Role of αArg^{145} and βArg^{263} in the active site of penicillin acylase of *Escherichia coli*

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The active site of penicillin acylase of *Escherichia coli* contains two conserved arginine residues. The function of these arginines, αArg^{145} and βArg^{263}, was studied by site-directed mutagenesis and kinetic analysis of the mutant enzymes. The mutants αArg$^{145} \rightarrow$ Leu (αArg145Leu), αArg145Cys and αArg145Lys were normally processed and exported to the periplasm, whereas expression of the mutants βArg263Leu, βArg263Asn and βArg263Lys yielded large amounts of precursor protein in the periplasm, indicating that βArg^{263} is crucial for efficient processing of the enzyme. Either modification of both arginine residues by 2,3-butanedione or replacement by site-directed mutagenesis yielded enzymes with a decreased specificity (k_{cat}/K_m) for 2-nitro-5-[(phenylacetyl)amino]benzoic acid, indicating that both residues are important in catalysis. Compared with the wild type, the αArg^{145} mutants exhibited a 3–6-fold-increased preference for 6-aminopenicillanic acid as the deacylating nucleophile compared with water. Analysis of the steady-state parameters of these mutants for the hydrolysis of penicillin G and phenylacetamide indicated that destabilization of the Michaelis–Menten complex accounts for the improved activity with β-lactam substrates. Analysis of pH–activity profiles of wild-type enzyme and the βArg263Lys mutant showed that βArg^{263} has to be positively charged for catalysis, but is not involved in substrate binding. The results provide an insight into the catalytic mechanism of penicillin acylase, in which αArg^{145} is involved in binding of β-lactam substrates and βArg^{263} is important both for stabilizing the transition state in the reaction and for correct processing of the precursor protein.

Key words: β-lactam, butanedione, pH-dependence, site-directed mutagenesis, transition-state stabilization.

INTRODUCTION

Penicillin acylase (PA) of *Escherichia coli* (EC 3.5.1.11) is a heterodimeric, periplasmic protein consisting of a small α-subunit and a large β-subunit. The enzyme is produced as an inactive preproprotein that contains a leader peptide directing the protein to the periplasm, and a spacer peptide separating the α- and β-subunits. The spacer peptide is removed during translocation to the periplasm, probably by an autocatalytic process, yielding the active form of the enzyme.

The enzyme belongs to the family of N-terminal nucleophile ('Ntn') hydrolases, a class of enzymes that share a common fold around the active site and contain a catalytic nucleophile on the N-terminal position [1]. In PA of *E. coli*, the catalytic nucleophile is a serine that is located on the N-terminal position of the large β-subunit. PA is used industrially to catalyse the hydrolysis of the amide bond in penicillin G, producing phenylacetic acid and the free β-lactam moiety 6-aminopenicillanic acid (6-APA). The enzyme is also used in the preparation of semi-synthetic β-lactam antibiotics, in which PA catalyses the reverse reaction, i.e. the condensation of 6-APA with a synthetic acyl donor [2].

The reaction catalysed by PA proceeds via an acyl-enzyme intermediate in which the catalytic serine residue is covalently bound to the acyl moiety of the substrate via an ester linkage. During formation and cleavage of the covalent intermediate, a tetrahedral intermediate occurs, of which the negatively charged carbonyl oxygen is stabilized by hydrogen bonds to the backbone amide of βAla^{69} and the side chain of βAsn^{241} (the amino acids are labelled to indicate the polypeptide chain, α or β, and the residue number in this chain) [3].

Structural and kinetic studies have suggested the involvement of two charged active-site arginine residues, αArg^{145} and βArg^{263}, in the catalytic cycle of PA [4–6]. In the native enzyme, the side chain of αArg^{145} points towards the active site, forming a hydrogen bond with the main chain oxygen of the active-site residue βPhe^{24} (Figure 1). X-ray structure analysis of the enzyme with the substrate penicillin G bound showed that αArg^{145} displays a large conformational change upon binding of the substrate, by moving away from the active site. The positional shift of αArg^{145} and the neighbouring αPhe^{146} is necessary to create space in order to accommodate the β-lactam moiety of the substrate. In this new conformation, the hydrogen bonds between αArg^{145}:NH2 and the main chain carbonyl oxygen of βPhe^{24} are replaced by new hydrogen bonds between αArg^{145}:NH2 and the carboxylate group of the substrate via two bridging water molecules [4] (Figure 1).

Arginine βArg^{263} is located near the oxyanion hole, and is hydrogen-bonded with its NH2 atom to the OG atom of βAsn^{241}, the residue stabilizing the transition state in the reaction. Morillas et al. [5] studied the pH-dependence of the hydrolysis of *p*-nitrophenylacetate and found a bell-shaped pH profile for the k_{cat} and k_{cat}/K_m parameters. It was suggested that the pK_a of the descending limb reflected the deprotonation of βArg^{263}, and that a positive charge on this position was necessary for activity.

In agreement with these structural and kinetic studies are the results obtained by Prabhune amd Sivaraman [7], who concluded from inactivation studies with the arginine-specific reagents 2,3-butanedione and phenylglyoxal that an arginine residue is involved in hydrolysis of penicillin G. Moreover, both

Abbreviations used: 6-APA, 6-aminopenicillanic acid; αArg145Leu, mutant bearing a substitution of αArg^{145} with leucine, etc.; NIPAB, 2-nitro-5-[(phenylacetyl)amino]benzoic acid; PA, penicillin acylase.

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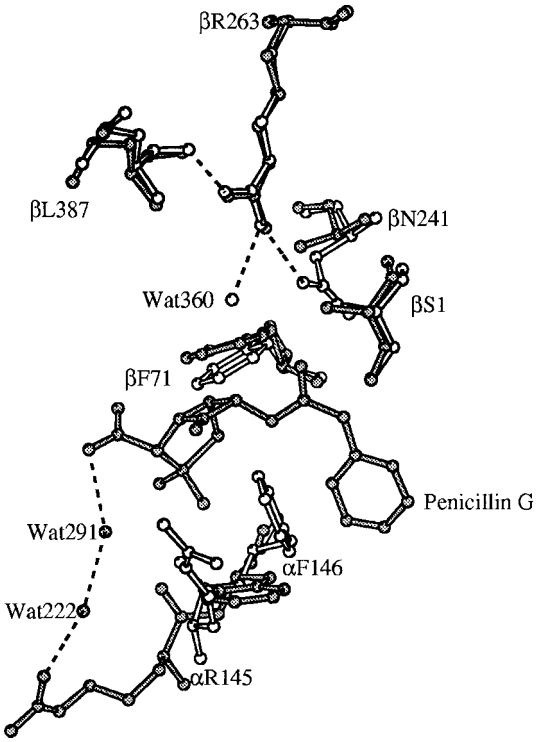

Figure 1 Location of αArg^{145} and βArg^{263} in the active site of *E. coli* PA

The structure of wild-type PA (shown in white; [3]) is superimposed on the structure of the βAsn241Ala mutant complexed with penicillin G, (shown in grey; [4]). The interaction of αArg^{145} with penicillin G via the two ordered water molecules (Wat222 and Wat291), and the interactions of βArg^{263} in the oxyanion hole, are shown with broken lines.

αArg^{145} and βArg^{263} are conserved in PAs of *E. coli*, *Kluyvera citrophila*, *Alcaligenes faecalis* and *Providencia rettgeri*, whereas βArg^{263} is also found in PAs of *Bacillus megaterium and Arthrobacter viscosus*, suggesting an important role for both arginine residues in PA [8–13].

Mutational analysis of these arginine residues, however, has not yet been undertaken, and the exact function of these residues in the catalytic cycle is still not fully understood. To study the role of αArg^{145} and βArg^{263} in the reactions catalysed by the PA of *E. coli*, these residues were changed by site-directed mutagenesis.

The results described in the present study show that the arginine at position β263 is necessary for efficient processing of the precursor protein, but that this arginine mayie be replaced by a lysine without losing catalytic activity. Kinetic analysis of the αArg^{145} mutant enzymes showed that removal of the arginine at this position leads to more efficient hydrolysis and synthesis of β-lactam substrates, indicating that the binding interactions between the arginine and the substrate in the wild-type enzyme lead to reduced catalytic efficiencies in the synthesis and hydrolysis of β-lactam antibiotics.

MATERIALS AND METHODS

Strains and plasmids

The plasmid pEC carrying the PA gene of *E. coli* A.T.C.C. 11105 was provided by DSM-Gist (Delft, The Netherlands), and was used for site-directed mutagenesis and overexpression of the recombinant enzymes, as described previously [4]. *E. coli* strain HB101 was used as a host.

Site-directed mutagenesis

Mutations were constructed by PCR. For introducing the αArg^{145} mutations, a fragment of 305 bp was amplified from the 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′-TGCCAGATT*ATCGAT*TTCGCTAGTACTATCAGAGAA NNNGTTTGCCAT-3′ was used. (In this primer, a *Cla*I site is shown in italics and the codon of amino acid α145 is underlined.) The codons ACA, CTT and CAA were used to substitute αArg^{145} for cysteine, lysine and leucine respectively. After amplification using *Pwo* polymerase (Boehringer Mannheim, Mannheim, Germany), the fragment was digested with *Cla*I and *Eco*RV and ligated into pEC that had been digested with the same enzymes. The forward primer used to construct the βArg^{263} mutant was 5′-CCCGCTTC*AGATCT*GTTTGCCTTTTTGTGGGGTG GTGCCGATNNNGTTACGGAG-3′. (In this primer, a *Bgl*II site is shown in italics and the codon for residue β263 is underlined.) The codons AAA, AAC and CTC were used to substitute βArg^{263} for lysine, asparagine and leucine respectively. As the reverse primer, 5′-TGTTCCACGGTTTTGATACTCCGC-3′ was used, which is located 30 bp downstream of an *Mlu*I site on the plasmid. The fragment was amplified using PCR and, after digestion with *Mlu*I and *Bgl*II, ligated into pEC that was digested with the same enzymes. The double mutant αArg145Lys/βArg263Lys was made by ligation of the *Bgl*II–*Mlu*I fragment containing the βArg263Lys mutation into the plasmid carrying the αArg145Lys mutation, that had been digested with *Bgl*II and *Mlu*I. Competent *E. coli* cells were transformed with ligation mixtures using standard protocols [14]. Transformants containing the desired fragment were sequenced in order to confirm the mutation and the absence of second site mutations introduced by PCR.

Enzyme isolation and purification

Wild-type and mutant PAs were obtained as described below. Strains of *E. coli* carrying the wild-type and mutant genes were grown at 17 °C with rotary shaking (150 rev./min). Cells were harvested in the late exponential phase by centrifugation at 5000 ***g*** for 10 min. The pellet was re-suspended in a one-tenth vol. 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 re-suspended in a one-tenth vol. 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. To the periplasmic extract, $(NH_4)_2SO_4$ was added to a final concentration of 1.5 M. The sample was loaded on to a Resource Phenyl column (Amersham Biosciences, Piscataway, NJ, U.S.A.), and eluted with a linear gradient of 1.5–0 M $(NH_4)_2SO_4$. The total protein concentration was determined with Coomassie Brilliant Blue staining. The concentration of purified enzyme was determined by measuring A_{280}, using a molar absorption coefficient (ϵ) of 210000 $M^{-1} \cdot cm^{-1}$. Determination of the concentration of active sites in periplasmic extracts was achieved using PMSF [15]. The purity of the purified enzyme was at least $\approx 95\,\%$, whereas the purity of PA in the periplasmic extracts was $\approx 40\,\%$. In neither sample was background activity of β-lactamases, amidases or esterases observed.

Enzyme assays

Kinetic parameters for the conversion of 2-nitro-5-[(phenylacetyl)amino]benzoic acid (NIPAB) were determined by measuring initial velocities at different substrate concentrations, and fitting the data using the program ENZFIT (Elsevier Biosoft, Cambridge, MA, U.S.A.). The hydrolysis of NIPAB was monitored by measuring the increase in absorbance at 405 nm, in a PerkinElmer spectrophotometer (PerkinElmer, Indianapolis, IN, U.S.A.). Rates were calculated using a $\Delta\epsilon$ of 9.09 $mM^{-1} \cdot cm^{-1}$ for the conversion of NIPAB. Unless stated otherwise, all measurements were performed at pH 7.0 and 30 °C. Conversion of other substrates was followed using HPLC, as described previously [4]. Steady-state kinetic parameters for αArg145Cys and αArg145Leu were determined using periplasmic extracts. For characterization of the other mutants, purified enzyme was used.

The pH-dependence of the steady-state kinetic parameters was measured using the following buffers: 50 mM sodium acetate (pH 4–5.5), 50 mM K_2HPO_4 (pH 5.5–8), 50 mM Tris/HCl (pH 8–9) and 50 mM $NaHCO_3$ (pH 9–11). Values of k_{cat}/K_m at pH 5.5, 8 and 9 were measured in two different buffers, and no changes in steady-state parameters resulting from buffer composition were observed.

Modification with 2,3-butanedione and phenylglyoxal

Enzyme was incubated with various concentrations of freshly distilled 2,3-butanedione or phenylglyoxal in 50 mM borate buffer, pH 8.0, at 25 °C. At various times, aliquots were withdrawn from the reaction mixture and assayed for enzymic activity using 250 μM NIPAB as the substrate.

Chemicals

NIPAB was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 6-APA, ampicillin and penicillin G were gifts from DSM-Gist (Delft, The Netherlands). Phenylglyoxal monohydrate, 2,3-butanedione and phenylacetic acid were from Janssen Chimica (Goirle, the Netherlands).

RESULTS

Site-directed mutagenesis and expression of the arginine mutants of PA

The function of the conserved arginine residues αArg^{145} and βArg^{263} in PA was investigated by site-directed mutagenesis. αArg^{145} was mutated to leucine, lysine and cysteine and βArg^{263} was mutated to leucine, lysine and asparagine. To study whether the mutations had an effect on the expression and processing of the precursor protein, periplasmic extracts of the mutants were analysed by SDS/PAGE. All αArg^{145} mutant enzymes were expressed and correctly processed, yielding concentrations in the periplasmic extracts that were similar to those of the wild type. Periplasmic extracts of the three βArg^{263} mutants, however, contained large amounts of precursor protein, indicated by the presence of a band of 90 kDa (Figure 2).

Only in the periplasmic extract of βArg263Lys were separate bands for the α- and β-subunits observed, showing that a part of the precursor protein had been correctly processed. In periplasmic extracts of βArg263Leu and βArg263Asn, no bands corresponding to the α- or β-subunit were visible. From the extract of βArg263Asn, no processed PA could be obtained, and from βArg263Leu only 0.1–0.2 mg of processed PA/litre of culture could be purified, whereas normally a yield of 20 mg of wild-type enzyme/litre of culture was obtained. These results suggest that transport of the preproprotein to the periplasm is not influenced by the mutations, but that an arginine on position β263 is required for correct processing of the precursor protein.

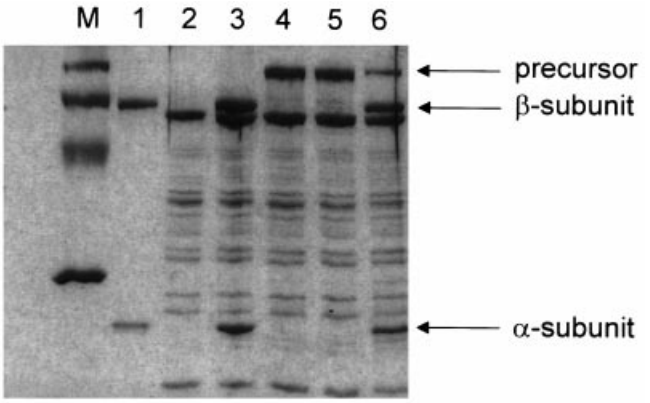

Figure 2 Expression of wild-type and βArg^{263} mutant enzymes in periplasmic extracts

Lanes were loaded as follows: lane M, molecular-mass marker proteins (94, 67, 43 and 30 kDa); lane 1, purified wild-type PA; lane 2, PA^- strain; lane 3, wild-type; lane 4, βArg263Asn; lane 5, βArg263Leu; lane 6, βArg263Lys. Proteins with an apparent molecular mass of 90 kDa are observed in periplasmic extracts containing the βArg^{263} mutant enzymes, indicating the presence of precursor protein. Only in the case of wild-type and βArg263Lys (lane 6) is processed protein in the form of the α- and β-subunits observed.

Table 1 Steady-state kinetic parameters of wild-type (WT), αArg^{145} and βArg^{263} mutants using NIPAB as the substrate

Values shown are the means for at least two independent experiments. Standard deviations were 10% or less from the mean value.

Enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
WT	16	11	1.45
αArg145Cys	0.4	60	0.007
αArg145Lys	0.6	22	0.027
αArg145Leu	1.5	30	0.05
βArg263Lys	32	30	1.1
βArg263Leu	0.1	152	0.00065
αArg145Lys/βArg263Lys	0.1	12	0.008

Chemical modification and kinetic analysis of the active-site arginine mutants

To study the importance of αArg^{145} and βArg^{263} in catalysis, the kinetic parameters of the mutant enzymes for the chromogenic model substrate NIPAB were measured. Furthermore, the sensitivity of both arginine residues towards chemical modification was determined.

All αArg^{145} mutants had 5–50-fold-reduced k_{cat}/K_m values for NIPAB (Table 1). The mutations predominantly affected k_{cat}, for which a 10–20-fold decrease was observed, whereas the K_m remained similar to that of the wild type. The kinetic parameters of βArg263Lys were similar to the wild-type values, whereas βArg263Leu displayed a more-than-1000-fold-decreased k_{cat}/K_m value for the hydrolysis of NIPAB, resulting from both a

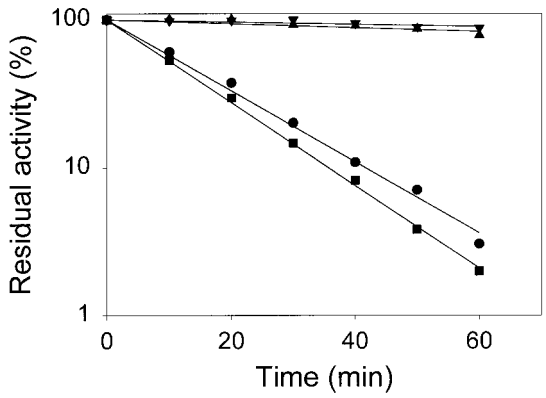

Figure 3 Modification of wild type and single arginine mutants with 2,3-butanedione

Symbols represent data and lines represent the best fit to the data, assuming first-order inactivation kinetics. Symbols: ●, WT; ■, βArg263Lys; ▲, αArg145Lys; ▼, αArg145Lys/βArg263Lys.

decreased k_{cat} and an increased K_m value. These data indicate that both arginine residues are important for efficient catalysis.

Wild-type enzyme and the single arginine mutants αArg145Lys and βArg263Lys were subjected to chemical modification using the arginine-modifying reagents phenylglyoxal and 2,3-butanedione. In both mutant enzymes, one of the active-site arginine residues was replaced with a lysine, allowing the characterization of the sensitivity towards chemical modification of the single remaining active-site arginine.

A time-dependent inactivation of the wild-type and single arginine mutants ($t_{1/2} \approx 30$ min) was observed upon incubation with 50 mM phenylglyoxal. However, a significant inhibition by phenylglyoxal was already observed at $t = 0$, preceding the time-dependent chemical modification. Further analysis of this phenomenon revealed that phenylglyoxal is a strong competitive inhibitor of the wild-type enzyme, with an inhibition constant of 6 μM. The severe competitive inhibition exerted by phenylglyoxal complicated further studies of the chemical modification, especially for αArg145Lys, which has a low activity for NIPAB. Therefore more detailed inactivation studies were performed with 2,3-butanedione, which does not competitively inhibit the enzyme. The wild-type enzyme was rapidly inactivated by 50 mM 2,3-butanedione, and no residual activity could be detected after prolonged incubation (Figure 3).

The same results were obtained with the βArg263Lys mutant enzyme, whereas αArg145Lys was only slowly inactivated. In all cases, the rate of the inactivation reaction could be described by first-order kinetics in terms of the enzyme concentration. In the double mutant in which both active-site arginine residues were removed, αArg145Lys/βArg263Lys, a slow time-dependent inactivation by 2,3-butanedione was also observed, suggesting that the slow modification of the αArg145Lys mutant enzyme probably reflects non-specific inactivation. These results indicate that βArg^{263} is shielded from the solvent and is not readily accessible to 2,3-butanedione, and that inactivation of the wild-type enzyme upon incubation with 2,3-butanedione is predominantly caused by modification of the guanidinium group of αArg^{145}. The findings from these inactivation experiments correlate well with the X-ray structure of the wild-type enzyme, in which αArg^{145} is exposed to the solvent and βArg^{263} is buried in the oxyanion 'hole', located in the interior of the enzyme and not very accessible to the solvent.

In combination with the kinetic analyses, the results show that both arginine residues are important for catalysis. Altering either of these residues by chemical modification or by site-directed mutagenesis yields, with the exception of βArg263Lys, mutants with decreased activity for NIPAB.

Interaction of αArg^{145} with 6-APA

Upon binding of penicillin G, PA undergoes a conformational change. During this conformational change, the enzyme switches to an 'open' conformation, in which the hydrogen bonds between αArg^{145} and βPhe^{24} are replaced by hydrogen bonds between αArg^{145} and the β-lactam group of the substrate [4]. To investigate the role of αArg^{145} in the interaction with the β-lactam moiety, the steady-state kinetic parameters of the αArg^{145} mutants for phenylacetamide and penicillin G were determined (Table 2). These substrates have the same phenylacetyl moiety, yielding the same acyl-enzyme intermediate during catalysis, but have as leaving groups ammonia or a β-lactam moiety respectively. The effect of the mutations on the steady-state kinetic parameters k_{cat} and K_m appeared indeed to be dependent on the type of leaving group of the substrate. The k_{cat} for phenylacetamide was reduced 10–50-fold in the mutants, whereas a 2–3-fold reduction was observed for the k_{cat} for penicillin G. For the latter substrate, a 3–11-fold-increased K_m was found, in contrast with only a 2-fold increase in K_m for phenylacetamide. In PA, the rate of acylation is much lower than the rate of deacylation of the acyl-enzyme [15]. This indicates that effects of the mutations on k_{cat} and K_m may be analysed in terms of the rate of acylation and K_s, i.e. binding of the substrate to the free enzyme [16]. These data therefore suggest that removal of αArg^{145} leads to a less tight binding of the β-lactam moiety to the enzyme, indicated by an increased K_m of all mutants for penicillin G, but also to an orientation that is more efficient for catalysis, indicated by the only slight effect of the mutations on k_{cat}.

Since the steady-state kinetic parameters of the αArg^{145} mutants showed that the mutations had an influence on the interaction of the enzyme with the 6-APA moiety of penicillin G in the hydrolytic reaction, we analysed how the mutations influenced the interaction with the free β-lactam nucleophile in the synthetic reaction. To this end, we performed a nucleophile competition experiment, in which the kinetics of synthesis of β-lactam antibiotics were measured [17]. Upon acylation by the acyl donor, the covalent intermediate can be deacylated by H_2O or 6-APA, yielding either the acid or the antibiotic. Using phenylacetamide as the acyl donor and 6-APA as the nucleophile, we measured the initial rates of production of penicillin G (V_s) and phenylacetic acid (V_h). It appeared that all αArg^{145} mutants showed an increased preference for 6-APA over water compared with the wild-type enzyme, indicated by increased values of V_s/V_h (Table 2). The largest change was observed with αArg145Leu, which exhibited a more-than-6-fold increase in the ratio between synthesis and hydrolysis compared with the wild type. Similar results were obtained when phenylglycine amide was used as the acyl donor, leading to the production of the semi-synthetic antibiotic ampicillin (Table 2). The fact that all mutants displayed an increased V_s/V_h suggests that the interaction of αArg^{145} with the β-lactam, as it exists in the wild-type enzyme, reduces the reactivity of 6-APA with the acyl-enzyme. These results are in line with the data from the hydrolytic reaction, which suggested that the interaction of the arginine with the β-lactam increased the affinity, but reduced the relative activity, for the substrate.

Table 2 Steady-state kinetic parameters for the hydrolysis and synthesis of β-lactam compounds by wild-type (WT) and αArg[145] mutants

The V_s/V_h ratio is defined as initial rate of antibiotic synthesis divided by the rate of production of acid at less than 5% conversion of the acyl donor. 6-APA (30 mM) was used as the β-lactam nucleophile, and phenylacetamide and phenylglycine amide (each at a concentration of 15 mM) were used as acyl donors for penicillin G and ampicillin synthesis respectively.

	Hydrolysis Phenylacetamide			Penicillin G			Synthesis (V_s/V_h)	
Enzyme	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 ($\mu M^{-1} \cdot s^{-1}$)	Penicillin G	Ampicillin
WT	50	160	0.31	42	5	8.4	0.78 ± 0.07	1.39 ± 0.03
αArg145Cys	3.5	348	0.01	19.2	88	0.22	3.27 ± 0.16	2.75 ± 0.2
αArg145Lys	3.2	355	0.009	10.6	32	0.33	2.14 ± 0.11	3.85 ± 0.13
αArg145Leu	5.5	85	0.065	17.1	16	1.1	4.84 ± 0.08	4.03 ± 0.04

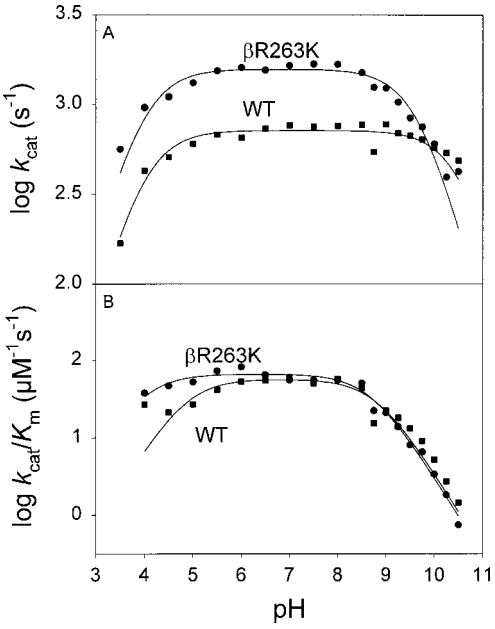

Figure 4 pH-dependence of k_{cat} (A) and k_{cat}/K_m (B) for the hydrolysis of NIPAB

Symbols represent the average of three independent measurements using the wild-type enzyme (■) and the βArg263Lys mutant enzyme (●). Lines represent the best fit to the data, using eqn (1).

pH profile of Arg263Lys

Replacement of βArg[263] with a positively charged lysine did not cause a loss of activity, whereas substitution with a neutral leucine led to a 1000-fold-reduced k_{cat}/K_m for NIPAB (Table 1). This indicates that a positively charged residue on position β263 is necessary for obtaining active enzyme. To test this hypothesis, the pH-dependence of the k_{cat} and k_{cat}/K_m parameters of wild-type and βArg263Lys enzymes for the hydrolysis of NIPAB was studied. If the protonation state of βArg[263] is important for the activity of PA, the lower pK_a value of the lysine side chain should be reflected in the pH profile of the βArg263Lys mutant enzyme by a shift of the descending limb to a lower pH. The pH-dependence of k_{cat} showed a typical bell-shaped curve, with an optimum occurring between pH 5 and 8, with ascending and descending slopes of 1 and −1 respectively, indicative of the presence of two ionizable groups on the enzyme where only the single protonated form of the enzyme is active (Figure 4).

To obtain the pK_a values of the ionizable groups, the data were fitted with eqn (1) [16].

$$k_{cat(pH)} = \frac{k_{cat}}{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}} \quad (1)$$

In this equation, K_1 is the ionization constant of the acidic group, K_2 is the ionization constant of the basic group and k_{cat} is the k_{cat} of the enzyme in the single protonated form. Fitting the data to eqn (1) yielded pK_1 values of 4.0 ± 0.2 and 3.9 ± 0.2, and pK_2 values of 10.7 ± 0.1 and 9.7 ± 0.1 for wild-type and βArg263Lys enzymes respectively. These values indicate that the βArg263Lys mutation did not influence the ionization constant of the acidic residue, but shifted the pK_a of the basic residue that is important for catalysis. The decrease of 1 pH unit may reflect the difference in pK_a values between free arginine and lysine, which are approx. 12.5 and 10.8 respectively [16]. These results indicate that, in pH profiles for the hydrolysis of NIPAB of wild-type and βArg263Lys, the descending limb reflects the pK_a of the βArg[263], and this residue has to be positively charged for catalysis.

The pH-dependence of k_{cat}/K_m, which reflects the ionization constant of the free enzyme and the free substrate [16], was similar for both the wild-type and βArg263Lys mutant enzymes. Fitting the data to eqn (1) yielded a pK_2 of 8.8 ± 0.2 for both enzymes, and a pK_1 of 4.7 ± 0.2 and 4.1 ± 0.2 for wild-type and βArg263Lys enzymes respectively (Figure 4). The latter pK might in principle reflect the pK of the substrate carboxylate group, but this is unlikely, given the similarity of pK_1 to the pK_1 for binding of NIPAB analogues lacking a carboxylate group [18], and therefore the pK_1 value determined probably reflects the pK_a of a group on the enzyme. These values also suggest that, in both the wild-type and mutant enzymes, the same basic residue is involved in binding of NIPAB, and that this is a residue other than βArg263. A role for αArg[145] in the binding of NIPAB seems unlikely, since mutation of αArg[145] did not lead to a large decrease in affinity for NIPAB (Table 1). The pH-dependence of k_{cat}/K_m on the hydrolysis of NIPAB by αArg145Leu yielded a pK_2 of 9.2 ± 0.1 (results not shown), which is close to the value of 8.8 obtained for wild-type and βArg263Lys, corroborating the hypothesis that the pK_2 of 8.8 for the k_{cat}/K_m of NIPAB hydrolysis does reflect the pK_a of another residue with a pK_a of approx. 9. An inspection of the active site of PA revealed βTyr[31] as a possible basic residue implicated in binding of NIPAB (see below).

DISCUSSION

On the basis of the structure of PA complexed with penicillin G and kinetic data, a role for αArg[145] and βArg[263] in the catalytic cycle was proposed [4,5]. The mutational analysis described in

the present paper provides an insight into the precise function of both arginine residues.

The first arginine, αArg^{145}, has two important interactions. In the native enzyme, the side chain of this arginine is hydrogen-bonded to the carbonyl oxygen of the active-site residue βPhe^{24}. When penicillin G binds, the enzyme switches to an open conformation, in which the hydrogen bonds between αArg^{145} and βPhe^{24} are replaced by hydrogen bonds between αArg^{145} and the β-lactam group of the substrate. It appeared that mutating αArg^{145} led to a 3–5-fold-increased activity (k_{cat}) of the mutant enzymes for penicillin G relative to phenylacetamide, compared with the wild type. If this difference is caused by a difference in transition-state stabilization in the conversion of both substrates, one would also expect a difference in the effect of the mutations on the k_{cat}/K_m for both substrates [16]. However, the k_{cat}/K_m for penicillin G is, in all mutants, approx. 20–30-fold higher than the k_{cat}/K_m for phenylacetamide, which is similar to the 27-fold difference observed for the wild type. The effect of the mutations on the k_{cat} for penicillin G and phenylacetamide can therefore only be explained by differences in ground-state stabilization of the enzyme–substrate (i.e. penicillin G or phenylacetamide) complex. A relatively less stable enzyme–penicillin G complex would require less energy to reach the transition state, leading to higher catalytic rates compared with the conversion of phenylacetamide, for which such a destabilization would not take place. This is in agreement with the 3–11-fold increase in K_m (which is equal to K_s in this case) for penicillin G compared with only a 2-fold increase in K_m for phenylacetamide with the αArg^{145} mutant enzymes.

An increased reactivity of the αArg^{145} mutants with β-lactam substrates was also observed in the deacylation reaction with 6-APA, which proceeds via the same transition state as the acylation reaction. This suggests that, also in the deacylation reaction, a ground-state destabilization mechanism is involved, although other mechanisms cannot be ruled out since the binding constant of the nucleophile to the acyl-enzyme cannot be determined and also the reactivity of the competing water nucleophile might have been affected by the mutations. Similar effects of the mutations on the reactivity with β-lactam substrates were observed in all αArg^{145} mutants, indicating that the removal of the arginine, rather than the introduction of another functional group (a lysine or a cysteine side chain), was the cause of the effects. This suggests that the interaction between the αArg^{145} and the β-lactam moiety in the wild-type enzyme leads to tight binding of the substrate to the enzyme, thus reducing the catalytic rate with β-lactam substrates compared with other substrates.

Similar results were obtained with an αPhe146Leu mutant of penicillin acylase. This mutant had a 20-fold-reduced affinity for the β-lactam moiety of the substrate, but only a 2-fold-decreased k_{cat} for penicillin G (compared with 10–1000-fold-decreased k_{cat} for other substrates), and a 6-fold-increased V_s/V_h ratio using 6-APA as the nucleophile [4]. A similar role in ground-state stabilization has been found for a conserved arginine (Arg^{244}) in class A β-lactamases, in which the arginine is also bound to the carboxylate group of the β-lactam moiety. Replacement of this arginine by a serine led to a mutant with a 100-fold-increased K_s for ampicillin, whereas the k_{cat} in this mutant was not affected [19].

The second active-site arginine, βArg^{263}, is located near the important oxyanion hole residue βAsn^{241}, which stabilizes the transition state by hydrogen-bonding with the negatively charged carbonyl oxygen. All mutants showed accumulation of the precursor protein in the periplasm, and only βArg263Lys showed a partial level of processing to produce mature enzyme. A similar impaired processing was observed during purification of another oxyanion hole mutant of PA, βAsn241Ala [4]. These results indicate that an intact oxyanion hole is necessary for efficient processing of the precursor enzyme, suggesting that maturation of PA is an autocatalytic process, as has been concluded in other studies published previously [1,20–22]. Recently, Hewitt et al. [22] described the structure of the precursor of PA, and suggested that a threonine residue in the spacer peptide would be involved in the first processing step, involving a cleavage of a Tyr–Pro bond in the spacer peptide (1E3A.PDB). The structure of the precursor protein shows that a hydrogen bond exists between βArg^{263} and the OG of this threonine, and it was proposed that this interaction would be needed to position the hydroxy group for nucleophilic attack [22]. These findings account for the impaired processing of the βArg^{263} mutant enzymes, since in these enzymes this hydrogen bond cannot be provided.

Analysis of the pH-dependence of k_{cat} for the hydrolysis of NIPAB, using wild-type and βArg263Lys enzymes, suggested that the residue on position β263 has to be protonated for catalysis. The electrostatic environment in the oxyanion hole is important for an efficient stabilization of the negative charge of the oxyanion [23,24], and it has been observed that charges as far as 15 Å (where 1 Å $\equiv$ 0.1 nm) away from the catalytic centre may influence the transition-state stabilization [25]. Although the negatively charged oxyanion in the transition state is stabilized by hydrogen bonds to βAsn^{241} and βAla^{69}, the increased polarity of the oxyanion hole, caused by the positively charged side chain of βArg^{263}, could nevertheless enhance further the stabilization of the transition state.

The pH-dependence of substrate binding indicated that βArg^{263} is not involved in binding of NIPAB, but that another ionizable residue, with a pK_a of approx. 9, performs this function. These results contradict conclusions made by Morillas et al. [5], who suggested that the positive charge on residue βArg^{263} was needed for both catalysis and substrate binding in the conversion of *p*-nitrophenyl acetate. The structural difference of both substrates, however, may account for the discrepancy. Inspection of the structure of the active site revealed βTyr^{31} as a candidate for substrate binding in the hydrolysis of NIPAB. This residue has been described as being important in substrate binding [4]. During the conformational change that takes place upon substrate binding, the phenylalanine on position α146 moves away from the active site towards the side chain of βTyr^{31}. Deprotonation of the polar hydroxy group on position βTyr^{31}, which has a pK_a of approx. 10, may well cause repulsion of the hydrophobic phenylalanine, thereby preventing the conformational change from taking place in the proper way and preventing productive binding of the substrate in the active site.

This work was financially supported by DSM Life Science Products and the Dutch Ministry of Economic Affairs.

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Received 11 October 2001/18 February 2002; accepted 9 April 2002