Role of Residue Lys315 in the Mechanism of Action of the *Enterobacter cloacae* 908R β-Lactamase†

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ABSTRACT: The role of the highly conserved Lys315 residue in the catalytic mechanism of a class C β-lactamase has been probed by site-directed mutagenesis. Lys315 has been replaced by a histidine in the *Enterobacter cloacae* 908R β-lactamase, thus introducing a tritratable group to probe the role of the positive charge, and by a glutamine. The effects of these mutations have been studied on the kinetics of penicillin G and cephalothin turnover and on the pre-steady-state kinetics with carbenicillin at different pH. Results showed that substrate binding was not impaired by the mutations, so that an interaction with the substrate-free carboxylate in the Henri–Michaelis complex could be ruled out. Lys315 must have a catalytic role as shown by the decreased acylation and deacylation rates observed with the mutant enzymes. The mutants exhibited a lower activity at acidic pH, and this observation could be correlated with a decreased affinity for (3-aminophenyl)boronate, a compound devoid of free carboxylate which binds to the active site and forms an adduct mimicking the tetrahedral intermediate. This suggested that Lys315 was somehow involved in accelerating the nucleophilic substitutions along the reaction pathway. The study was extended to modified substrates where the free carboxylate had been esterified. Neither acylation nor deacylation seemed severely impaired with these compounds, showing that the interaction between the enzyme and the substrate-free carboxylate did not play a major role in catalysis.

The active-site serine β-lactamases and DD-peptidases are members of a large family of penicillin-recognizing enzymes. They exhibit homologous 3D structures consisting of one α and one α/β domain where a few conserved residues can be identified (Joris *et al.*, 1988, 1991). Among these is a lysine (exceptionally an Arg in some β-lactamases and a His in the *Streptomyces* R61 DD-peptidase) located on the innermost strand of the β-pleated sheet that borders the active site. The ammonium group of this Lys residue (234 in class A and 315 in class C) was assumed to be the enzyme group most suitably located to interact with the substrate carboxylate and thus play an essential role in substrate binding (Herzberg & Moult, 1987; Kelly *et al.*, 1989).

In the *Bacillus licheniformis* and TEM I, class A β-lactamases (Ellerby *et al.*, 1990; Lenfant *et al.*, 1991) substitutions of the Lys234 residue by Glu, Ala, Arg, or Thr had more influence on the k_{cat} than on the K_m values, and it was thus proposed that formation of a salt bridge between this residue and the substrate carboxylate mainly contributed to the binding of the transition states. Besides, studies performed with modified substrates (Varetto *et al.*, 1991; Laws *et al.*, 1993) underlined the importance of their carboxylate negative charge but did not support any clear interaction between this group and a specific residue of the enzyme.

In order to study a possible interaction between the substrate carboxylate and Lys315 of the *Enterobacter cloacae* 908R β-lactamase, the effects of enzyme and substrate modifications on steady-state and pre-steady-state kinetics were investigated. By site-directed mutagenesis, residue Lys315 was successively replaced by His and Gln. The role of Lys315 in the hydrolysis and transacylation of noncyclic depsipeptides was investigated with the help of the same mutants. This activity is probably reminiscent of a common evolutionary precursor of DD-peptidases and β-lactamases (Pratt & Govardhan, 1984; Govardhan & Pratt, 1987; Adam *et al.*, 1990).

EXPERIMENTAL PROCEDURES

Strains and Plasmids. The *Escherichia coli* strains used were the same as those described by Dubus *et al.* (1993).

Nucleic Acid Techniques. M13 has phage was grown and single-stranded DNA prepared as described by Messing (1983). Mutagenesis was an adaptation of the method devised by Taylor *et al.* (1985), where T7 DNA polymerase (Sequenase) and T4 DNA polymerase were used for the first and second extensions, respectively, with no change in the described buffers. This increased the efficiency of the method and reduced the overall duration of the procedure. Sequencing was done according to the dideoxy-chain-termination method using the Sequenase DNA sequencing kit (United States Biochemical, Cleveland, OH). Other DNA manipulations were done according to standard techniques (Sambrook *et*

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al., 1989). The procedure for *E. coli* transformation was described by Hanahan (1983).

Enzymes and Chemicals. T4 DNA ligase and polymerase were purchased from Boehringer Mannheim GmBH (Brussels, Belgium) or New England Biolabs (Beverly, MA) and used in the buffer supplied by the manufacturer. [^{35}S]dATPαS (10 mCi/mL) was purchased from Amersham Inc. (Bucks., U.K.), dCTPαS was a product of PL Biochemicals (Pharmacia, Uppsala, Sweden), and other dNTPαS were from Boehringer. The 8-bp *Xho*I DNA linker was from Pharmacia. The oligonucleotides were purchased from SYN-TEC AB (Umeå, Sweden) and the Protein Chemistry Lab at the department of Biochemistry and Molecular Biophysics (Washington University, St. Louis, MO).

Mutagenesis. The construction of plasmids pNU602 (*ampC*) and pNU608 (*ampC* and *ampR*) has been described (Dubus *et al.*, 1993). A 710-bp *Kpn*I/*Xho*I DNA fragment encoding the C-terminal part of the *ampC* gene was cloned into a M13mp18 phage modified by a *Xho*I linker previously introduced into the *Hinc*II restriction site of the polylinker. This construct was used to provide single-stranded DNA (ssDNA) for the site-directed mutagenesis reactions.

The oligonucleotides AGAGCCCGTGTGATGGACCCA and GCCCGTTTGATGGACCC were used to change the lysine codon (AAA) into those corresponding to histidine (CAC) and glutamine (CCA), respectively. From each mutagenesis reaction, several (5–10) phage plaques were selected and ssDNA was prepared and sequenced. Those which exhibiited the expected mutation were further sequenced to ensure that no other unwanted mutation was present. Subsequently, M13 RF DNA was prepared, and the fragment(s) of interest were subcloned into pNU602 to yield plasmids pNU609 (K315H) and pNU639 (K315Q). Subsequently, an *Apa*I/*Xho*I DNA fragment from these plasmids was subcloned into pNU608 and transformed into *E. coli* SNO302 for enzyme production.

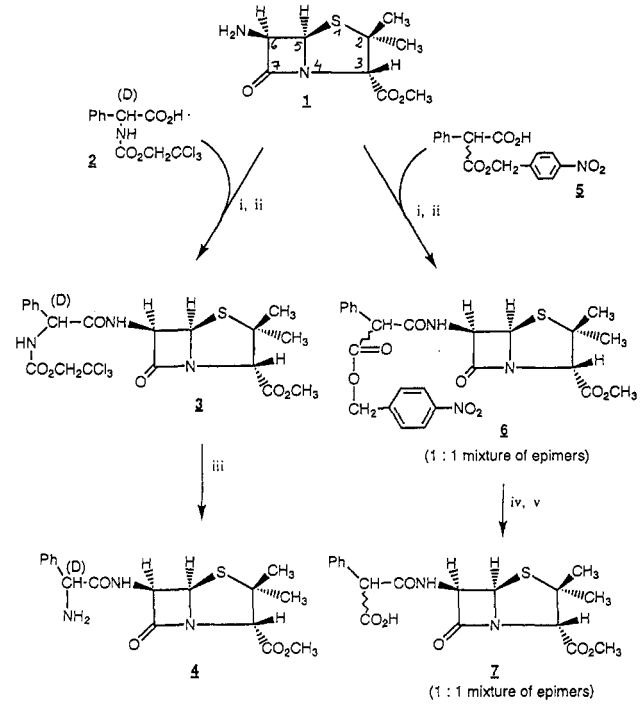
Antibiotics. Benzylpenicillin was from Rhône-Poulenc (Paris, France), ampicillin was from BristolMyers-Squibb (Brussels, Belgium), and carbenicillin was from Glaxo Group Research (Greenford, Middx., U.K.). Cephalothin was from Eli Lilly & Co. (Indianapolis, IN). These compounds were kind gifts of the respective companies. Nitrocefin was purchased from Oxoid (Basingstoke, Hants., U.K.).

Substrates. Hippuryl phenyllactate was purchased from Sigma (St. Louis, MO). The synthesis of hippuryl thioglycolate was previously described (Adam *et al.*, 1990). The methyl ester of benzylpenicillin was prepared as described by Jaszberenyi and Gunda (1975).

Methyl 6-Aminopenicillinate (1) (Scheme 1). The *p*-toluenesulfonic salt of methyl 6-aminopenicillinate was prepared according to Manhas *et al.* (1983). Treatment with triethylamine (1 equiv) in CH_2Cl_2 for 2 h at 20 °C followed by washing with cold water, drying ($MgSO_4$), and concentration at 10 °C gave the free amine **1**: IR (film) ν 3400–3330 (w), 1780 (s), 1752 (s) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.49 (s, 3H), 1.65 (s, 3H), 1.90 (br s, 2H, NH_2), 3.78 (s, 3H, CO_2CH_3), 4.40 (s, 1H, H-3), 4.59 (d, 1H, J = 4.4 Hz, H-6), 5.52 (d, 1H, J = 4.4 Hz, H-5).

Methyl Ampicillinate (4) (Scheme 1). D-Phenyl-2-[[[(trichloroethyl)oxy]carbonyl]amino]acetic acid (**2**) was coupled to **1**, in a CH_2Cl_2 solution at 0 °C, in the presence of diisopropylcarbodiimide (1 equiv) and (dimethylamino)-pyridine as catalyst. After 4 h at room temperature, the mixture was filtered and washed with cold 0.05 N HCl and brine. Drying on $MgSO_4$ and concentration under vacuum gave crude methyl D-6-[phenyl-2-[[[(trichloroethyl)oxy]carbonyl]amino]acetamido]penicillinate (**3**) which was purified by flash chromatography on silica gel (eluent = EtOAc:CH_2Cl_2, 1:5): IR (film) ν 3320 (br, m), 1789 (s), 1742 (br, s), 1685 (m), 1525 (br, m) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.42 (s, 3H), 1.52 (s, 3H), 3.76 (s, 3H, CO_2CH_3), 4.39 (s, 1H, H-3), 4.70 (ABq, 2H, OCH_2CCl_3), 5.29 (d, 1H, J = 5.5 Hz, PhCH-), 5.46 (d, 1H, J = 4.1 Hz, H-5), 5.62 (dd, 1H, J = 4.1 and 8.8 Hz, H-6), 6.41 (d, 1H, J = 5.5 Hz, NH carbamate), 6.60 (d, 1H, J = 8.8 Hz, NH amide), 7.42 (s, 5H).

Scheme 1[a]



[a] (i) iPrN=C=NiPr, DMAP (catalyst), CH_2Cl_2, 0–20 °C, 4 h; (ii) flash chromatography on SiO_2; (iii) Zn, HOAc, EtOAc, 0 °C, 4 h; (iv) H_2 (40 psi), Pd–C, $NaHCO_3$, 1 equiv, H_2O–CH_3CN, 2 × 2 h, 20 °C; (v) HCl, 1 equiv, H_2O, CH_2Cl_2, 0 °C.

Selective deprotection of the side-chain amino function was conducted as usual (Chauvette *et al.*, 1971), in the presence of zinc dust and acetic acid (5 equiv) in ethyl acetate solution at 0 °C for 4 h. Filtration, neutralization, separation of the organic layer, drying ($MgSO_4$), and concentration yielded methyl ampicillinate (**4**). The product was purified by precipitation in ether–pentane: IR (CH_2Cl_2) ν 3400–3300 (w), 1792 (s), 1755 (m), 1690 (m), 1505 (m) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.49 (s, 3H), 1.65 (s, 3H), 1.86 (br s, 2H, NH_2), 3.78 (s, 3H, CO_2CH_3), 4.46 (s, 1H, H-3), 4.57 (s, 1H, PhCH-), 5.55 (d, 1H, J = 4 Hz, H-5), 5.68 (dd, 1H, J = 4 and 9 Hz, H-6), 7.35 (br s, 5H), 8.07 (d, 1H, J = 9 Hz, NH amide); MS (FAB, + Q1MS) 364 (M + 1, 5), 347 (M $-NH_3$, 23), 231 (methyl 6-aminopenicillinate, 5), 214 (methyl penicillinate, 14), 174 ($C_7H_{12}NO_2S$, 95), 136 (C_8H_9NO, 23), 114 ($C_6H_{10}O_2$, 42), 106 (PhCH=NH_2, 100).

Methyl Carbenicillinate (7) (Scheme 1). (±)-Phenyl-2-[[[(*p*-nitrobenzyl)oxy]carbonyl]acetic acid (**5**) was coupled to **1**, as previously described, to yield methyl 6-[phenyl-2-[[(*p*-nitrobenzyl)oxy]carbonyl]acetamido]penicillinate **6** (1:1 mixture of diastereoisomers): IR (CH_2Cl_2) ν 3350 (br, w), 1790 (m), 1760 (br, s), 1690 (m), 1615 (w), 1535 (s) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.46, 1.47, 1.52, and 1.58 (4s, 6H), 3.77 and 3.78 (2s, 3H, CO_2CH_3), 4.42 and 4.46 (2s, 1H, H-3), 4.65 and 4.68 (2s, 1H, PhCH-), 5.28 and 5.30 (2s, 2H,

CH_2PhNO_2), 5.53 and 5.54 (2d, 1H, J = 4.2 Hz, H-5), 5.67 (dd, 1H, J = 4.2 and 8.9 Hz, H-6), 7.24 and 7.65 (2d, 1H, J = 8.9 Hz, CONH), 7.38–7.45 (sharp m, 7H), 8.18 (d, 2H, J = 7.8 Hz).

Selective deprotection of the side-chain carboxylic function was performed as usual (Ernest *et al.*, 1978), by hydrogenolysis in a Parr apparatus at 20 °C (p = 2.6 kg/cm^2), in an acetonitrile–aqueous $NaHCO_3$ (1.3 equiv) solution and with 10% palladium on activated carbon as catalyst. After two runs (2 × 2 h), the mixture was filtered and the catalyst was rinsed with CH_3CN–H_2O. The organic solvent was smoothly evaporated under vacuum. The aqueous phase was acidified at 0 °C and extracted twice with CH_2Cl_2 to give methyl carbenicillinate (7) (1:1 mixture of diastereoisomers). The product was purified by precipitation in ether–pentane: IR (CH_2Cl_2) ν 3300–3400 (br, w), 1790 (s), 1755 (br, s), 1690 (m), 1650 (w), 1528 (m), 1440 (m) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.43, 1.45, 1.47, and 1.49 (4s, 6H), 3.76 (s, 3H, CO_2CH_3), 4.41 and 4.44 (2s, 1H, H-3), 4.57 and 4.59 (2s, 1H, PhCH-), 5.52–5.65 (m, 2H, H-5 + H-6), 7.17 (d, 0.5H, J = 7 Hz, CONH), 7.39 (sharp m, 6.5H, Ph + CONH); MS (FAB, +QIMS) 393 (M + 1, 7), 349 (M − CO_2 + 1, 5), 231 (methyl 6-aminopenicillinate, 4), 174 ($C_7H_{12}NO_2S$, 100), 136 (CH_9NO, 28), 114 ($C_6H_{10}O_2$, 10).

IR spectra were recorded on a Perkin-Elmer 681 apparatus calibrated with polystyrene (1601 cm^{-1}). ^{1}H NMR spectra were recorded at 200 MHz on a Varian Gemini 200 spectrometer; tetramethylsilane (TMS) was used as internal reference. Mass spectra were recorded on a Finnigan-Mat TSQ-70 spectrometer in FAB mode (Xe, 8 kV).

Enzyme Production and Purification. The plasmid was transformed in *E. coli* SNO302 for constitutive production. Bacteria were grown for 16 h at 37 °C in 15 L of Terrific Broth supplemented with 10 mg/L tetracycline. The cells were pelleted by centrifugation (4000g, 20 min), suspended in 10 mM sodium phosphate, pH 6.8, containing 1% (v/v) phenethyl alcohol (Janssen Pharmaceutica NV, Belgium), 5 mM EDTA, 25% (w/v) sucrose, and 0.01 mg/mL lysosyme, and submitted to three freeze–thaw cycles. The cells were spun down at 15000g for 20 min, and the conductivity of the supernatant was adjusted to 45 μS (measured with a Metrohm E527 conductometer of cell constant 11.6 cm^{-1}) before batch adsorbtion on 1 L of CM-Sepharose (Pharmacia) previously equilibrated in 10 mM sodium phosphate, pH 6.8. The gel was then poured in a 50- × 5-cm column. The enzyme was eluted with a NaCl gradient (0–0.25 mM) in the same buffer.

Kinetic Measurements. Experiments were performed at 30 °C in the following buffers, all added with 0.2 M NaCl: 20 mM sodium cacodylate/HCl, pH 5.0–6.0; 20 mM sodium phosphate, pH 7.0; 20 mM HEPES/HCl, pH 8.0; 20 mM glycine/NaOH, pH 9.0–10.0. Absorbance measurements were performed with a Uvikon 860 spectrophotometer linked to a Copam microcomputer and rapid kinetics with a BioLogic SFM-3 stopped-flow apparatus. The results were analyzed on the basis of the double-displacement mechanism generally accepted for the active-site serine β-lactamases:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES^* \xrightarrow{k_3} E + P$$

where ES* represents the acylenzyme. With class C enzymes, k_3 is usually much smaller than k_2 and the acylenzyme is the predominant catalytic intermediate at the steady state. The kinetic methods have been described in detail before (Galleni & Frère, 1988; Monnaie *et al.*, 1992). For the pH dependencies, curve fitting was performed with the help of the Enzfitter program (Leatherbarrow, 1987).

Table 1: Thermal Stability of the Wild-Type and Mutant Proteins[a]

	$t_{1/2}$ (min)		
	pH 6.0	pH 7.0	pH 8.0
wild-type (60 °C)	10 ± 1	9.4 ● 1	13 ± 1
Lys315His (60 °C)	1.9 ± 0.1	2.0 ± 0.1	1.5 ± 0.1
Lys315Gln (60 °C)		0.25 ± 0.05	
Lys315His (55 °C)	13.2	12.7	12.0

[a] SD values did not exceed 10%.

RESULTS

Thermal Stability of the Mutants. As shown by Table 1, the Lys315His mutation did not severely affect the stability of the enzyme, indicating the absence of gross structural modifications. By contrast, the Lys315Gln mutation significantly decreased the stability of the protein, which might reflect more important, although not dramatic, alterations of the protein conformation.

Steady-State Experiments. The steady-state parameters at pH 7.0 for the interaction between β-lactams and the wild-type and mutant enzymes are listed in Table 2. In all cases, a linear increase of k_{cat} with increasing methanol concentrations was observed, indicating that k_{cat} corresponded to the deacylation rate constant (k_3). Both substitutions yielded enzymes with reduced k_{cat} and k_{cat}/K_m values (particularly for the Lys315Gln mutant) and, except for cephalothin, increased K_m values.

pH Dependency. The pH dependencies of the steady-state parameters k_{cat}, K_m, and k_{cat}/K_m for the hydrolysis of cephalothin and benzylpenicillin by the wild-type and modified enzymes are shown in Figures 1–3 and some of the most striking features summarized in Table 3. In several cases, fitting the kinetic parameter values to simple partition equations such as those given in the legends of Figures 1 and 2 was not expected to yield significant pK values. Moreover, utilization of different fitting procedures (simple vs proportional) sometimes yielded large errors in different pH ranges. More details can be found in the legends of the figures. Nonetheless, the following features emerged from the analysis of these data.

The K_m values of the Lys315His mutant exhibited a novel pH dependency which might be attributed to the titration of the His side chain if one assumes that K_m directly reflects the dissociation constant of the noncovalent ES complex. But surprisingly, the k_{cat} values similarly increased with pH so that the variations of k_{cat}/K_m in the same pH range were much less spectacular. That k_{cat} corresponded to the deacylation rate constant (k_3) over the whole pH range was again demonstrated by an increase of the reaction rates in the presence of methanol, so that if the k_2 and K' (where $K' = (k_{-1} + k_2)/k_1$) values were not strongly modified in the studied pH range (but see below), the increases of k_{cat} and K_m of the Lys → His mutant at high pH values might be explained by the sole increase of k_3 (since $k_{cat} = k_3$ and $K_m = k_3K'/k_2$). It is evident that such a decreased transition-state stabilization at acidic pH is in complete disagreement with an efficient participation of the protonated His residue in the last step of the catalytic phenomenon (deacylation). This was confirmed by the drastically decreased k_{cat} and k_{cat}/K_m values recorded at pH 5–6 for the Lys → His mutant when compared to the wild-type enzyme (see Table 4). Moreover, the k_{cat} of the Lys315Gln mutant exhibited a pH profile similar to that of Lys315His, confirming that the titration of the histidine residue

Table 2: Steady-State Parameters of the Wild-Type, Lys315His, and Lys315Gln 908R β-Lactamases at pH 7.0 and 30 °C[a]

	wild-type			Lys315His			Lys315Gln		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (mM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (mM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (mM^{-1} s^{-1})
benzylpenicillin	14	0.5	28000	0.8	40	20	0.025	5	5
ampicillin	0.5	0.4	1300	0.030	5	6	0.004	50	0.08
cephalothin	216	4.2	51000	0.7	4.3	160	0.15	3.5	40
cephalexin	70	10*	7000	6	500*	12	0.10	300*	0.33
nitrocefin	830	33*	25000	>100	>400*	250§	>12	>400*	31§
hippuryl thioglycolate	ND	ND	5.8§	ND	ND	0.4§	ND	ND	ND
hippuryl phenyllactate	ND	ND	19§	ND	ND	0.014§	ND	ND	ND

[a] The K_m values were determined as K_i values with nitrocefin as reporter substrate or measured from the progress curves of the hydrolysis of the substrate (*). Values of k_{cat}/K_m were calculated from the individually measured k_{cat} and K_m values or obtained from first-order time courses at low substrate concentrations (§). SD values did not exceed 10%.

Table 3: Comparison of the Kinetic Parameters between pH 9.0 and 6.0 (8.0 and 6.0)

	ratio pH 9.0/pH 6.0 (pH 8.0/pH 6.0)		
	$k_{cat} = k_3$	K_m	k_{cat}/K_m
benzylpenicillin			
wild-type	1 (1)	1 (1)	1 (1)
Lys315His	29 (15)	8.5 (6)	3.5 (1.3)
Lys315Gln	38 (16)	1.6	20
cephalothin			
wild-type	0.76 (1)	0.5 (0.8)	1.5 (1.3)
Lys315His	24 (16)	18 (9)	1.3 (1.7)
Lys315Gln	14	0.7 (0.65)	20

was not related to the k_{cat} variations. By contrast, the K_m values for the Lys315Gln mutant were not strongly pH dependent, a situation similar to that observed for the wild-type enzyme. Table 2 also shows that the K_m values for the Lys315Gln enzyme were generally higher than those for the wild-type protein, but, as stated above, the K_m values depend upon those of several individual rate constants, and, in consequence, it was important to determine the pH dependencies of the pre-steady-state parameters k_2 and K'.

Pre-Steady-State Parameters. For technical reasons [see Monnaie *et al.* (1992)], the individual k_2 and K' values could be obtained only for carbenicillin, a poor substrate, and in the pH range 6–8. It is, however, unlikely that the behavior of good substrates might be significantly different, as indicated by the relative invariability of the k_{cat}/K_m ratios for the wild-type enzyme with cephalothin and benzylpenicillin within the same pH range, which clearly reflects the similar behavior of the k_2/K' ratio for carbenicillin. Several striking features emerge from the results shown in Table 5: i. With the wild-type enzyme, the k_2 and K' values do not exhibit important variations. ii. Similarly, the K' values for the Lys315Gln mutant remain fairly constant, but k_2 significantly increases with pH. iii. The k_2 value of Lys315His exhibits a pH dependency similar to that of Lys315Gln, but with this latter mutant, K' increases in a parallel manner between pH 6 and 8 so that k_2/K' remains constant.

Since all the k_2 values are rather low, it can safely be assumed that $K' = K = k_{-1}/k_1$ and that the poor catalytic efficiency of the mutants can be attributed more to an impaired acylation (k_2) than to a failure to recognize the substrate (K'). Indeed, and somewhat surprisingly, the K' values at low pH are nearly the same for the three proteins. The pH dependency of the K_m value for the Lys315His mutant appears to reflect that of K', but this is due to the fact that k_2 and k_3 exhibit similar pH dependencies.

Inhibition by (3-Aminophenyl)boronic Acid (m-H_2N-C_6H_4-$B(OH)_2$). This compound, devoid of carboxylic acid side chain, binds to the active-site serine forming an adduct whose structure mimics that of the tetrahedral intermediate (Matthews *et al.*, 1975; Beesley *et al.*, 1983). While the dissociation constant of the complex formed with the wild-type enzyme remained constant between pH 6 and 9, that for the Lys315Gln mutant significantly decreased at higher pH values (Table 6). This result suggested a decrease of the active serine nucleophilic potency in the mutant, which could be partially restored by increasing the pH.

Substrates Devoid of a Free Carboxylate. In contrast to several other penicillin-recognizing enzymes, class C β-lactamases rather efficiently hydrolyze penicillins and cephalosporins where the carboxylate group at C_3 or C_4 has been esterified (Varetto, 1991). The electrostatic potential map of the benzylpenicillin methyl ester was not significantly different from that of the free acid. The study of the interaction between the wild-type enzyme and the methyl esters of benzylpenicillin, ampicillin, and carbenicillin indicated that the disappearance of the negative charge of the carboxylate had similar effects for the three compounds (Table 7) and that these were far from dramatic. If the structure of the side chain, which bears a negative charge in carbenicillin and a partial positive charge in ampicillin, has a distinct influence on the interaction with the enzyme, the modification of the C_3 carboxylate does not appear to result in additional specific modifications for any of the three compounds. The interactions with the mutants were thus only studied with the benzylpenicillin derivative (Table 8). Strikingly, both mutants exhibited better catalytic efficiencies with the ester than with benzylpenicillin itself, in contrast with the wild-type enzyme which shows a low but significant preference for benzylpenicillin. In addition, the pH dependencies of k_{cat} for the hydrolysis of the benzylpenicillin methyl ester by the wild-type and mutant enzymes were analogous (not shown), indicating that the same catalytic functions were affected in the interactions with both substrates.

Transfer Properties. Class C β-lactamases also hydrolyze some noncyclic depsipeptides of general structure C_6H_5-CO-NH-CH_2-CO-X-CHR-COO^-. Two such compounds were examined, hippuryl thioglycolate (X = S, R = H) and hyppuryl phenyllactate (X = O, R = C_6H_5-CH_2-). Hydrolysis of the former by the Lys315His mutant was significantly less impaired than that of the latter (Table 2). In the presence of a suitable D-amino acid, the wild-type enzyme also catalyzes a transfer reaction of the hippuryl moiety onto the amino group of this "acceptor" D-amino acid (Pratt & Govardhan, 1984). With the Lys315His mutant and hippuryl thioglycolate, no such reaction could be detected in the presence of 150 mM D-valine or 20 mM D-phenylalanine, conditions under which transfer/hydrolysis ratios of 1.3 and 1.8 were obtained with the wild-type enzyme.

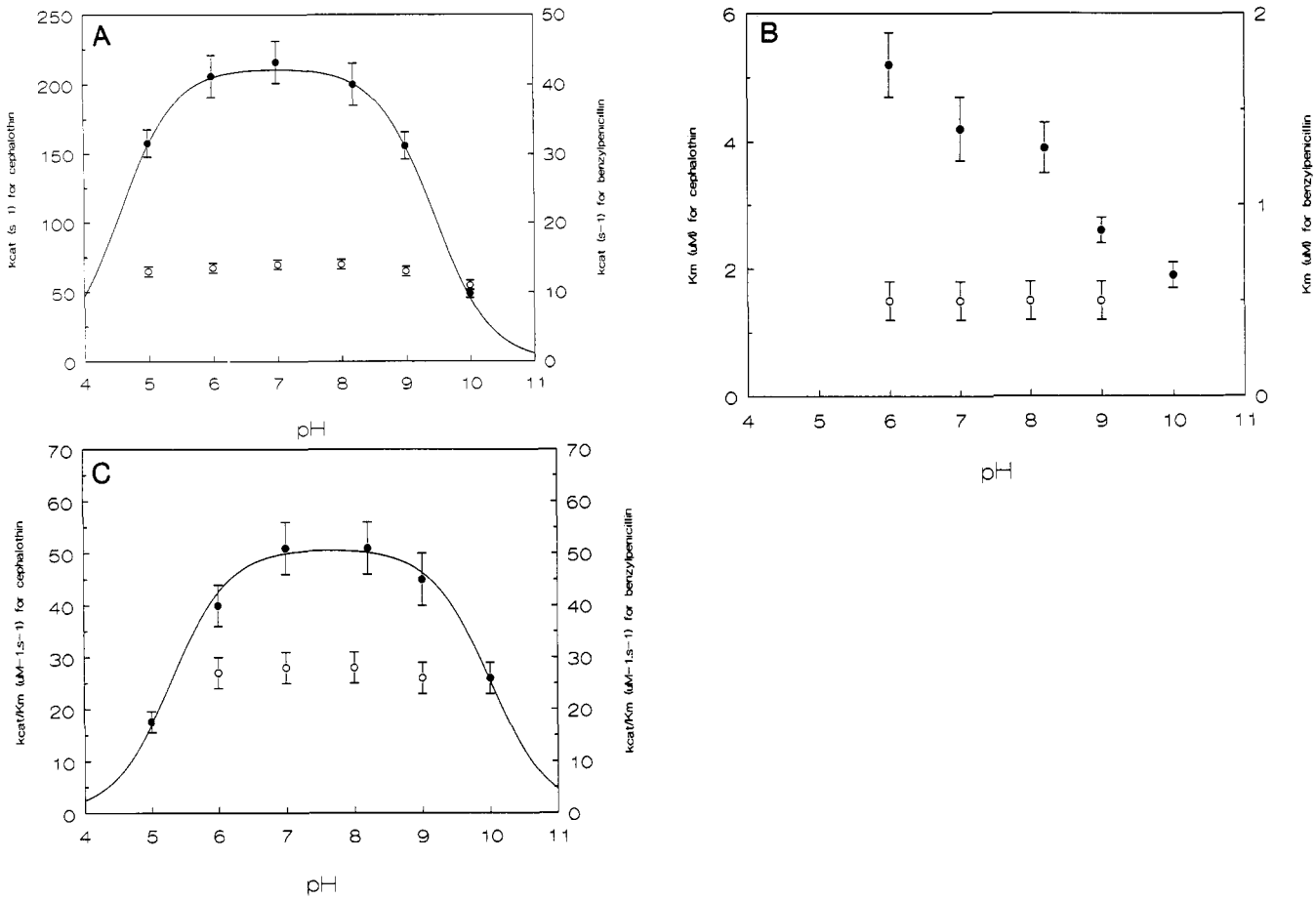


FIGURE 1: pH dependencies of the steady-state parameters (A, k_{cat}; B, K_m; C, k_{cat}/K_m) for the wild-type enzyme: filled symbols, cephalothin; open symbols, benzylpenicillin. For cephalothin, the k_{cat} and K_m values could be fitted to a simple equation: $y = y_{max}/(1 + 10^{pK_1-pH} + 10^{pH-pK_2})$. The theoretical curves thus obtained are shown as solid lines yielding the following values. i. k_{cat}: $(k_{cat})_{max} = 214\ s^{-1}$, $pK_1 = 4.5$, $pK_2 = 9.5$. ii. k_{cat}/K_m: $(k_{cat}/K_m)_{max} = 51 \times 10^6\ M^{-1}\ s^{-1}$, $pK_1 = 5.3$, $pK_2 = 10$.

DISCUSSION

The Positive Charge on Lysine 315. Sequence and X-ray crystallographic data indicate that a basic residue is found in all active serine penicillin-recognizing enzymes in a position analogous to that of Lys315 in class C β-lactamases: Lys in most proteins, Arg in some class A β-lactamases, and His in the sole *Streptomyces* R61 extracellular DD-peptidase. The importance of this side chain has been underlined by the fact that substitutions obtained at this position by side-directed mutagenesis have resulted in severe decreases of the catalytic or penicillin-binding efficiencies of the proteins. The Lys234His mutation in the class A β-lactamase of *Streptomyces albus G* has been particularly interesting. In an acidic environment, the k_{cat} values for the mutant were around 50% of those for the wild-type protein (which showed that neither the k_2 nor k_3 values were severely decreased), but the deprotonation of the imidazole group resulted in a dramatic decrease of the enzyme activity. The fact that the newly introduced histidine was the sole such residue in the mutant protein also allowed an NMR titration experiment yielding a pK_a value which nicely corresponded to that deduced from the pH–activity curves (Brannigan *et al.*, 1991).

The results obtained in the present study with the same mutation in a class C β-lactamase reflect a very different situation. Indeed, at pH 6.0, both the k_{cat} (corresponding to k_3) and k_2 values were decreased by a factor of at least 40. The k_{cat}/K_m values were similarly (cephalothin) or even more affected (benzylpenicillin; see Table 4). In consequence, it appears that, in contrast with the class A enzyme, a positively charged histidine side chain cannot successfully replace the positive lysine side-chain ammonium in the catalytic process of the class C enzyme. One could, however, argue that the pK of the His315 side chain might be grossly altered in this enzyme. Surprisingly and again in striking contrast to the behavior of the class A Lys234His mutant, the k_{cat} (= k_3) and k_2 values for the class C Lys315His mutant increased more than 20-fold between pH 6 and 9. This behavior is very different from that of the wild-type enzyme, with which these two first-order rate constants did not significantly change in the same pH range. Interestingly, the k_{cat} pH profile of the Lys315Gln mutant closely paralleled that of the Lys315His mutant, confirming the irrelevance of the His protonation state in the catalytic phenomenon, and moreover, it is also reminiscent of the behavior of several Lys234 mutants of class A enzymes (Lys234 → Glu, Ala, Thr, Arg) with which the k_{cat} values were also more affected at a lower pH. This might indicate the intervention of nonspecific phenomena (increased deprotonation of the active-site Ser residue and direct involvement of the hydroxyl ions) at high pH (see also below).

Binding of the Substrates (K'). The absence of a direct ionic interaction between the positive lysine side chain and the negative carboxylate of the substrate was underlined by the behaviors of the methyl esters. Strikingly, with the wild-type enzyme, the K' parameter for carbenicillin was only marginally smaller than that for its methyl ester (Table 7).

On the other hand, the disappearance of the positive charge of the side chain of residue 315 had little bearing on the substrate-recognizing process. Indeed, the noncovalent bind-

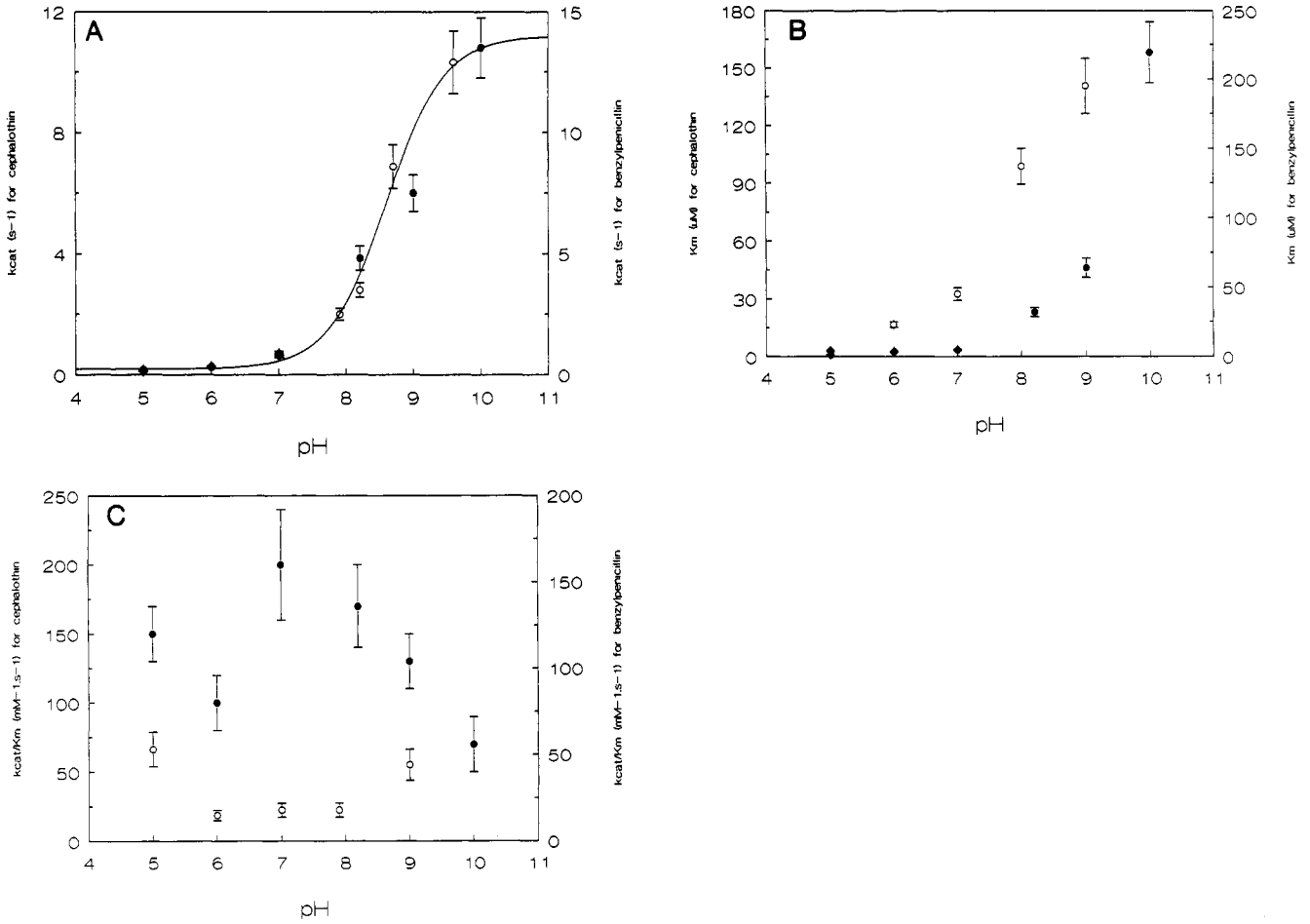


FIGURE 2: pH dependencies of the steady-state parameters for the Lys315His mutant. Symbols are as in Figure 1. It was attempted to fit the k_{cat} values to the simple equation, $y = y_{min} + (y_{max} - y_{min})/(1 + 10^{pK} - 10^{pH})$ but in most cases, fitting on the basis of "simple weighting" (absolute errors) yielded very large relative errors for the data in the low-pH range, while fitting on the basis of "proportional weighting" yielded large errors in the high-pH range. Only for the benzylpenicillin k_{cat} values were good results obtained with both weighting methods (pK = 8.6–8.8, $(k_{cat})_{min}$ = 0.24 s^{-1}, $(k_{cat})_{max}$ = 14.1 s^{-1}; solid curve in Figure 2A). Nonetheless, all fittings yielded pK values between 8.1 and 8.8. However, it is important to note that the evidence for a plateau at high pH values was not very strong, but as stated in the text, the k_{cat} values are certainly not proportional to [OH^-]. No conclusions could be drawn from the k_{cat}/K_m pH dependencies.

Table 4: Influence of pH on the Relative Values of the Kinetic Parameters of the Mutant Enzymes[a]

	$(k_{cat})_{WT}/k_{cat}$		$(k_{cat}/K_m)_{WT}/(k_{cat}/K_m)$	
	pH 6.0	pH 9.0	pH 6.0	pH 9.0
benzylpenicillin				
Lys315His	40	1.5	1800	590
Lys315Gln	2700	68	27000	1300
cephalothin				
Lys315His	820	26	400	460
Lys315Gln	9800	140	6500	240

[a] SD values did not exceed 10%.

ing of carbenicillin by the Lys315Gln mutant and the wild-type enzyme exhibited similar affinities.

In this respect, the increase of the K' value at higher pH's observed with the Lys315His mutant, and which is probably responsible for the similar increase of the K_m values, appears to be due to a fortuitous interference of the nonprotonated imidazole group with the substrate binding more than to a favorable interaction with the protonated form.

Acylation. The major conclusions to be drawn from the pre-steady-state measurements were that the mutations mainly decreased the k_2 values and that, in consequence, the Lys315 side chain contributed to increasing the nucleophilic character of the active serine hydroxyl group. This hypothesis was confirmed by the strongly impaired formation of the tetrahedral intermediate analog upon interaction between the Lys315Gln mutant and (3-aminophenyl)boronic acid. Interestingly, with the mutant, the K_i values were significantly decreased at a higher pH, while they remained unchanged with the wild-type enzyme, suggesting again a nonspecific (or a less specific) favorable effect of a high pH on the formation of the tetrahedral intermediate. Several additional observations point into that direction: i. The Lys315His mutation only results in a 20-fold decrease of the catalytic efficiency ($k_{cat}/K_m = k_2/K'$) versus hyppuryl thioglycolate compared to more than 1000-fold with the very similar hippuryl phenyllactate, which probably reflects the higher intrinsic reactivity of the thiol ester. ii. Accordingly, the k_{cat}/K_m values for the methyl ester of benzylpenicillin are significantly less affected for the mutant proteins than for the wild-type enzyme. It has been shown (Varetto *et al.*, 1991) that the methyl ester is about 16-fold more susceptible to a nucleophilic attack by OH^- ions than benzylpeniciillin itself. Laws and Page (1989) have defined the enzyme rate enhancement factor (EREF) as the ratio between the enzyme efficiency (k_{cat}/K_m) and the k_{OH^-} value which reflects the intrinsic reactivity of the compound. For the wild-type enzyme, the EREF is 80-fold larger for benzylpenicillin than for its methyl ester. Conversely, with the mutants, the EREF's for benzylpenicillin are not very different from those of the methyl ester (Table 8), indicating an increased influence of the intrinsic properties

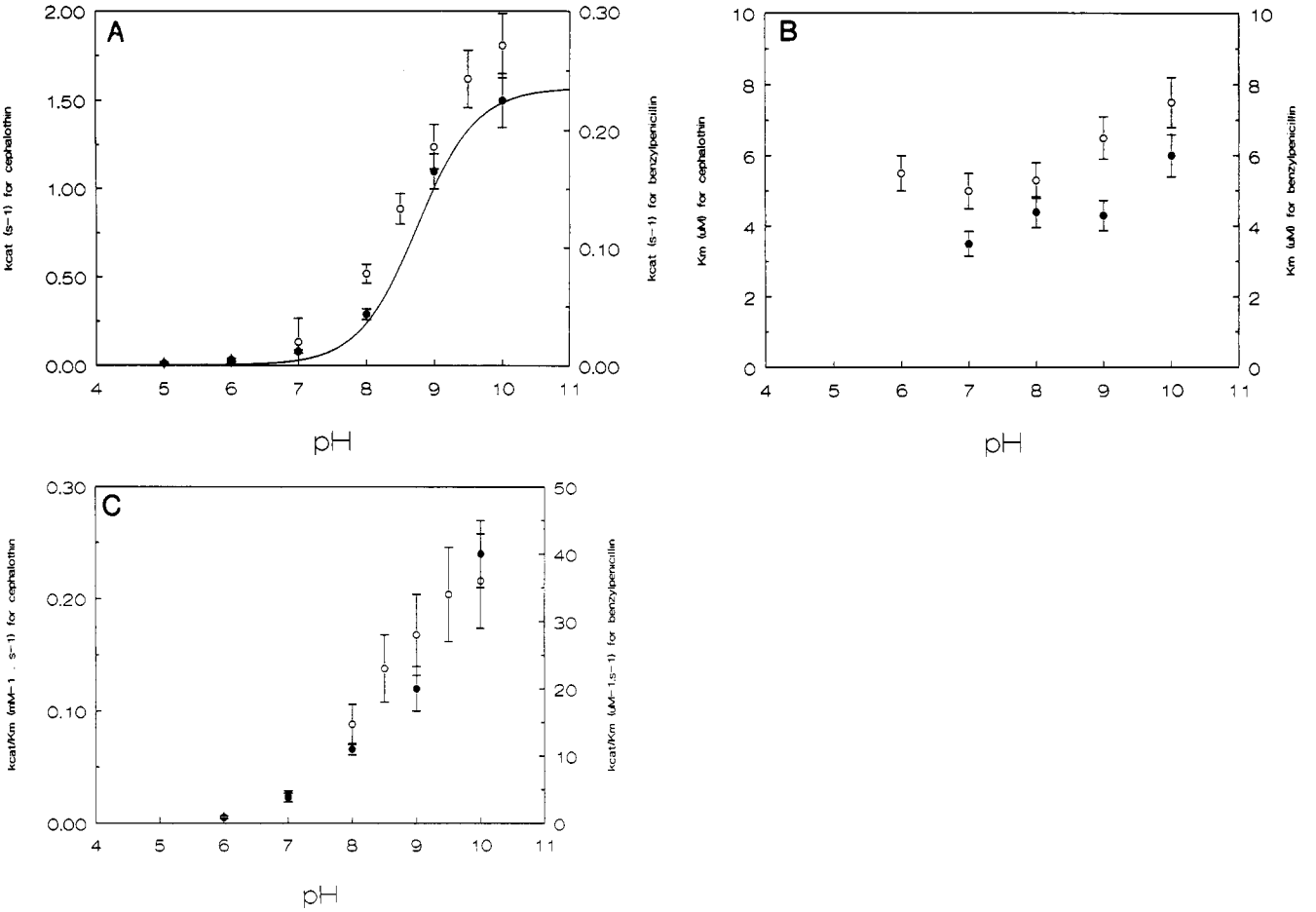


FIGURE 3: pH dependencies of the steady-state parameters for the Lys315Gln mutant. Symbols are as in Figure 1. As in Figure 2A,C, the values in the low-pH range are sometimes superimposed for both compounds. Fitting of the cephalothin k_{cat} values to the equation given in the legend of Figure 2 yielded a reasonably good agreement with the proportional weighting method (solid curve in Figure 3A), and the following values were found, pK = 8.52, $(k_{cat})_{min}$ = 0.001 s^{-1}, and $(k_{cat})_{max}$ = 1.46 s^{-1}. A similar pK of 8.2 was found with benzylpenicillin, but the fitting was much poorer and yielded a nonrandom pattern of residuals. Note that the remarks at the end of the legend to Figure 2 also apply here.

Table 5: Pre-Steady-State Parameters for the Interaction with Carbenicillin Measured by the Reporter Substrate Method

		pH 6.0	pH 7.0	pH 8.0	pH 8.0/pH 6.0
wild type:	k_2 (s^{-1})	4.1	3.5	3.9	0.95
	K' (μM)	45	15	14	0.3
	k_2/K' (mM^{-1} s^{-1})	90	230	280	3.1
Lys315His:	k_2 (s^{-1})	11×10^{-3}	24×10^{-3}	135×10^{-3}	12
	K' (μM)[a]	50	125	500	10
	k_2/K' (mM^{-1} s^{-1})[b]	0.22	0.20	0.27	1.22
Lys315Gln:	k_2 (s^{-1})	$<2 \times 10^{-3}$	6×10^{-3}	20×10^{-3}	>10
	K' (μM)[a]	75	85	110	1.5
	k_2/K' (mM^{-1} s^{-1})[b]	<0.03	0.07	0.18	>6

[a] Determined in a competitive inhibition experiment. [b] Calculated from the individual values. SD values did not exceed 10%.

Table 6: Competitive Inhibition Constants K_i (μM) for the Interaction with (3-Aminophenyl)boronic Acid Hemisulfate[a]

	pH 6.0	pH 7.0	pH 8.0	pH 9.0
wild-type	25	ND	35	35
Lys315Gln	2500	900	350	170

[a] K_i values were measured using nitrocefin as substrate at 30 °C. SD values did not exceed 10%.

of the substrates with the modified proteins, in agreement with a more important role of nonspecific factors with these enzymes.

Deacylation. A similar analysis of the k_{cat} (= k_3) values is more difficult, since the intrinsic stabilities of the various penicilloyl or cephalosporoyl esters are not known. But the following observations indicate an identical emergence of a less specific process on deacylation at high pH values: i. With the wild-type enzyme, the k_{cat} value for benzylpenicillin is less affected at pH 10 than that for cephalothin but the highest values, obtained at pH 7–8, are also 15-fold larger with the latter substrate. ii. At pH 10, the k_{cat} values for the hydrolysis of benzylpenicillin by the wild-type enzyme and the Lys315His mutant are nearly identical and with cephalothin, the mutant k_{cat} value is up to 20% of that of the wild-type. iii. The pH profile of k_{cat} for the hydrolysis of the benzylpenicillin methyl ester by the Lys315Gln mutant was analogous to that for benzylpenicillin, showing that the same catalytic functions were affected in the hydrolysis of both substrates.

The possible involvement of additional less specific processes at high pH values might also explain the impossibility of fitting most of the k_{cat} and k_{cat}/K_m pH dependencies to simple

Table 7: Influence of the Esterification of the C_3 Carboxylate on the Kinetic Parameters of the Wild-Type Enzyme[a]

	k_2 (s^{-1})	K' (μM)	k_2/K' (μM^{-1} s^{-1})	k_3 (s^{-1})
carbenicillin	3.5	15	0.23	4×10^{-3}
carbenicillin methyl ester	2.8	75	0.04	0.5×10^{-3}
ampicillin	150	100	1.5	0.8
ampicillin methyl ester	>50	>300	0.16	0.8[c]
benzylpenicillin	>150	>5	25[b]	14
benzylpenicillin methyl ester	>150	>30	5[b]	23

[a] Pre-steady-state parameters k_2 and K' were determined by the reporter substrate method. The k_3 values were determined as k_{cat} values for benzylpenicillin and ampicillin and by reactivation for carbenicillin. All experiments were performed at pH 7.0 and 30 °C. SD values did not exceed 10%. [b] Determined as k_{cat}/K_i values. [c] Determined by extrapolation of the inactivation pseudo-first-order rate constant at [I] = 0.

Table 8: Steady-State Kinetic Parameters for the Interaction between Benzylpenicillin and Benzylpenicillin Methyl Ester and the Wild-Type, Lys315His, and Lys315Gln Enzymes at pH 7.0[a]

	benzylpenicillin	benzylpenicillin methyl ester
wild-type:		
k_{cat} (s^{-1})	14	23
K_m (μM)[b]	0.5	4
k_{cat}/K_m (s^{-1} μM^{-1})	28	6
EREF	220×10^6	2.6×10^6
Lys315His:		
k_{cat} (s^{-1})	0.7	1.2
K_m (μM)[b]	40	1.25
k_{cat}/K_m (s^{-1} μM^{-1})	18×10^{-3}	1
EREF	140×10^3	470×10^3
Lys315Gln:		
k_{cat} (s^{-1})	0.025	0.025
K_m (μM)[b]	5	0.75
k_{cat}/K_m (s^{-1} μM^{-1})	5×10^{-3}	33×10^{-3}
EREF	38.5×10^3	15.5×10^3

[a] SD values did not exceed 10%. EREF = enzyme rate enhancement factor (Laws & Page, 1989). [b] The K_m values were determined as K_i.

equations resting on the ionization of one or two enzyme or acylenzyme functional group(s) (mainly with benzylpenicillin). It should likewise be noted that the k_{cat} pH profiles for the mutants do not directly reflect the OH^- concentrations, in which case a 1000-fold increase of the k_{cat} values should be expected between pH 7 and 10.

The Role of Lysine 315 and of the Equivalent Residues in Other Enzymes. It can safely be concluded that the major role of the Lys315 side chain appears to consist in facilitating the nucleophilic attack of the substrate by the active-site serine residue and of the acylenzyme by the water molecule in the acylation and deacylation processes, respectively. Moreoever, the specific impairment of the transacylation reaction with the K315H mutant underlines an additional role for this residue. This mutant can be compared to the wild-type *Streptomyces* R61 DD-peptidase in which a histidine is found in the equivalent position. This residue was assumed to be involved as a general base catalyst in the transpeptidation reaction because transpeptidation appeared to be dependent upon a pK_a of about 7 (Frère *et al.*, 1973). Accordingly, when this histidine was replaced by Lys or Gln, the enzyme behaved almost exclusively as an hydrolase (Hadonou *et al.*, 1992). In consequence, it appears that each enzyme has optimized the transpeptidation mechanism using a different residue and that the Lys and His side chains cannot be interchanged since they are unlikely to exhibit similar pK_a values.

One is forced to conclude that if these two side chains play an equivalent role in both enzymes, it is probably not a direct activation of the acceptor molecule by proton withdrawal but they might indirectly facilitate the nucleophilic attacks by (i) the active serine residue in the acylation step and (ii) the acceptor or the water molecule in the transfer or hydrolysis reactions, respectively. Alternatively, the two enzymes might have diverged so much that these structurally equivalent residues would no longer play similar roles in the catalytic processes. This latter hypothesis indeed receives additional support from the very different behavior of the class A Lys234His mutant.

One major question remains completely unsolved at the end of the present discussion: why is the Lys315His mutant consistently better than its Lys315Gln counterpart? This observation is quite surprising if one considers that protonation of the His side chain does not improve the catalytic properties of the former protein. As noted above, the decreased stability of the Lys315Gln mutant might reflect a somewhat altered conformation, but the low K' value for carbenicillin, similar to that observed with the wild-type enzyme, seems to be in good agreement with the absence of significant modifications of the active-site structure. One can only hope that attempts to solve the crystal structure of the three proteins studied here will be successful and will supply an answer coherent with the kinetic data.

In the meantime, it can safely be concluded that the Lys315 side-chain major role consists in facilitating nucleophilic attacks by other participants in the catalytic process. If it does contribute to the binding of the substrate carboxylate through an electrostatic interaction, this factor can be considered, at best, as very minor. These conclusions are in complete disagreement with those of Tsukamoto *et al.* (1990) who, on the basis of the properties of the Lys315Arg mutant of another class C β-lactamase from *Citrobacter freundii*, had suggested that the main role of the Lys315 side chain was to electrostatically interact with the substrate carboxylate as proposed by Oefner *et al.* (1990). It should, however, be emphasized that their studies only involved a small number of substrates, that no effort was made to determine the values of the individual rate constants, and that the analysis of the pH dependency of the kinetic parameters remained rather incomplete. Nonetheless, they also observed rather high k_{cat} values for the mutant in the high-pH range. However, their wild-type enzyme consistently exhibited k_{cat} values 2 orders of magnitude lower than those observed here with the wild-type *E. cloacae* enzyme or by Galleni *et al.* (1988) for a nearly identical *C. freundii* class C β-lactamase. One can thus wonder about the meaning of the conclusions drawn from modifications of an already severely impaired wild-type protein.

How Can Lys315 Facilitate Nucleophilic Substitutions? Lys315 could be catalytically effective in the formation of a positive electrostatic field in the enzyme active site or, alternatively, in stabilizing the basic form of an ionizable group acting as a general base catalyst. On the basis of crystallographic observations, Oefner *et al.* (1990) proposed that Tyr150 was suitably located to act as a general base but it would mean that the pK_a of this tyrosine is lowered by at least 6 units and this mechanism can hardly be generalized to class A β-lactamases where a serine is found in that position.

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