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Characterization of the β-lactam binding site of penicillin acylase of *Escherichia coli* by structural and site-directed mutagenesis studies

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The binding of penicillin to penicillin acylase was studied by X-ray crystallography. The structure of the enzyme–substrate complex was determined after soaking crystals of an inactive βN241A penicillin acylase mutant with penicillin G. Binding of the substrate induces a conformational change, in which the side chains of αF146 and αR145 move away from the active site, which allows the enzyme to accommodate penicillin G. In the resulting structure, the β-lactam binding site is formed by the side chains of αF146 and βF71, which have van der Waals interactions with the thiazolidine ring of penicillin G and the side chain of αR145 that is connected to the carboxylate group of the ligand by means of hydrogen bonding via two water molecules. The backbone oxygen of βQ23 forms a hydrogen bond with the carbonyl oxygen of the phenylacetic acid moiety through a bridging water molecule. Kinetic studies revealed that the site-directed mutants αF146Y, αF146A and αF146L all show significant changes in their interaction with the β-lactam substrates as compared with the wild type. The αF146Y mutant had the same affinity for 6-aminopenicillanic acid as the wild-type enzyme, but was not able to synthesize penicillin G from phenylacetamide and 6-aminopenicillanic acid. The αF146L and αF146A enzymes had a 3–5-fold decreased affinity for 6-aminopenicillanic acid, but synthesized penicillin G more efficiently than the wild type. The combined results of the structural and kinetic studies show the importance of αF146 in the β-lactam binding site and provide leads for engineering mutants with improved synthetic properties.

Keywords: β-lactam binding site/penicillin acylase/site-directed mutagenesis/substrate-induced conformational change/X-ray structure

Introduction

Penicillin acylase (PA) of *Escherichia coli* ATCC 11105 (EC 3.5.1.11) is a heterodimeric periplasmic enzyme that can catalyse the hydrolysis of penicillin G to phenylacetic acid and 6-aminopenicillanic acid (6-APA) (Cole, 1969) (Figure 1). The enzyme is of biotechnological interest since it can be used both for the hydrolysis of β-lactam antibiotics and for the preparation of semi-synthetic antibiotics (Bruggink *et al.*, 1998). PA belongs to the family of N-terminal-nucleophile hydrolases, comprising enzymes that share a common fold around the active site and that contain a catalytic nucleophilic residue at an N-terminal position (Brannigan *et al.*, 1995). The catalytic residue in PA is the N-terminal serine of the β-subunit, βS1 (the amino acids are labelled to indicate the polypeptide chain, α or β, and the residue number in this chain) (Duggleby *et al.*, 1995). The OG of this serine is activated by its own amino group, possibly via a bridging water molecule, after which the carbonyl carbon of the substrate is attacked. The oxyanion that is formed in the transition state is stabilized by hydrogen bonds to the ND1 of βN241 and the backbone amide of βA69. Collapse of the tetrahedral intermediate under expulsion of the leaving group gives an acyl-enzyme intermediate, which can be cleaved by water or a different nucleophile yielding the acid or the condensation product, respectively (Figure 2).

The acyl group binding site of PA is a pocket made up of several hydrophobic residues, rendering the enzyme highly specific for the phenylacetyl group of penicillin G. A variety of groups can replace the β-lactam nucleus as the leaving group in the hydrolysis reaction (Huang *et al.*, 1963; Cole, 1969; Svedas *et al.*, 1996; Stambolieva *et al.*, 1998) (Figure 1) and no high-affinity site for the leaving group has been identified. A structure of the enzyme complexed with an intact penicillin molecule has not been reported. The fact that PA is capable of accepting a wide range of structurally different compounds including phenylacetylated derivatives of anilines, alcohols, α- and β-amino acids and several β-lactams, indicates that a possible leaving group binding site should have a low specificity. It was suggested that a positively charged group would be present close to the entrance of the hydrophobic phenylacetyl binding pocket where it could have an interaction with the negatively charged carboxylate group of β-lactam nuclei and amino acids (Svedas *et al.*, 1996). The observed inhibition constants of 10–100 mM for 6-APA and 7-ADCA indicate that the β-lactam nucleophiles have a binding site on the enzyme (Balasingham *et al.*, 1972; Kutzbach and Rauenbusch, 1974; Ospina *et al.*, 1992) and kinetic evidence for a nucleophile binding site has been obtained by Kasche *et al.* (1984). However, the geometry of the proposed binding site and the function of specific amino acid residues in binding of leaving groups and alternative nucleophiles is still unclear.

Binding of the β-lactam nucleus is important for the application of PA in the production of semi-synthetic antibiotics. In order to obtain structural information on the nucleophile binding site, we constructed the inactive βN241A mutant and soaked crystals of this mutant with penicillin G. The structural data indicated an important role for the residues αR145 and αF146 in binding of the β-lactam moiety of penicillin G. The function of αF146 was further investigated by site-directed mutagenesis and kinetic characterization of mutants. The results showed that substituting the αF146 residue can lead to

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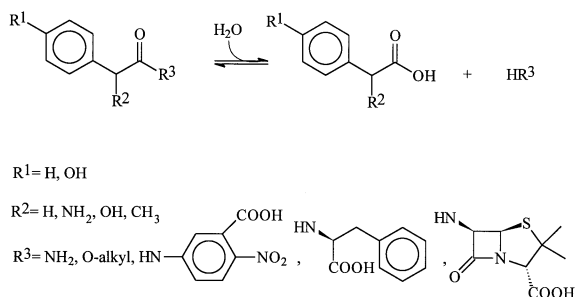

Fig. 1. Reaction catalysed by PA. Only small substituents are accepted on the *para* position (R^1) and the Cα position (R^2) of the phenylacetic acid moiety. A large variety of leaving groups (R^3) are accepted, ranging from small amides and alkyl esters to amino acids and β-lactam nuclei (Huang *et al.*, 1963; Cole, 1969; Svedas *et al.*, 1996).

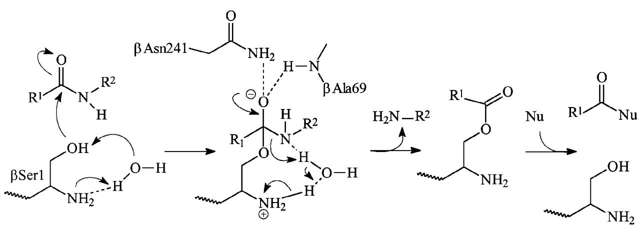

Fig. 2. Mechanism of PA-catalysed reactions as suggested by Duggleby *et al.* (1995). The nucleophilic serine attacks the carbonyl carbon of the peptide bond. The oxyanion, formed in the tetrahedral transition state, is stabilized by the ND1 of βN241 and the backbone of βA69. After collapse of the transition state, the acyl-enzyme is formed and the first product (H_2NR^2) is released from the active site. Cleavage of the covalent intermediate by a second nucleophile (Nu) occurs via the same transition state after which the second product is released. R^1 is a phenylacetic acid derivative whereas R^2 may vary, as depicted in Figure 1.

mutants which are improved with respect to their use in the synthesis of β-lactam antibiotics.

Materials and methods

Strains and plasmids

For site-directed mutagenesis and expression of the recombinant enzymes, the plasmid pEC was used, which was provided by DSM – Gist (Delft, The Netherlands). This plasmid carries the penicillin acylase gene of *E.coli* ATCC 11105, but has a βV148L mutation compared with the GenBank sequence 42247. *E.coli* HB101 was used as a host.

PCR mutagenesis

Mutants were made by PCR. For introducing the αF146 mutations, a fragment of 305 bp was amplified from plasmid pEC using the oligonucleotide 5′-GAAGTGCTTGGCAAA-3′ as the forward primer. This primer anneals 21 bp upstream of an *Eco*RV site in the PA gene. As the reverse primer 5′-GCCAGATT*ATCGAT*TTCGCTAGTACTATCAGA<u>XXX</u>-GCGGTTT-3′ was used. In this primer a *Cla*I site is shown in italics and the codon for amino acid αF146 is underlined. The codons GTA, GGC and GAG were used to change αF146 to Tyr, Ala and Leu, respectively. After amplification using Pwo polymerase (Boehringer Mannheim), the fragment was digested with *Cla*I and *Eco*RV and ligated into pEC that was digested with the same enzymes.

The reverse primer used to construct the βN241A mutant was 5′-AAAGGCAAAC*AGATCT*GAAGCGGGATAATCTT-TTTGGGGAGAATT<u>CGC</u>CCAGTTAGC-3′ in which a *Bgl*II site is shown in italics. Introduction of the underlined codon for the βN241A mutation simultaneously creates an *Eco*RI restriction site. As a forward primer, 5′-CTGACCAGACGA-CACAAACGGCTTA-3′ was used which binds 30 bp upstream of an *Nhe*I site in the PA gene. The 382 bp fragment was amplified using PCR and after digestion with *Nhe*I and *Bgl*II ligated into pEC that was digested with the same enzymes.

Competent *E.coli* cells were transformed with ligation mixtures using standard protocols (Sambrook *et al.*, 1989). Transformants containing the desired fragment were sequenced in order to confirm the mutation and the absence of second site mutations.

Purification

Wild-type and mutant PAs were obtained as follows. Recombinant *E.coli* was grown at 17°C in LB medium (Sambrook, *et al.*, 1989) at 150 r.p.m. and induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested in the late exponential phase by centrifugation at 5000 *g* for 10 min. The pellet was resuspended in 1/10 volume of ice-cold osmotic shock buffer A (20% sucrose, 100 mM Tris–HCl, pH 8.0, 10 mM EDTA) and centrifuged for 10 min at 5000 *g*. The pellet was then resuspended in 1/10 volume of ice-cold osmotic shock solution B (1 mM EDTA) and centrifuged for 10 min at 5000 *g*. Potassium phosphate buffer (1 M, pH 7.0) was added to the supernatant (periplasmic extract) to a final concentration of 50 mM, then $(NH_4)_2SO_4$ was added to a final concentration of 1.5 M. The sample was loaded on a Resource-Phe column (Amersham Pharmacia Biotech) and eluted with a linear gradient of 1.5–0 M $(NH_4)_2SO_4$ in 50 mM phosphate buffer, pH 7.0. The PA of *E.coli* eluted at a concentration of 1 M $(NH_4)_2SO_4$.

This preparation was used for kinetic experiments but was further purified for crystallization experiments. Enzyme eluted from the Resource Phenyl column was pooled and desalted by dialysis against 2×1 l of 50 mM Tris–HCl, pH 8.5. The desalted protein was then applied to a HiTrap-Q column (Amersham Pharmacia Biotech) mounted on an Äkta explorer (Amersham Pharmacia Biotech). A gradient from 0 to 250 mM NaCl in 50 mM Tris–HCl, pH 8.5, was used to elute the enzyme. PA eluted from the column at 37 mM NaCl.

Crystallization and data collection

Purified PA was crystallized as described earlier, with small modifications (McVey *et al.*, 1997). After purification, the buffer was exchanged for 100 mM MOPS, pH 7.5, and the sample was concentrated using a Microsep 30 filter (Filtron) to a final concentration of 10 mg/ml. Crystals were obtained with the hanging drop vapour diffusion technique at 4°C. Enzyme solution (2.5 μl, 8 mg/ml) was mixed with 2.5 μl of precipitant containing 12–15% PEG MME 2000 and 50 mM MOPS, pH 7.2, and crystals with a bad morphology (two-dimensional intergrown plates) typically appeared within 1–2 weeks. These crystals were used for streak seeding as described by McVey *et al.* (1997). Crystals from streak seeding showed better morphology and were used for data collection.

Crystals with bound penicillin G and crystals with bound phenylacetic acid were obtained by soaking crystals of βN241A PA with 10 and 1 mM penicillin G in mother liquor [20% (w/v) PEG MME 2000, 4.4% (v/v) glycerol and 50 mM MOPS, pH 7.2] for 22 min and 5.5 h, respectively.

Data were collected from single flash-frozen crystals (120 K). For this, the crystals were transferred from the drop to a mother liquor containing 20% (w/v) PEG MME 2000, 4.4% (v/v) glycerol and 50 mM MOPS, pH 7.2. Prior to freezing, the crystals were dipped in a cryo solution, containing 35%

Table I. Data collection and refinement parameters

	PA βN241A Pen G (22 min soak)	PA βN241A Pen G (5.5 h soak)
Collected at	DIP2030	Hamburg X31
a (Å)	50.93	51.01
b (Å)	63.97	64.08
c (Å)	64.19	64.23
α	72.85	72.92
β	73.77	73.91
γ	74.06	73.54
Resolution	20–2.25	20–1.97
Unique reflections	32510	50127
Completeness (%)[a]	94.4 (79.9)	97.3 (95)
I/σ^a	11.8 (3.2)	15.7 (5.1)
$R_{work}{}^b$	0.187	0.181
$R_{free}{}^b$	0.236	0.215
R.m.s.d. bond lengths (Å)	0.0055	0.0048
R.m.s.d. bond angles (°)	0.98	0.94
R.m.s.d. improper angles (°)	0.73	0.72
PDB accession number	1FXV	1FXH

[a]In parentheses: outer-shell values.
[b]R_{free} and R_{work} as implemented in X-PLOR/CNS (Brünger, 1992; Brünger *et al.*, 1998).

(w/v) PEG MME 2000, 6.5% (v/v) glycerol and 50 mM MOPS, pH 7.2. The crystals showed a large variation in size but only small crystals with typical dimensions of 0.2×0.1×0.04 mm could successfully be frozen. Larger crystals tended to give double spots and smeared diffraction images.

Diffraction data were collected using Cu Kα radiation from a rotating anode source and a MacScience DIP2030 area detector. The 5.5 h penicillin G soak of βN241A was measured at beamline X31 of the EMBL outstation at DESY, Hamburg. Data were processed using Denzo and Scalepack (Otwinowsky, 1993).

Molecular replacement and model refinement

The unit cell of PA crystals in this study deviates slightly from previously published unit cells (Hunt *et al.*, 1990; Duggleby *et al.*, 1995) (Table I). Therefore, molecular replacement was used to obtain initial phases. The wild-type structure (1PNK.PDB) was used as a search model in the program AMORE (CCP4, 1994). Electron density of phenylacetic acid and penicillin G was already clearly visible after rigid body refinement. The obtained solution was used for further refinement in X-PLOR (Brünger, 1992), including Powell energy minimization and overall and individual *B*-factor refinement. After four rounds of refinement the ligands were modelled with O in the improved electron density of phenylacetic acid and penicillin G, respectively (Jones *et al.*, 1991). Final refinement cycles were done in CNS (Brünger *et al.*, 1998) using the maximum likelihood function of this program. Data collection and refinement statistics are presented in Table I. Penicillin G was present with both the thiazolidine and the β-lactam ring intact as has been observed in the electron density of isopenicillin N (Burzlaff *et al.*, 1999).

Models of the βN241A/αF146Y and βN241A/αF146L mutants were built using the InsightII modelling package (Biosym/MSI, San Diego, CA) and used for calculation of the α146–β31 distances and the distances between the α146 residue and the substrate.

Determination of kinetic parameters

Steady-state kinetic parameters were determined by measuring initial velocities at different substrate concentrations and fitting the data using the program Enzfit (Elsevier Biosoft, 1987). The hydrolysis of 2-nitro-5-[(phenylacetyl)amino]benzoic acid (NIPAB) and D-2-nitro-5-[(phenylglycyl)amino]benzoic acid (NIPGB) was followed by measuring the increase in absorbance at 405 nm in a Perkin-Elmer spectrometer. Rates were calculated using a Δε of 9.09 $mM^{-1}\cdot cm^{-1}$ for the conversion of NIPAB and NIPGB. The inhibition constants of the β-lactam nuclei were determined by measuring the K_m for the hydrolysis of NIPGB in the presence of various concentrations of inhibitor. 6-APA was found to be a competitive inhibitor and the inhibition constant was calculated using

$$K_{m,app} = K_m\{1 + ([I]/K_i)\} \quad (1)$$

where $K_{m,app}$ is the K_m in the presence of inhibitor, [I] the inhibitor concentration and K_i the inhibition constant. Conversion of phenylacetamide and penicillin G was followed by reversed-phase HPLC using a Chrompack C_{18} column with Jasco PU-980 pumps and a Jasco MD-910 detector set at 214 nm. All compounds were eluted isocratically using a solution containing 340 mg/l SDS, 5 mM phosphate, 30% acetonitrile, which was adjusted to pH 3.0 with dilute phosphoric acid. All enzymatic conversions were carried out in a 50 mM phosphate buffer at pH 7.0 at 30°C.

Chemicals

NIPAB was purchased from Sigma Chemical and NIPGB from Syncom (Groningen, The Netherlands). Penicillin G was a gift of DSM – Gist (Delft, The Netherlands). PEG MME 2000 was purchased from Hampton Research.

Results

Crystallization of an inactive βN241A mutant with penicillin G

Binding constants of wild-type penicillin acylase for β-lactam nuclei are of the order of 10–100 mM (Kutzbach and Rauenbusch, 1974; Kasche *et al.*, 1984; Ospina *et al.*, 1992). Since the solubility of these compounds is approximately the same, co-crystallization or soaking crystals with β-lactam nuclei was expected to be difficult. Since co-crystallization or soaking with the natural substrate penicillin G results in hydrolysis, due to catalytic activity in the crystals (Duggleby *et al.*, 1995), we studied the interaction of the enzyme with the β-lactam moiety by soaking crystals of an inactive mutant with penicillin G. An inactive mutant was made by replacing asparagine β241 by an alanine, thereby removing the side chain amide that stabilizes the oxyanion that is formed in the transition state. The βN241A mutant was about 10 000-fold less active than the wild type using NIPAB as the substrate. When periplasmic extracts of clones overexpressing this mutant protein were analysed on SDS–PAGE gels, a prominent band with a molecular weight of ~90 kDa was seen, indicating the presence of large amounts of precursor protein. This suggests that export of the precursor protein to the periplasmic space takes place, but that processing is severely impaired in this mutant, although not completely blocked. After incubation for 48 h at room temperature, the precursor protein precipitated, whereas the processed mutant protein that was present remained soluble and could be purified as described. After crystallization, the βN241A mutant was soaked with penicillin G. Soaking the crystals for 22 min with penicillin G revealed the intact

substrate in the active site, although not fully occupied as indicated by higher *B*-factors of the penicillin G (Figure 3A). Soaking for 5.5 h resulted in crystals with an intermediate structure, where the active site was occupied with phenylacetic acid, showing that slow hydrolysis of the amide bond still can take place without the stabilizing side chain of the asparagine in the oxyanion hole.

A comparison of the structure of the wild type with phenylacetic acid and the βN241A structure with penicillin G in the active site showed that the catalytic residues βS1 and βA69 and the residues that are close to βN241, such as βR263 and βW240, have not shifted position (Figure 3D). The position of the side chain of the mutated asparagine is now occupied by an H_2O molecule (H_2O_{175}). This indicates that the mutation does not cause major structural changes in the active site and that the loss of activity is indeed caused by the removal of the asparagine side chain and not by the incorrect folding of the active site.

Upon binding of the penicillin G to the enzyme, the position of the phenyl ring of the phenylacetic acid moiety changes as compared with the enzyme–phenylacetic acid complex (Figure 3B). Because of this shift, the carbonyl carbon of the bond that is to be hydrolysed in penicillin G is at a distance of 3.3 Å from the OG of the nucleophilic serine, whereas in the structure with phenylacetic acid this carbon atom is located at 2.9 Å from the OG.

A significant difference between the structures of the enzyme–penicillin G and the enzyme–phenylacetic acid complex is the position of the side chains of αR145 and αF146 (Figure 3B). The side chain of αR145 is protruding into the solvent and is at a distance of 8 Å from the carboxyl group of the ligand. Two water molecules are positioned in such a way that hydrogen bonding between the NH1 atom of αR145 and one of the oxygen atoms of the carboxylate group can take place. The side chain of αF146 has moved away from the binding pocket towards βY31, thereby opening the active site in order to bind penicillin G. The CD1 and CG atoms of the side chain of αF146 in this open conformation are located at 3.1 and 3.5 Å from the 2β-methyl group of the thiazolidine ring of penicillin G. If penicillin G were bound to the enzyme without this conformational change taking place, the CD1 atom would be at a distance of 1.8 Å from this methyl group, creating steric hindrance. This suggests that the αF146 has to shift position in order to accommodate the large β-lactam nucleus in the active site. The short distance between the CD1 and the 2β-methyl group of the thiazolidine ring of penicillin G indicates that in this conformation αF146 assists in binding of the substrate by van der Waals interactions with the β-lactam moiety of the substrate.

The side chain of βF71 moves slightly upon binding of penicillin G towards a staggered stacked conformation with the thiazolidine moiety of the substrate. In this conformation the CZ atom of βF71 is located at 3.2 Å from the sulfur atom and the C5 atom of the thiazolidine moiety. The backbone carbonyl oxygen of the βQ23 residue is hydrogen bonded to the carbonyl oxygen of the phenylacetic acid moiety via an H_2O molecule in the active site. This H_2O molecule is only observed in the structure with penicillin G and might be the nucleophilic water that deacylates the acyl enzyme. Van der Waals interactions of the main chain oxygen of βQ23 with the carbonyl oxygen of the β-lactam ring, which is at 3.4 Å, may also take place (Figure 3C).

The results indicate that both van der Waals interactions and hydrogen bonding play a role in binding of the β-lactam moiety of penicillin G. The fact that the distances between the enzyme and penicillin G are shorter than expected for van der Waals interactions may be caused by the low occupancy of penicillin G in the active site.

Activity of the site-directed mutants

The structure of the penicillin G–enzyme complex showed a positional shift of αF146. This phenylalanine closes the active site, shielding the hydrophobic binding site from the solvent (Figure 3B) and upon binding of the substrate the phenyl ring assists in binding of the β-lactam moiety (Figure 3C). The phenylalanine on this position is conserved in penicillin acylases of *E.coli*, *Alcaligenes faecalis* and *Kluyvera citrophila* (Verhaert *et al*., 1997). In order to study the role of this residue in more detail, it was mutated to an alanine, leucine or tyrosine. The effect of the mutations was studied by measuring the steady-state kinetic parameters for the chromogenic substrate NIPAB as well as for penicillin G and phenylacetamide (Table II).

The αF146Y mutant enzyme had similar kinetic parameters for phenylacetamide and NIPAB, but the k_{cat} for the hydrolysis of penicillin G was about 50-fold lower than the wild-type value. For the αF146L mutant the reverse effect was observed. Whereas the k_{cat} values for phenylacetamide and NIPAB were 10–100-fold lower, the k_{cat} for penicillin G was only halved compared with the wild type. The αF146A mutant showed a reduced activity for all substrates tested. The K_m values of the αF146Y were similar to wild-type values whereas for the αF146L and αF146A enzymes, in which the aromatic ring was removed, the affinity had decreased 10–30-fold. These results suggest that an aromatic ring on position α146 is necessary for efficient binding of the substrate. It has been observed that in PA-catalysed hydrolysis reactions, the rate-limiting step in the catalytic cycle is the formation of the covalent intermediate, which is much slower than the hydrolysis of the acyl-enzyme (Roa *et al*., 1996; Morillas *et al*., 1999). The k_{cat} values in the αF146 mutants are strongly dependent on the type of leaving group, which indicates that also in these mutants the rate-limiting step is the formation of the covalent intermediate. The effect of the leaving group on the k_{cat}, however, is different for each mutant, suggesting an interaction of the α146 residue with this part of the substrate. This is also suggested by the values of the inhibition constants of 6-APA, which increased about 5-fold in the αF146A and αF146L mutant as compared with the wild-type enzyme and the αF146Y mutant (Table II). These findings are in line with the

Fig. 3. Structures of the βN241A mutant complexed with penicillin G. (A) Simulated annealed omit map of penicillin G density contoured at 3σ after soaking for 22 min. (B) Overlay of the mutant βN241A soaked for 22 min with 10 mM penicillin G and wild-type PA soaked with penicillin G (hydrolysed to phenylacetic acid) (1PNL.PDB) (Duggleby *et al*., 1995). (C) Mutant βN241A soaked for 22 min with 10 mM penicillin G, showing the open conformation of the enzyme and the residues involved in binding of the substrate. Distances between the enzyme–substrate complex and the water molecules are as follows, αR145–H_2O_{222}, 2.7 Å; H_2O_{222}–H_2O_{291}, 2.9 Å; H_2O_{291}–PenG, 2.9 Å; βQ23–H_2O_{290}, 2.6 Å; H_2O_{290}–PenG, 2.6 Å. (D) Overlay of the mutant βN241A soaked for 22 min with 10 mM penicillin G and wild-type PA soaked with penicillin G (hydrolysed to phenylacetic acid) (1PNL.PDB) (Duggleby *et al*., 1995), showing the oxyanion hole and neighbouring residues. This figure was produced using BOBSCRIPT (Esnouf, 1997).

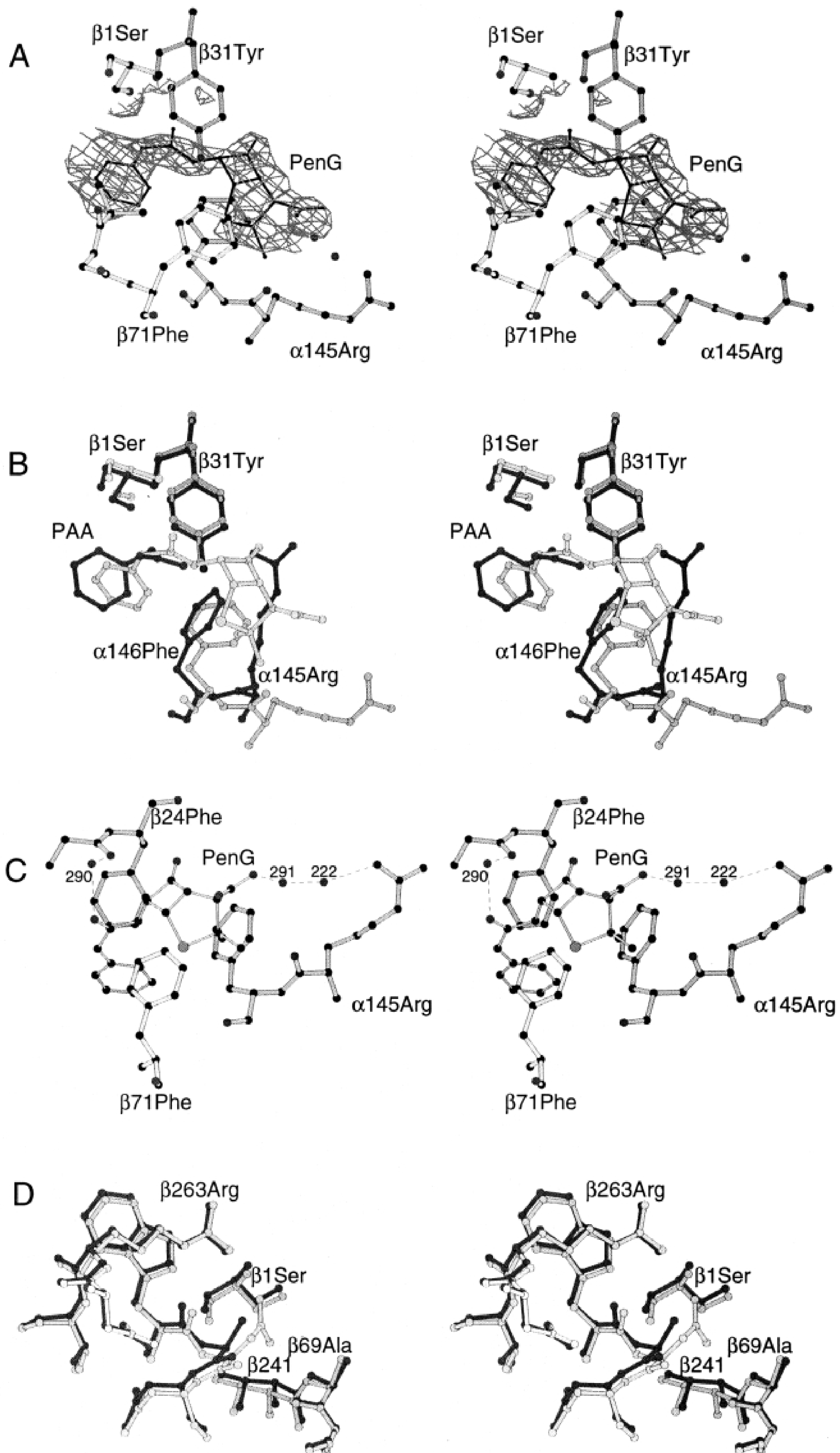
A
β1Ser
β31Tyr
PenG
β71Phe
α145Arg
B
β1Ser
β31Tyr
PAA
α146Phe
α145Arg
C
β24Phe
PenG
290
291
222
α145Arg
β71Phe
D
β263Arg
β1Ser
β69Ala
β241

Table II. Steady-state kinetic parameters of WT and αF146 mutants of penicillin acylase.

	Phenylacetamide			NIPAB			Penicillin G			6-APA
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	K_i (mM)
WT	50	160	0.31	16.2	11.3	1.43	42	7	6	32
αF146Y	22	44	0.5	1.8	4.8	0.375	1.5	5	0.3	28
αF146A	1	1.7×10^3	5.8×10^{-3}	1.5×10^{-2}	55	3×10^{-4}	2.3	141	0.023	180
αF146L	4	2.3×10^3	1.7×10^{-3}	3.8×10^{-2}	25	1.5×10^{-3}	25	147	0.41	116

Values are means of at least two independent experiments. Standard deviations are ≤10% from the mean value.

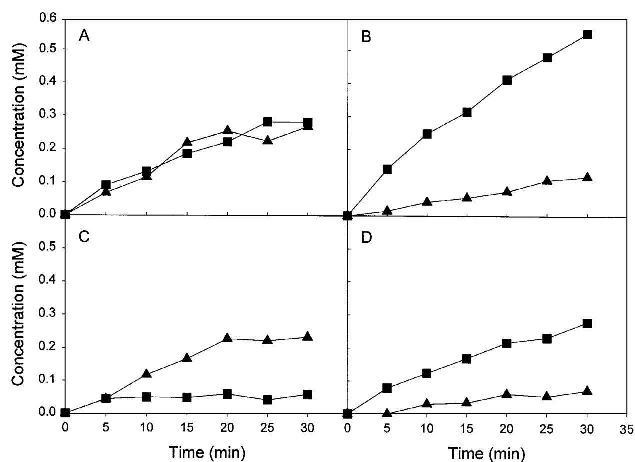

Fig. 4. Synthesis of penicillin G (■) and formation of phenylacetic acid (▲) from 15 mM phenylacetamide and 25 mM 6-APA by wild type and αF146 mutants. (A) 10 nM wild type; (B) 100 nM αF146L; (C) 10 nM αF146Y; (D) 500 nM αF146A.

data obtained from the structure of the penicillin G-bound enzyme.

Interaction of αF146 with the β-lactam nucleus

The activity measurements with the mutant enzyme suggested that the interaction of the mutant enzyme with substrates was dependent on the type of leaving group. To study the interaction of β-lactam substrates with the acyl enzyme of these mutants, we measured the kinetics of the synthesis of penicillin G, using 6-APA as the nucleophile and phenylacetamide as the acyl donor. The acylated enzyme can be deacylated by H_2O or 6-APA, yielding either phenylacetic acid or penicillin G.

All mutant enzymes differed markedly from the wild-type enzyme in their ability to synthesize penicillin G (Figure 4). It appeared that 6-APA was no longer able to deacylate efficiently the covalent intermediate of αF146Y and small amounts of penicillin G were synthesized. The rapid production of phenylacetic acid is in agreement with the observed steady-state parameters for the hydrolysis of phenylacetamide and indicate that H_2O is still able to deacylate the αF146Y enzyme efficiently. Since the wild-type and the αF146Y mutant enzyme have a similar affinity for 6-APA (Table II), this difference in synthesizing capacities seems to be caused by a difference in reactivity of bound 6-APA rather than by differences in the degree of saturation of the acyl enzyme with 6-APA. Using the αF146L and αF146A mutants, much more penicillin G than phenylacetic acid was produced, indicating that 6-APA very efficiently deacylates the αF146L and αF146A mutant enzymes, even though binding of 6-APA is weaker. The results indicate that the synthesizing capacity of penicillin acylase, i.e. the ratio of aminolysis by a β-lactam nucleophile vs hydrolysis by H_2O, can be improved by mutations that influence the interaction of the enzyme with the substrate in the β-lactam binding site.

Discussion

Structural analysis of the βN241A mutant soaked with penicillin G revealed the presence of a β-lactam binding site, which is only formed after binding of the substrate. Upon binding of penicillin G the enzyme undergoes a conformational change in which the side chains of the amino acid residues αR145 and αF146 shift away from the active site. Comparison of the structure of the penicillin-bound enzyme with the wild-type structure thus indicates that binding proceeds via an induced fit mechanism. In the wild-type structure, the binding site for the β-lactam moiety is occupied by the side chain of αF146. The CD1 atom of the phenylalanine in the position of the native enzyme would be at 1.8 Å from the β-lactam group. This would lead to steric hindrance between this residue and the thiazolidine ring of the substrate if no conformational chance took place. In the structure of wild-type penicillin acylase with 3,4-dihydroxyphenylacetic acid bound to the enzyme (1AI4.PDB), the same outward motion of αR145 and αF146 takes place. In this structure the CD1 atom of αF146 in the closed (native) conformation would be at a distance of 1.85 Å from the Cα atom of the ligand, causing steric hindrance with the ligand (Done *et al.*, 1998). These findings suggest that when a ligand is bound in the active site in such a way that steric conflicts with the side chain of αF146 arise, the enzyme switches to a new low-energy conformation, in which the phenylalanine has moved away from the active site towards βY31 and αR145 towards the solvent (open conformation).

Three amino acid residues assist in binding of the β-lactam moiety of the substrate. The side chain of αR145 is 8 Å away from the carboxylate group of the β-lactam nucleus but has an interaction with an oxygen of this carboxylate group via two bridging water molecules. This is in line with observations made by Svedas *et al.* (1996), who concluded that a positively charged residue in or near the active site might play a role in binding nucleophiles by interacting with the negatively charged carboxylate group. For this interaction to take place, the enzyme has to be in the open conformation, since in the closed form the side chain of this αR145 is pointing towards the active site and forms a hydrogen bond with the carbonyl oxygen of βF24.

Upon binding of penicillin G, the phenylalanine on position β71 moves slightly towards a stacked conformation with the thiazolidine ring of penicillin G and is probably involved, by means of hydrophobic interactions, in binding of the hydrophobic β-lactam nucleus. Roa *et al.* (1994) identified an

F360V mutant of penicillin acylase of *K.citrophila*, which was obtained after random mutagenesis and showed that it had altered substrate specificity. The mutant was able to hydrolyse glutaryl-L-leucine and NIPAB, but could not cleave the corresponding β-lactam substrates with the same acyl group, glutaryl-7-ACA and penicillin G. Since F360 is equivalent to βF71 in the *E.coli* enzyme, this indicates that also in PA of *K.citrophila* this phenylalanine is involved in binding of the β-lactam moiety.

The most important residue for interaction with the leaving group of the substrate is the phenylalanine on position α146. Kinetic data indicate that removing the aromatic function on position α146 decreases the affinity for the phenylacetylated substrates. Svedas *et al.* (1996) have already proposed that hydrophobic interactions are the most important factor governing substrate binding. Moreover, it seems that an aromatic function is needed for correct positioning of the substrate relative to the catalytic serine in the active site, since also the k_{cat} for penicillin G in the αF146L and αF146A mutants has decreased significantly.

Mutating αF146 to a tyrosine could influence the binding of β-lactam substrates in the following way: when the phenylalanine moves to the open conformation, the CZ position is located 3.2 Å away from the hydroxyl group of βY31, whereas in the closed conformation the distance is 4.6 Å. A tyrosine on position αF146 would place the hydroxyl groups of both tyrosine residues at 4.0 Å from each other in the closed structure, but this distance would decrease to 2.1 Å in the open conformation of the enzyme. Therefore, steric hindrance of the phenyl ring of residue α146 of the αF146Y mutant may occur when the enzyme switches to the open conformation, which may explain the reduced k_{cat} value of the αF146Y mutant for hydrolysis of the β-lactam substrate penicillin G. In contrast to the αF146Y mutant, a conformational change in the αF146L mutant would place the side chain of the leucine at a distance of 4.6 Å from βY31. This may explain the fact that the αF146L mutant is still able to hydrolyse and synthesize penicillin G efficiently.

The observation that soaking the βN241A mutant with penicillin G yielded a structure with intact substrate in the active site confirms the crucial role of the asparagine residue in catalysis by means of stabilizing the oxyanion (Duggleby *et al.*, 1995). However, a low but significant activity for NIPAB was observed and also prolonged incubation of the crystals with penicillin G resulted in a structure with phenylacetic acid in the active site. Probably also the backbone amide of βA69 plays a role in stabilizing the negative charge of the oxyanion, as suggested by Duggleby *et al.* (1995), allowing catalysis to take place, albeit at a much lower rate.

The βN241A mutant is also impaired in processing, as indicated by the large amount of precursor protein formed. This indicates that an intact oxyanion hole is necessary for maturation of the enzyme, suggesting that this is an autocatalytic process as proposed for the family of Ntn-hydrolases (Brannigan *et al.*, 1995). The structure of the precursor protein might give more insight into the precise details of the processing events (Hewitt *et al.*, 1999).

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