RESEARCH COMMUNICATION

The role of lysine-67 in a class C β-lactamase is mainly electrostatic

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By using site-directed mutagenesis, the conserved Lys-67 residue situated three positions after the active-site Ser of a class C β-lactamase was replaced by Arg or Gln. The Lys-67–Gln protein was nearly inactive. Although severely impaired, the Lys-67–Arg mutant exhibited an appreciable activity above pH 7.5 and, for some poor substrates of the wild-type enzyme, the $k_{cat.}$ values were even increased. The properties of the Lys-67–Arg mutant were studied by both steady-state and transient-state kinetic methods with a variety of compounds representing distinct classes of available substrates. With β-lactam substrates, the $k_{cat.}/K_m$ values reflecting the efficiency of the acylation step (k_{+2}/K) were decreased 25–100-fold. When the individual values could be measured, k_{+2} was not significantly altered, but K was found to be strongly increased, a result most likely explained by a corresponding increase in the k_{+1}/k_{-1} ratio. These results, combined with the much stronger impairment of the Lys-67–Gln mutant, can be interpreted by attributing an electrostatic role to the positive ammonium group of the Lys-67 side chain.

INTRODUCTION

In all active-site-serine penicillin-recognizing enzymes, the active serine is invariably followed by a Xaa-Xaa-Lys sequence (Joris et al., 1988), and in the known three-dimensional structures lies at the N-terminus of a long helix, so that the side chain of the Lys residue, just one turn downstream, also points into the catalytic cavity, where it participates in a dense network of hydrogen bonds (Herzberg and Moult, 1987; Oefner et al., 1990; Lamotte-Brasseur et al., 1991). Not surprisingly, site-directed mutagenesis experiments performed with the *Streptomyces* R61 penicillin-sensitive DD-peptidase and β-lactamases of both classes A and C have shown that replacement of this Lys by other residues resulted in significant decreases in the enzyme efficiencies (Hadonou et al., 1992; Gibson et al., 1990; Tsukamoto et al., 1990). In the present paper, a detailed analysis of the behaviour of the Lys-67–Arg and Lys-67–Gln mutants of the class C *Enterobacter cloacae* 908R β-lactamase indicates that the role of the side chain of residue 67 might be mainly electrostatic.

EXPERIMENTAL

Escherichia coli strains and plasmids are described by Dubus et al. (1993). The oligodeoxynucleotides designed for the mutations were GAAGGTTTGACTTATAG and TGAAGGTTCTACTT-ATA to mutate the lysine codon (AAA) to glutamine (CAA) and arginine (AGA) respectively. Both oligodeoxynucleotides were purchased from Syn-Tek (Umeå, Sweden). The mutagenesis method and other DNA manipulations were essentially the same as previously described by Dubus et al. (1993). Two plasmids expressing the mutated enzymes were obtained, pNU657 (Lys-67–Gln) and pNU658 (Lys-67–Arg).

Enzyme production and purification were performed as described previously (Monnaie et al., 1994). Steady- and transient-state kinetic experiments were performed as described by Galleni and Frère (1988) and Galleni et al. (1988), and Monnaie et al. (1992), respectively. The various β-lactams were of the same origin as in these previous studies.

RESULTS

The fluorescence spectra of the modified Lys-67–Arg and Lys-67–Gln enzymes were not significantly different from that of the wild-type protein. However, the rate of thermal denaturation of the Lys-67–Arg mutant was significantly increased, with an apparent first-order rate constant of $(3 \pm 0.2) \times 10^{-3}$ s^{-1} at 67 °C, compared with $(1 \pm 0.2) \times 10^{-3}$ s^{-1} and $(0.7 \pm 0.1) \times 10^{-3}$ s^{-1} for the Lys-67–Gln mutant and for the wild-type respectively.

The Lys-67–Gln mutant

The enzyme was severely impaired. The $k_{cat.}/K_m$ value for cephalothin was about 1100 M$^{-1} \cdot$s^{-1}, i.e. 5000-fold lower than that of the wild-type. Its activity on benzylpenicillin and cefuroxime was too low to allow an accurate determination of the kinetic parameters. Moreover, attempts to estimate the K_m values for these two compounds by utilizing them as competitive inhibitors of cephalothin hydrolysis yielded non-linear Dixon graphs, and in consequence the kinetic study was restricted to the analysis of the pH-dependence of the cephalothin kinetic parameters (Table 1).

The Lys-67–Arg mutant

The results are summarized in Table 2. The $k_{cat.}/K_m$ values were decreased 20–1000-fold with penicillins and cephalosporins, generally because of dramatic increases in the K_m values. Indeed, the $k_{cat.}$ values were not significantly decreased; they even increased in the cases of the poor substrates cefotaxime, cefuroxime and cefoxitin. By contrast, for the ester and thiolester substrates, the $k_{cat.}/K_m$ values were barely affected.

The K_m increases became even more significant under more acidic conditions (Table 1), but the nature of the buffer seemed to be involved, the K_m value being 3-fold higher with phosphate than with Hepes at pH 7.5. This appeared to be due to a competitive inhibition by the phosphate ions (since the $k_{cat.}$ values did not appear to be affected), a phenomenon that was not observed with the wild-type protein. Conversely, the $k_{cat.}$ values

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Table 1 pH-dependence of the kinetic parameters for the hydrolysis of cephalothin

The experiments were done at 30 °C in 50 mM cacodylate[a], 50 mM Na_2HPO_4/NaH_2PO_4[b], 50 mM Hepes[c] or 50 mM glycine[d]. All buffers were supplemented with 0.2 M NaCl. S.D. values did not exceed 10%.

pH	6.0[a]	6.5[b]	7.0[b]	7.5[b]	7.5[c]	8.0[c]	8.5[c]	9.0[d]	9.5[d]	10.0[d]
Wild-type										
$k_{cat.}$ (s^{-1})	206	–	215	–	–	200	–	156	–	49
K_m (μM)	5.2	–	4.2	–	–	3.9	–	2.6	–	1.9
$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)	40000	–	51000	–	–	51000	–	45000	–	26000
Lys-67–Arg										
$k_{cat.}$ (s^{-1})	–	65	120	150	150	150	150	130	90	50
K_m (μM)	–	1200	700	400	120	70	70	40	30	20
$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)	–	55	170	375	1250	2200	2200	3200	3000	2500
Lys-67–Gln										
$k_{cat.}$ (s^{-1})	–	–	0.07	0.07	–	0.08	0.08	0.09	0.22	0.5
K_m (μM)	–	–	900	550	–	70	70	40	75	160
$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)	–	–	0.08	0.12	–	1.1	1.1	2.2	2.9	3.1

Table 2 Steady-state kinetic parameters ($k_{cat.}$, K_m and $k_{cat.}/K_m$)

Experiments were done in 50 mM Hepes, pH 8.0, containing 0.2 M NaCl and at 30 °C. S.D. did not exceed 10%.

	Wild-type			Lys-67–Arg		
	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)
Nitrocefin	830	25	33000	> 240	> 400	600
Cephalothin	220	4.2	51000	150	70	2200
Cephaloridin	800	100	8000	250	> 1000	250
Cefazolin	> 2000	> 1000	2000	> 50	> 1000	50
Cefotaxime	0.015	0.01*	1500†	0.15	25	6
Cefuroxime	0.05	0.016*	3100†	0.13	5*	26†
Cefoxitin	0.06	0.024*	2500†	0.2	25	8
Benzylpenicillin	14	0.5*	28000†	14	500	28
Ampicillin	0.5	0.4*	1300†	0.2	15*	13†
Hippuryl-thioglycollate	> 7	> 1000	7	> 3	> 1000	3
Hippuryl-phenyl-lactate	> 19	> 1000	19	> 10	> 1000	10

* Determined as a K_i value in a competitive-inhibition experiment.
† Calculated from the $k_{cat.}$ and K_m values.

Table 3 Pre-steady-state kinetic parameters (k_{+2}, K', k_{+2}/K' and $k_{cat.}/K_m$) for wild-type and Lys-67–Arg enzymes

Experiments were done in 50 mM Hepes, pH 8.0, or in 50 mM Na_2HPO_4/NaH_2PO_4, pH 7.0,* containing 0.2 M NaCl, and at 30 °C. N.D., not determined.

	Wild-type				Lys-67–Arg			
	k_{+2} (s^{-1})	K' (mM)	k_{+2}/K' ($mM^{-1} \cdot s^{-1}$)	$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)	k_{+2} (s^{-1})	K' (mM)	k_{+2}/K' ($mM^{-1} \cdot s^{-1}$)	$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)
Carbenicillin	3.3 ± 0.2	0.015 ± 0.002	220 ± 20	N.D.	0.6 ± 0.03	3 ± 0.5	0.2 ± 0.02	N.D.
Ampicillin	120 ± 30*	0.1 ± 0.02*	1200 ± 100*	1800 ± 200*	> 25	> 1.5	15 ± 2	15 ± 1
Cefuroxime	> 100*	> 0.03*	3600 ± 400*	3100 ± 100*	> 10	> 0.4	27 ± 2	26 ± 3

for the mutant were not strongly pH-dependent, as observed with the wild-type enzyme.

Class C β-lactamases hydrolyse β-lactams according to the now classical three-step pathway:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} ES^* \xrightarrow{k_{+3}} E + P$$

where ES* is the acyl-enzyme. Generally k_{+3} is lower than k_{+2} (Knott-Hunziker et al., 1982; Monnaie et al., 1992) for the wild-type enzyme. That this remained so for the Lys-67–Arg mutant was demonstrated by the fact that the protein could be covalently labelled by [^{14}C]benzylpenicillin, as shown by SDS/PAGE and fluorography and that the $k_{cat.}$ values for cephalosporins increased linearly in the presence of increasing methanol con-

centrations. Moreover, with carbenicillin, ampicillin and cefuroxime the reporter-substrate method indicated a progressive, concentration-dependent, inactivation of the enzyme corresponding to the accumulation of the acyl-enzyme (see below).

The Lys-67–Arg mutant: transient kinetics

With the use of nitrocefin as a reporter substrate (De Meester et al., 1987), the progressive inactivation of the mutant could be monitored by stopped flow and the deduced parameters compared with those of the wild-type enzyme (Table 3). As previously (Monnaie et al., 1992), better substrates could not be studied for technical reasons, the $k_{cat.}$ values being too high. The observed k_{+2}/K' values [where $K' = (k_{-1}+k_{+2})/k_{+1}$] nicely agreed with the $k_{cat.}/K_m$ values determined under steady-state conditions, confirming that the mutant protein was pure and that all the molecules were active. Indeed, the computation of $k_{cat.}/K_m$ involves the enzyme concentration, whereas that of k_{+2}/K' does not (Monnaie et al., 1992). From the results in Table 3, it appeared that the decreased acylation efficiencies were mainly due to increased K' values and that the k_{+2} values remained high and similar to those of the wild-type protein.

DISCUSSION

A positive charge on the side chain of residue 67 appears to be essential for the catalytic activity of the *Enterobacter cloacae* 908R class C β-lactamase. The replacement by Gln produced a nearly inactive enzyme, resulting probably from a complete breakdown of the hydrogen-bond network whose presence was deduced from the three-dimensional structure of the nearly identical *Enterobacter cloacae* P99 enzyme (Lobkovsky et al., 1993). By contrast, the Lys-67–Arg mutant retained an appreciable proportion of the wild-type activity, but the consequences of the mutation on the $k_{cat.}$ and $k_{cat.}/K_m$ parameters were very different. The $k_{cat.}$ values, generally reflecting those of k_{+3}, were barely affected, and even increased in a few cases, whereas the $k_{cat.}/K_m$ values decreased 20–1000-fold with penicillins and cephalosporins. Since $K_m = k_{+3}K'/(k_{+2}+k_{+3})$, the consistently increased K_m values might be due to a strong and systematic impairment of the acylation process characterized by dramatically lowered k_{+2} values. When the experiments could be performed, it appeared, however, that k_{+2} was not significantly different for the mutant and wild-type enzymes, but that the former exhibited strongly increased K values. With carbenicillin, the value of k_{+2} is low for both enzymes, and it can be safely assumed that $K' = k_{-1}/k_{+1} = K_S$. Although one might argue that it is dangerous to extrapolate the results obtained with poor substrates to the best ones, all the data obtained in the present study can be explained on the basis of increased K' values, due to corresponding increases in the k_{-1}/k_{+1} ratios.

Our results are in qualitative agreement with those obtained by Tsukamoto et al. (1990), who studied the effects of the same mutation in another class C β-lactamase. Although some of their results are rather preliminary and difficult to analyse (see Monnaie et al., 1994, for more details), they also observed significantly increased K_m values for the Lys-67–Arg mutant and a nearly complete disappearance of activity for their Lys-67–Thr and Lys-67–Glu counterparts, where the side-chain positive charge had also been eliminated.

The Lys residue of the Ser*-Xaa-Xaa-Lys sequence, where Ser* is the active serine, is one of the very few strictly conserved residues in all active-site-serine penicillin-recognizing enzymes (Joris et al., 1988). If one assumes that the acylation reaction rests on very similar mechanisms in all these enzymes, it becomes a leading candidate for acting as a general base in the activation of the Ser* residue, as proposed by Strydnaka et al. (1992) for a class A β-lactamase. Accordingly, the corresponding Lys-73–Arg and Lys-67–Arg mutations in the class A β-lactamase of *Bacillus cereus* (Gibson et al., 1990) and the *Streptomyces* R61 DD-peptidase (Hadonou et al., 1992), respectively, yielded strongly impaired enzymes. But the consequences of the mutations on the individual parameters were very different, at least for the *Bacillus cereus* class A β-lactamase. Indeed, the K_m values remained nearly unchanged, and the poorer activity was due to a lowered $k_{cat.}$ value, resulting, in the case of benzylpenicillin, from a 70-fold decrease in the k_{+2} value. With the peptidase, the acylation rate by benzylpenicillin decreased 20000-fold and became nearly negligible. By contrast, the catalytic efficiency towards the peptide and thiolester substrates decreased only 200-fold, with, for the latter, a little affected $k_{cat.}$ and strongly increased K_m values, a situation reminiscent of that observed here. Indeed, acylation by the thiolester was even less affected with the *Enterobacter cloacae* 908R β-lactamase than with the DD-peptidase, a result which might result from the rather low catalytic efficiency of the wild-type β-lactamase towards this substrate, the fitting of which in the deformed active site of the mutant would not be much more difficult than into that of the wild-type.

If Lys-67 were to act as a general base in the acylation step, its pK_a should be decreased to 6 or lower, but one would expect the corresponding pK_a of the Arg mutant to be at least 2.5–3 pH units higher. The $k_{cat.}/K_m$ pH-dependency of the mutant might reflect a pK_a value of about 7.5, but this seems to be extremely low for an Arg side chain. Moreover, as stated above, at pH 8.0 the k_{+2} values for carbenicillin and ampicillin were not significantly lower than for the wild-type, and the k_{+2}/K' decrease could be mainly attributed to a higher K' value.

Another hypothesis was proposed for the mechanism of the acylation step in class A β-lactamases: the carboxylate of Glu-166 acting as the general base would abstract the proton of Ser-70 via a conserved water molecule (Lamotte-Brasseur et al., 1991). This latter hypothesis was supported by experimental data showing that cefoxitin and other compounds which bear a methoxy group on C-6 (penicillin) or C-7 (cephalosporins) are orders of magnitude less efficient than the analogous compounds devoid of such side-chains in acylating class A enzymes, and by computer modelling studies showing that the major difference between the two families of compounds was that the methoxy group of the former displaced the water molecule, rendering the proton-transfer system ineffective (Matagne et al., 1993). By contrast, class C β-lactamases are readily acylated by cefoxitin (Table 2), but deacylation is slow. This comparison underlines the possibility that the mechanisms of class A and class C β-lactamases might be rather different, as also illustrated by the diverging effects of the Lys→Arg mutations on the two types of enzymes.

In the class C protein, the role of the Lys-67 side chain appears to be mainly electrostatic, which would explain why it can be replaced by Arg without a complete loss of the enzymic properties. However, the active site of the Arg mutant must be distorted, so that binding of the substrates becomes more difficult. But this distortion would not greatly affect the catalytic machinery itself, and the bound substrate would be normally processed. A similar situation was encountered with the Lys-234–His mutant of the class A β-lactamase of *Streptomyces albus* G (Brannigan et al., 1991), for which it could be shown that a positively charged His residue could successfully replace Lys-234 and that titration of the His side chain resulted in a complete loss of activity at high pH (Lys-234 is another active-site residue assumed to bear a positive charge). Moreover, the positive charge appeared to be mainly involved in transition-state stabilization, since the major

effect was on $k_{cat.}$. By contrast, the corresponding Lys-315–His mutant of the *Enterobacter cloacae* 908R β-lactamase was strongly impaired, and did not recover activity when the pH was lowered below 6.5 (Monnaie et al., 1994). The results involving the second positively charged residue in the cavity also argue in favour of different roles for similarly positioned residues in class A and class C enzymes. It has been suggested that in the latter the general base might be Tyr-150, also exhibiting a drastically lowered pK_a (Oefner et al., 1990), but, although several well-resolved structures are available, the catalytic mechanism of β-lactamases remains enigmatic. This is even more true for DD-peptidases, where the search for a general base has also failed to produce convincing results (J.-M. Wilkin and J.-M. Frère, unpublished work).

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