βS1, βA69 and βN241 and in the acyl binding site βF24, βV56 and βS67 are not affected by the conformational change.

In contrast to the well-defined open position of the side chain of αR145, the side chain of αF146 has multiple conformations (Figure 4), depending on the nature of the bound ligands. In the enzyme–penicillin G complex the phenyl ring has van der Waals contacts with the β-lactam moiety of the substrate. In the complexes with substituted phenylacetic acids, the phenyl

ring shifts to give an optimal configuration for aromatic–aromatic interactions (Done *et al*., 1998). The plasticity of αF146 provides an explanation for the ability of (*S*)-α-ethylphenylacetic acid still to bind to the enzyme, even though the structure of the wild-type enzyme shows that steric hindrance with αF146 would occur. The conformational change in PA seems to serve two purposes. One function is to create additional space in the active site to allow binding of substrates and inhibitors that do not fit in the pre-aligned active site. Second, αF146 and αR145 reorient to provide additional substrate binding interactions.

The kinetic and structural data presented in this paper have provided insight into the binding of Cα-substituted phenylacetic acids, which are used as acyl donors in the synthesis of semi-synthetic β-lactam antibiotics. Our results show that the size of the substituent and hydrophobicity of the active site and also induced fit mechanisms are the major determinants for substrate specificity in PA. The double mutant αF146Y/βF24A showed a 20-fold reduced inhibition by PAA and an almost 3-fold increased specificity for the substrate phenylglycinamide, indicating that site-directed mutagenesis is a promising way to tailor enzymatic activity towards synthetic substrates that are used in the production of β-lactam antibiotics.

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