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To cite this version:
Françoise Lenfant, Roger Labia, Jean-Michel Masson. Replacement of lysine 234 affects transition state stabilization in the active site of beta-lactamase TEM1. Journal of Biological Chemistry, American Society for Biochemistry and Molecular Biology, 1991, 266 (26), pp.17187-17194. hal-02713231

HAL Id: hal-02713231
https://hal.inrae.fr/hal-02713231
Submitted on 1 Jun 2020

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Replacement of Lysine 234 Affects Transition State Stabilization in the Active Site of \(\beta\)-Lactamase TEM1*

(Received for publication, March 25, 1991)

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Lysine 234 is a residue highly conserved in all \(\beta\)-lactamases, except in the carbenicillin-hydrolyzing enzymes, in which it is replaced by an arginine. Informational suppression has been used to create amino acid substitutions at this position in the broad spectrum *Escherichia coli* \(\beta\)-lactamase TEM-1, in order to elucidate the role of this residue which lies on the wall at the closed end of the active site cavity. The mutants K234R and K234T were constructed and their kinetic constants measured.

Replacement of lysine 234 by arginine yields an enzyme with similar activity toward cephalosporins and most penicillins, except toward the carboxybenzylpenicillins for which the presence of the guanidine group enhances the transition state binding. The removal of the basic group in the mutant K234T yields a protein variant which retains a low activity toward penicillins, but loses drastically its ability to hydrolyze cephalosporins. Moreover, these two mutations largely decreased the affinity of the enzyme for penicillins (10-fold for K234R and 50-fold for K234T). This can be correlated with the disruption of the predicted electrostatic binding between the C3 carboxylic group of penicillins and the amine function of the lysine.

Therefore, lysine 234 in the *E. coli* \(\beta\)-lactamase TEM-1 is involved both in the initial recognition of the substrate and in transition state stabilization.

The most common mechanism of resistance to \(\beta\)-lactams is the production of \(\beta\)-lactamases, in both Gram+ and Gram− bacteria. Understanding the molecular details of the interactions between the \(\beta\)-lactamases and their substrates, the \(\beta\)-lactam antibiotics, could help draw a precise picture of the structure-function relationships within the active site. This should prove an invaluable tool for the design of new antibiotics.

On the basis of comparison of the primary structures of a few \(\beta\)-lactamases, the different enzymes have been grouped into four classes (1), A, B, C, and D, of which the \(\beta\)-lactamases of the class A are the most commonly encountered in clinical isolates. The sequence alignments have defined seven highly conserved "boxes" (2). The crystallographic data of the class A \(\beta\)-lactamases from Gram-positive bacteria *Staphylococcus aureus* PC1 (3), *Streptomyces albus* G (4), and more recently the high resolution structure of *Bacillus licheniformis* 749/C \(\beta\)-lactamase at 2.0 Å (5) suggest strongly that the sequence homologies of class A \(\beta\)-lactamases can be related to high tertiary structural and functional similarities.

The \(\beta\)-lactamases involve an active site serine residue, Ser-70 (using Ambler’s numbering) and the hydrolysis of the \(\beta\)-lactam ring is catalyzed via an acyl-enzyme intermediate (6). It has been suggested that both lysine 73 and glutamic acid 166 play a part in the mechanism. Oligonucleotide-directed mutagenesis performed at these positions resulted in protein variants with drastic loss of activity (7, 8).1 The role of the Ser-Asn-Asn region has just been investigated (9). On the \(\beta\) strand delimiting the active site, residues were also identified as playing a role in substrate binding or in substrate specificity (10–12). Lysine 234 is located on this \(\beta\) strand, in the highly conserved triad Lys-Ser-Gly or Lys-Thr-Gly (box VII) both in \(\beta\)-lactamases and in most penicillin-binding proteins. The crystallographic data (3, 5) have shown that this highly conserved amino acid is localized in the wall at the closed end of the active site depression, and its contribution to the initial binding of the antibiotic in the catalytic cavity was postulated. Recently, site-directed mutagenesis was used by Ellerby et al. (13) to substitute the lysine 234 with glutamic acid or alanine in the \(\beta\)-lactamase from *B. licheniformis*, demonstrating its importance in catalysis. However, in most known carbenicillin-hydrolyzing enzyme sequences (14), this lysine is replaced by an arginine. All the models that are proposed on the interaction of the class A enzymes with their substrate are built from \(\beta\)-lactamases from Gram− organisms and penicillin. It remains to be seen whether this can be fully applied to enzymes from Gram+ bacteria, which usually exhibit a broader substrate spectrum including cephalosporins.

To investigate further the role of lysine 234 in the binding and catalysis of \(\beta\)-lactam hydrolysis by one such broad spectrum \(\beta\)-lactamase, the enzyme TEM-1, we used informational suppression. This is a powerful tool to investigate the effects of various amino acid substitutions at a given position in a protein. With the new extended set of amber suppressor strains (15–17), this method has been used recently to create variants of the lac repressor (18), to investigate the thermostability of \(\alpha\)-amylase (19) or to study new substrate specificity in TEM-1 \(\beta\)-lactamase (11). We likewise generated multiple amino acid substitutions of this lysine 234 in the \(\beta\)-lactamase TEM-1 and investigated the phenotype of the variants producing strains. This informational suppression experiment suggested to us the substitution of lysine 234 by a threonine, in addition to the more conservative substitution by an arginine.

1 M. Delaire, F. Lenfant, R. Labia, and J. M. Masson, unpublished results.
MATERIALS AND METHODS

E. coli Strains and Plasmids—Bacterial strains used were E. coli Xac-1; E. coli lacI, lacZ, recA, proA, proD (Hfr) (McNeil, 1965), naa rif argE6, and its derivatives expressing a trNA_DUA suppressor gene. They have been described previously (11). Suppressor plasmids are 81, 82, 89, 83, 84, and GlyU inserting serine, glutamine, tyrosine, leucine, and glycine, respectively, and the synthetic suppressor genes AlgB, trNA_DUA, Cys-trNA_DUA, GlnA-trNA_DUA, His-trNA_DUA, Lys-trNA_DUA, Pro-trNA_DUA, Phe-trNA_DUA, Thr-trNA_DUA, and Val-trNA_DUA. The Arg-trNA_DUA is made from a modified phenylalanine inserting gene (20). To provide compatibility with any plasmid having a ColE1 replicative origin, the HindIII-HincII fragment from pGFB1 was cloned in the plasmid pCT1 by the same fragment from plasmid pT28C2 encoding the mutant bla gene T284A (23). The resulting plasmid was called pCT3.

Media, Antibiotics, and Enzymes—Cells were grown in LB medium supplemented with the appropriate antibiotics: 100 µg/ml ampicillin (Sigma), 30 µg/ml chloramphenicol (Serva), and 12 µg/ml tetracycline (Boehringer Mannheim). [35S]UTP was provided by Amersham Corp. All enzymes for genetic engineering were obtained from Pharmacia LKB Biotechnology Inc.

Antibiotic powders were a gift from their respective manufacturers: benzylpenicillin (Rhone-Poulenc), amoxicillin, ticarcillin, carbenicillin, clavulanic acid (Beecham), piperacillin (Lederle), cephalothin (Lilly), cephaloridine (Glaxo), cefoperazone (Pfizer), cefotaxime (Rooussel-Uclaf). Phenoxymethylpenicillin was purchased from Sigma.

Bacterial strains producing the different protein variants were assayed for growth in the presence of various β-lactam antibiotics. Assays were first done using the disc diffusion technique on LB agar plates. Paper discs (Diagnostics Pasteur) impregnated with the indicated amount of antibiotic (25 µg of amoxicillin, 20 µg of amoxicillin + 10 µg of clavulanic acid, 75 µg of ticarcillin, 75 µg of ticarcillin + 10 µg of clavulanic acid, 30 µg of cephalothin, 30 µg of cephaorozone, and 30 µg of cefotaxime) were placed on a lawn of growing cells, and the area of inhibited growth around the discs was measured.

Recombinant DNA Techniques—Cloning techniques were based on Maniatis et al. (24). DNA sequencing was carried out using the Pharmacia T7 polymerase sequencing kit. The preparation of competent E. coli cells and subsequent transformation with the inserts were carried out according to the protocol of Hanahan (25).

Oligonucleotides and Site-directed Mutagenesis—Synthetic oligonucleotides were made as trityl derivatives on an Applied Biosystem 380B DNA synthesizer using phosphoramidite chemistry and purified on Nensorb Prep Columns as specified by the manufacturer (I. E. Du Pont de Nemours & Co., Inc.). The following oligonucleotides were used:

- K234am 5'ACCGCGTCCTCATTATCAGCCTGAT ATTAC 3'
- K234R 5'ACGGCTCCAGCGGATCCAGCAGAT 3'
- K234T 5'ACCGCTCCAGGTCAGGATCCAGCAGAT 3'

The codon downstream from the amber codon was changed from TCT to AGT to ensure a better suppressor context without changing the protein sequence.

Oligonucleotides were phosphorylated as previously described (15). The single-stranded pCT1 DNA was isolated after infection by the helper phage M13K07 (Pharmacia). The site-directed mutagenesis was then accomplished using Eckstein's method (26). The mutant colonies were selected for their loss of resistance to ampicillin. Multiple revertants were obtained by the same manner, starting from the amber mutated genes. The mutations were confirmed by sequencing the entire gene to check that no undesired mutation had been introduced. This sequencing was performed using single-stranded pCT1 DNA and six internal oligonucleotides as primers.

β-Lactamase Assay and Purification—The expression vector was introduced pCT3. Bacterial cells were grown at 30 ºC in LB medium supplemented with 0.02% glycine, 20 mM MgSO4, 100 µg/ml ampicillin, and 12 µg/ml tetracycline. β-Lactamase was extracted by osmotic shock, as previously described (27).

The β-lactamase was then purified from 1 liter of culture by preparative electrofocusing using the Multiphor II system (LKB) on a 4-6.5 pH gradient. After elution with 10 ml of 50 mM sodium phosphate buffer, pH 7, gel filtration on a Sephacryl HR100 (Pharmacia) column was further performed to reach a high level of purity. The fractions containing the enzyme were detected using the nitrocefin assay (28) in 50 mM buffer, at 37 ºC (ε = 20,500 M⁻¹cm⁻¹). Protein concentration was determined by absorbance measurements at 280 nm, with ε = 18,200 M⁻¹cm⁻¹ obtained for the wild type enzyme. The homogeneity of the protein can be estimated to be >85% on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The isoelectric point (pI) was determined on analytical isoelectric focusing revealed by the iodine procedure, in agar gel with benzylpenicillin as substrate (29).

Determination of the Kinetic Parameters of the Mutant Enzymes—The kinetic constants kcat and Km for various substrates were determined by computerized microsometry (30). When the Km value was too high, only kcat/Km could be obtained by using a nonlinear least square fit regression for one exponential curve at [S] ≪ Km.

The affinity of the enzyme for the inhibitor, which is expressed as the inhibition constant Ki, was measured using competition procedures with benzylpenicillin. It is determined from the extrapolated rate, at the time when the inhibitor is added. Inhibition by clavulanic acid was tested by incubation with the enzyme for 10 min at 37 ºC before measuring the remaining enzymatic activity. KI value was defined as the concentration of inhibitor causing 50% inhibition of enzymatic activity. The enzyme was incubated with highly purified proteins, the value of KI was used to calculate the stoichiometry of the reaction in order to access to the turnover number. The inactivation constant kina was also deduced from incubation of the inhibitor with the enzyme for various times. A large excess of benzylpenicillin was thus added and the remaining rate was monitored. The measure of the half-life of inactivation then allowed us to calculate the kina value (kina = ln2/t½ when [S] ≫ Km).

One unit of β-lactamase activity is defined as the amount of enzyme hydrolyzing 1 µmol of substrate/min at pH 7 and 37 ºC.

RESULTS

Multiple Amino Acid Substitutions of Lysine 234 and Expression in E. coli

To perform the multiple amino acid substitutions of lysine 234, the amber codon “TAG” was first introduced at the corresponding codon “AAA” into the bla gene TEM-1 by oligonucleotide-directed mutagenesis. After the insertion of the highly efficient promoter Pa+Pb of T284A gene in front of the mutated bla gene, the plasmid pCT3 was introduced in the 14 available strains harboring an amber suppressor gene. Fourteen protein variants at the position 234 were thus generated. The activity of each protein variant was then characterized phenotypically by antibiotic disk assays with respect to five different antibiotics: two penicillins, amoxicillin and ticarcillin, alone or in combination with clavulanic acid; a first generation cephalosporin, cephalothin; two third generation cephalosporins, cefoperazone and cefotaxime (Fig. 1).

Table I reports the values of the inhibition diameters as determined by antibiograms. It can first be observed that the strain expressing the mutated gene in the absence of suppressor presented no β-lactamase activity. Immunoblot revealed no detectable amount of the enzyme (data not shown), indicating that the truncated unfunctional protein was unstable or could not be exported to the periplasmic space. In other respects, the strain overexpressing the amber mutated gene was found to grow very slowly. The substitution of lysine 234 by the different amino acids yielded a large number of mutants demonstrating a very low but detectable activity. The strains producing the protein variants K234G, K234E, K234C, K234Y, and K234S exhibited some activity toward amoxicillin but far from a true resistance phenotype. Surprisingly, these β-lactamase mutants correspond to substitutions by amino acids with polar chains. Suppression efficiency can vary widely depending both on the type of suppressor used and the
Replacement of Lysine 234 in TEM-1 β-Lactamase

context of the amber codon to be suppressed, thus resulting in variable amounts of the protein being made. However, previous measures (11) showed that as little as 1/10,000 of the activity of the wild type is readily detected with the antibiotic disc assays and actually still confers resistance to the selected penicillin. In the case of the aforementioned substitutions, one can assume from the inhibition diameters that the residual activity of these protein variants does not fall even within that range.

Of the 14 protein variants obtained at position 234, only three enzymes were significantly active: the ones obtained with the lysine, arginine, or threonine inserting suppressor tRNAs. Unfortunately, the threonine suppressor inserts both threonine and lysine (17), thus making the corresponding results in Table I inconclusive. To study further these substitutions, the amber mutation was reverted by oligonucleotide-directed mutagenesis, to the missense codons CGG and ACG for arginine and threonine, respectively. The pUC bla promoter was then replaced by the stronger promoter from T284. No difference in the level of expression between K234R, K234T β-lactamase, and the wild type enzyme was observed by immunoblots. The antibiotic disc assays (Table II) did not reveal major differences for the protein variant K234R compared with the wild type. This is not the case for the protein variant K234T; the inhibition diameters show a relative decrease in ticarcillin resistance and a totally susceptible phenotype toward cephalosporins. Moreover, the inhibition diameter for ticarcillin combined with clavulanic acid (timentin) was surprisingly small compared with the value for ticarcillin alone.

These first results suggest that these protein variants had enough activity to be analyzed in more detail. The enzymes were then purified almost to homogeneity, as described under “Materials and Methods.”

The Mutant Enzyme K234R

Kinetic Parameters—Detailed kinetic studies were then performed using the microacidometric method on a large variety of substrates: amino-, carboxy-, and ureidopenicillins and first and third generation cephalosporins. The kinetic constants for the enzymes are given in Table III.

The $k_{cat}$ values for K234R and TEM-1 β-lactamases for

| Penicillins | Cephalosporins |
|-------------|----------------|
| Benzylpenicillin | Cephalothin |
| Phenoxymethylpenicillin | Cephaloridine |
| Amoxycillin | Cefoperazone |
| Ticarcillin | Cefotaxime |
| Carbenicillin | Inhibitor |
| Piperacillin | Clavulanic acid |

**Fig. 1.** β-Lactam antibiotics used in this study.
benzylpenicillin are similar. However, the mutation K234R causes significant changes in the catalytic profile toward the other penicillins. The mutant enzyme presents a greater ability to hydrolyze the carboxypenicillins and the ureidopenicillin tested (6-20-fold increase). In contrast, the affinity has been decreased 10-20-fold for all the penicillins, including the carboxypenicillins. Thus, the mutant K234R exhibits significant decrease in catalytic efficiency ($k_{cat}/K_m$) toward benzylpenicillin and amoxicillin, whereas the catalytic efficiency toward carboxypenicillin is conserved. In contrast, the hydrolysis of all the cephalosporins tested is unmodified by the mutation. Both the $k_{cat}$ and the affinity constant $K_m$ remain similar for the K234R mutant and TEM-1 enzyme.

**Interactions with Clavulanic Acid**—Clavulanic acid is the first inhibitor effective in vitro as well as in vivo when combined with amoxicillin or ticarcillin. The competitive and irreversible inhibition can be fitted to Scheme 1 (31, 32):

$$k_1 E + I \rightarrow (E, I) \rightarrow (E, I)^* \rightarrow E - 1 \downarrow$$
$$k_1 E + P$$

**Scheme 1.**

Where $(E,I)$ is the noncovalent Michaelis complex, $(EI^*)$ the acyl-enzyme, $(E-I)$ an inactivated form of the enzyme still covalently linked to the inhibitor, and $P$ a degradation product of the clavulanic acid. The different parameters relative to the inhibition are reported in Table IV. The ratio $k_i/k_r$ (or turnover number, $t_o$) represents the number of molecules metabolized before the $(EI^*)$ complex is rearranged into its inactivated form. Therefore, a variation of the $k_i/k_r$ ratio can be explained by a variation of the deacylation rate constant $k_r$ or by an opposite variation in the value of $k_i$. Starting from the expression of the rate of inactivation in terms of the instantaneous concentration of $EI^*$ given by Equation 1:

$$d[E - I]/dt = k_1 [EI^*]$$

The rate of formation of $EI^*$ is then given by Equation 2:

$$d[EI^*]/dt = k_d[E,I] - k_d[EI^*] - k_d[EI^*]$$

Applying Bodensteins’s steady state approximation, which in the context of Equation 2 sets $d[EI^*]/dt = 0$, we obtain

$$0 = k_d[E,I] - (k_i + k_d)[EI^*]$$

which can be rearranged into

$$[EI^*] = [E,I]k_2/(k_3 + k_d).$$

Since $E,I$ is the instantaneous concentration of the non-

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**Table I**

Values of inhibition diameters given by antibiotic disk assays for the XaC-1 strain and the 14 suppressor strains expressing the wild type enzyme or the β-lactamase mutant gene K234am

In the first column, we reported the amino acid inserted by suppression. The values of the inhibition diameters are given in millimeters; R, no inhibition zone (i.e. diameter < 7); AMX, amoxicillin; AMC, augmentin (amoxicillin + clavulanic acid); TIC, ticarcillin; TCC, timentin (ticarcillin + clavulanic acid); CF, cefotaxime; CFP, cefoperazone; and CTX, cefotaxime.

| Amino acid inserted by the suppressor | AMX | AMC | TIC | TCC | CF | CFP | CTX |
|--------------------------------------|-----|-----|-----|-----|----|-----|-----|
| Ala                                  | 19  | 22  | 25  | 31  | 34 | 20  | 31  | 34 |
| Arg                                  | 12  | 24  | 21  | 33  | 31 | 18  | 31  | 35 |
| Cys                                  | 11  | 25  | 25  | 33  | 32 | 20  | 32  | 35 |
| Gln                                  | 12  | 25  | 25  | 33  | 32 | 20  | 30  | 35 |
| Glu                                  | 16  | 25  | 28  | 33  | 32 | 20  | 32  | 35 |
| His                                  | 20  | 25  | 28  | 33  | 32 | 19  | 32  | 40 |
| Leu                                  | 23  | 23  | 28  | 32  | 28 | 20  | 28  | 34 |
| Lys                                  | 14  | 25  | 25  | 33  | 32 | 21  | 32  | 35 |
| Phe                                  | 19  | 26  | 25  | 33  | 32 | 21  | 30  | 35 |
| Pro                                  | 9   | 25  | 14  | 33  | 23 | 21  | 31  | 35 |
| Ser                                  | 22  | 22  | 27  | 19  | 26 | 24  | 26  | 34 |
| Thr                                  | 12  | 25  | 14  | 33  | 22 | 31  | 35  | 35 |
| Tyr                                  | 19  | 25  | 28  | 33  | 20 | 31  | 31  | 34 |
| K234am                               | 12  | 14  | R   | R   | R | 34  | 34  | 34 |
| TEM-1                                | 10  | 12  | 12  | 9   | R | 34  | 34  | 34 |
| K234T                                | 11  | 12  | 14  | 19  | 29 | 34  | 34  | 34 |
| K234am                               | 15  | 25  | 28  | 33  | 35 | 22  | 31  | 34 |

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**Table II**

Antibiogram disk assays for the protein variants K234R and K234T compared with the wild type enzyme TEM-1

The diameters are given in millimeters; R, no inhibition zone (i.e. diameter < 7). Other abbreviations are defined in Table I legend.

|                  | AMX | AMC | TIC | TCC | CF | CFP | CTX |
|------------------|-----|-----|-----|-----|----|-----|-----|
| K234R            | R   | 10  | R   | 12  | 9  | R   | 34  |
| K234T            | R   | 11  | 12  | 14  | 19 | 29  | 34  |
| TEM-1            | R   | 10  | 14  | R   | R  | 34  | 34  |
| K234am           | 15  | 25  | 28  | 33  | 35 | 22  | 31  |

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**Table III**

Kinetic constants of the protein variants K234R and K234T compared with the wild type enzyme TEM-1

The $k_{cat}$ and the $K_m$ values were determined as described under "Materials and Methods." The $k_{cat}/K_m$ were calculated (ND), not determined. Asterisks indicate that the values could not be determined.

|                  | TEM-1 | K234R | K234T |
|------------------|-------|-------|-------|
| $k_{cat}$        | $s^{-1}$ | $K_m$ | $s^{-1}$ | $K_m$ | $s^{-1}$ | $K_m$ |
| Penicillins      |       |       |       |       |       |       |
| Benzylpenicillin | 1200  | 24    | 5.0·10³ | 1000  | 24    | 4.2·10⁶ | 25    | 900  | 2.7·10⁹ |
| Penicillin G     |      | 1400  | 25    | 4.5·10³ | 500   | 100   | 5.0·10⁶ | ND    | ND   | ND    |
| Amoxicillin      |      | 1008  | 43    | 2.3·10³ | 600   | 155   | 3.8·10⁶ | 7.5    | >1000 | <7.3·10⁹ |
| Ticarcillin      |      | 36    | 10    | 3.6·10⁶ | 600   | 240   | 2.5·10⁶ | 1.125  | >1000 | <11.1·10⁹ |
| Carbenicillin    |      | 120   | 14    | 8.5·10⁵ | 700   | 180   | 3.8·10⁶ | ND    | ND   | ND    |
| Piperacillin     |      | 144   | 260   | 5.5·10⁹ | 950   | >250  | <3.8·10⁻⁵ | ND    | ND   | ND    |
| Cephalosporins   |      |       |       |       |       |       |       |       |       |       |
| Cephalothin      |      | 144   | 350   | 4.1·10³ | 90    | 250   | 3.6·10⁻⁵ | <0.5  | ND   | ND    |
| Cephaloridine    |      | 2040  | 612   | 3.3·10⁶ | 1700  | 800   | 2.1·10⁻⁵ | ND    | ND   | ND    |
| Cephalorazone    |      | 492   | 240   | 2.1·10⁶ | 800   | 600   | 1.3·10⁻⁵ | <0.5  | ND   | ND    |
| Cefotaxime       |      | 3.6   | ND    | ND    | 1     | ND    | ND    | **   | **   | **    |

*Phenoxymethylpenicillin.*
covalent Michaelis complex, the factor $k_2/k_1/(k_1 + k_2)$ is the rate constant for inactivation of the enzyme, on the assumption of the irreversible reaction Scheme 1. This inactivation rate constant, $k_{\text{inac}}$, can be approximated to $k_2/k_1/k_3$, as $k_4 \ll k_3$. The turnover number can then be correlated with the $k_{\text{cat}}/k_{\text{inac}}$ ratio.

We observed some difference in the interaction with clavulanic acid between the mutant K234R and TEM-1. The affinity of K234R for clavulanic acid is reduced 5–8-fold, in the same proportion as its affinity for penicillins. Nevertheless, the inactivation constant $k_{\text{inac}}$ is slightly increased, the inactivation then becomes a little more efficient. As the catalytic constant $k_{\text{cat}}$ is lower, this behavior could explain in part the decrease of the turnover number. This decrease of the turnover is probably due to a decrease of the deacetylation rate constant $k_2$ more than $k_4$.

In the mutated enzyme K234R, the reversible formation of the enzyme-inhibitor complex characterized by the affinity constant $K_i$ is reduced compared with the wild type, whereas the rearrangement into the inactivated complex appears to be more efficient.

**Effects of pH on Kinetics Properties—**Replacement of lysine 234 by an arginine in β-lactamase TEM-1 does not modify the isoelectric point of the enzyme, and the titration curve does not reveal a difference in charge, over the pH range 3–9 (data not shown). This is not so surprising, as both the lysine and the arginine are protonated in this range of pH. Nevertheless, we studied the pH dependence of the kinetic constants for benzylpenicillin and cephalothin of the K234R mutant compared with the wild type.

The plots for $k_{\text{cat}}$ against pH over the range 5.5–8.5 for hydrolysis of benzylpenicillin and cephalothin are reported in Fig. 2. The optimum pH for hydrolysis of both substrates by K234R is one unit lower than that of the wild type. For the $K_m$ values, which are plotted in Fig. 3, there is a clear dependence on the pH. The affinity is conserved at low pH range (pH < 6.5) and at higher pH, the constant $K_m$ increases with pH both for benzylpenicillin and cephalothin. Although the affinity constants differ by one order of magnitude, the $K_m$ values for benzylpenicillin for K234R and the wild type follow the same profiles, and the discrepancy between the curves remains the same over the pH range. For cephalothin, the $K_m$ curves present essentially the same pattern, with a slightly better affinity for the mutant K234R than the wild type. This behavior corroborates the $k_{\text{cat}}$ values (Table III), which showed that the hydrolysis of penicillins is more perturbed than that of cephalosporins with the mutant enzyme K234R. The guanidine function of arginine, with its side chain 1 Å longer, is probably in a spatial location which still remains correct for the hydrolysis of cephalosporins.

The curve for $k_{\text{cat}}/K_m$ (Fig. 4) for K234R compared with the wild type shows that the apparent alkaline $pK_a$ for the free enzyme was unmodified. These indications strongly suggest that the lysine is not directly responsible for the pH dependence of $k_{\text{cat}}/K_m$.

### Table IV

| Enzyme       | $K_m$ (μM) | $k_{\text{cat}}/k_4$ | $k_{\text{inac}}$ |
|--------------|------------|----------------------|-------------------|
| TEM-1        | 0.08       | 100                  | 0.02              |
| K234R        | 0.5        | 30                   | 0.032             |
| K234T        | 300        | 300,000              | <0.00019          |

**Fig. 2.** pH dependence of the relative $k_{\text{cat}}/K_m$ for wild type (O), K234R (○), and K234T (△) enzymes. The hydrolysis of benzylpenicillin (A) or cephalothin (B) was measured at 37°C by computerized microacidometry using [5] $K_m$. The ordinate represents the rate constant $k_{\text{cat}}$ relative to its maximum value over the pH range tested.

### The Mutant Enzyme K234T

**Kinetic Parameters—**The conversion of lysine 234 to a threonine had a drastic effect on activity toward penicillins. The $k_{\text{cat}}$ for benzylpenicillin was reduced about 50-fold, and the $K_m$ was increased by a comparable amount ($K_m = 900$ μM). For the other penicillins, the affinities were so drastically decreased that the $K_m$ values cannot be measured. Thus, the catalytic efficiency $k_{\text{cat}}/K_m$ was very low (1000-fold reduction). Moreover, the $k_{\text{inac}}$ values for all the cephalosporins dropped 300–1000-fold compared with the wild type enzyme. Consequently, the mutation resulted in more than 99.7% reduction of its activity (Table III) toward cephalosporins, but the enzyme still retained a significant activity toward penicillins.

**Interactions with Clavulanic Acid—**The suicide inhibitor clavulanic acid was also tested on the mutant K234T. In contrast to the mutant K234R, K234T presents an original behavior toward clavulanic acid. The $K_m$ constant and the turnover number were respectively 3000- and 306-fold higher than for the TEM-1 enzyme. This mutant seemed unable to bind the clavulanic acid in the active site; the interactions became unspecífic. The $k_{\text{inac}}$ was similarly reduced (approximately 1000-fold). This indicates that this mutation results in a great reduction in efficiency of activation. The entire activation process is altered.

**Effects of pH on Kinetics Properties—**The $p$H value for K234T is 5.35. Moreover, the titration curves (data not shown) reveal a small difference of charge at pH > $p$I between K234T and RTEM-1 and none between K234R and the wild type. This small decrease in the isoelectric point for the mutant K234T was expected based on the change from the positively charged ammonium group of lysine 234 to the uncharged group of the threonine.

Interestingly, the mutant K234T kept the same optimum pH (Fig. 2A), but it was essentially inactive at pH = 6 and reached 10% of the wild type activity at pH = 8. The basic environment at pH = 8 therefore enhanced the catalytic activity.
as the mutant showed close to 10-fold increase in $K_m$ for the penicillins. Both the amine function of the lysine or the guanidine function of the arginine are highly basic with respective $pK_a$ values of 10.53 and 12.48. In the range of pH values compatible with a good stability for the enzymes and the $\beta$-lactams (i.e. 5–9), both residues are fully protonated. Nevertheless, arginine is more bulky than lysine, and the increase in side-chain length can be estimated to about 1 Å. The resulting steric hindrance could be responsible for the increase in $K_m$ for penicillins.

Comparison of three-dimensional structures of various $\beta$-lactam antibiotics (33) showed that the distance between the oxygen atom of the amide group and the carbon of the carboxylic group of “active” $\beta$-lactams is in the range of 3.0–3.9 Å. This distance is 3.9 Å for ampicillin and 3.2 Å for cephaloridine, which makes a 0.7-Å difference. In relation to the position of the $\beta$-lactam ring in the active site of $\beta$-lactamases, the carboxylic groups are not in the same spatial location for penicillins and cephalosporins. If the postulated salt bridge between the C3 carboxylic group of penicillins and the amine function of the lysine is disrupted in the mutant $\beta$-lactamase K234R, this cannot be the case with cephalosporins where the C4 carboxylic group probably cannot make such an ionic interaction with the wild type protein.

This last hypothesis is also supported by work from Laws and Page (34), who have compared the second order rate constants for the hydrolysis of esters and lactones of penicillins and cephalosporins catalyzed by Bacillus cereus I and II $\beta$-lactamases. They have shown that the hydrolysis is 50 times better for a cephalosporin lactone than for an analogous cephalosporin. It appears that the two oxygen atoms of the lactone in the cephalosporin lactone carry considerable negative charge and could interact with a suitably placed positive charge such as lysine 234.

The results obtained with the mutant K234T, in which the basic group is removed, support the importance of the binding of the carboxyl group of the penicillins with the basic lysine residue 234 for the positioning of the substrate in the active site cavity. This modified enzyme exhibited a very low affinity toward penicillins, representative of a severe decrease of the binding of the substrates in the active site, although it retained a noticeable hydrolysis capability toward most penicillins. This activity varied largely with pH; the residual activity for mutant K234T represents 2% of that of the wild type at pH = 7 and shifted to 10% at higher pH. In contrast, the removal of the basic group was critical for the activity toward cephalosporins; the enzyme lost >99.7% of its activity toward these substrates. This mutant K234T can be compared with the mutated Bacillus enzymes K234E and K234A studied by Ellerby et al. (13).

On the other hand, this mutant presents a very interesting point; its susceptibility to clavulanic acid is so reduced that we can consider that this compound is no longer an inhibitor of the enzyme. The kinetic constants indicate that the clavulanic acid reacts very slowly with the K234T protein variant. Thus, the inactivation is no more effective although most penicillins can still be hydrolyzed. On the contrary, we have shown that inhibition of the K234R mutant by clavulanic acid is barely modified, compared with the wild type. The inactivation of $\beta$-lactamase TEM-1 or K234R mutant by clavulanic acid is rapid and effective. This inhibitor presents structural similarities to penicillins, with steric hindrance largely reduced on account of the lack of lateral chain substitutions on the 6-aminopenicillanic acid nucleus. So, the drastic reduction in inactivation efficiency for the mutant enzyme K234T is essentially due to the basic group removal. Conse-

**DISCUSSION**

Systematic amino acid replacement of lysine 234 in $\beta$-lactamase TEM-1 with nonsense suppressors enabled us to select quickly two mutants with interesting enzymatic activity: K234R and K234T. Kinetic study of these mutants yields valuable information on the role of this conserved residue in the active site box VII.

The mutant K234R compared with the wild type exhibits a similar catalytic constant $k_{cat}$ for penicillin G. However, the catalytic efficiency $k_{cat}/K_m$ toward penicillins was modified,
quently, the basic group appears to be essential for the recognition and binding of inhibitors and substrates in the active site.

Moreover, the mutation Lys-234 → Arg extends the substrate profile of the enzyme to carboxypenicillins. An amino acid sequence comparison of the class A carbenicillin-hydrolyzing enzymes or CARBs (14) shows that these enzymes contain an unusual arginine instead of a lysine in the Lys-Ser-Gly box. This was shown for most of these enzymes: PSE-4 (35), CARB-3, and CARB-4 (14), but not the PSE-3 enzyme (36). The high hydrolytic activity of these enzymes for carbenicillin, ticarcillin, and related compounds, which have been characterized previously (37), could be in part correlated with the presence of the arginine at position 234. These penicillins possess, on the 6-aminopenicillanic acid nucleus, a side chain substituted by a carboxylic acid. This strongly suggests that the basic guanidine group of the arginine residue, in contrast to the amine group of the lysine, places this side chain in a spatial location where new interactions are favorable.

Nevertheless, although the mutant K234R exhibits a high hydrolytic activity toward carbenicillin and ticarcillin, the affinity constant remains very high. The sequence alignments of the TEM and SHV enzymes with the carbenicillin-hydrolyzing enzymes suggest that the carbenicillinase character could be associated with two events: the presence of an arginine at the Lys-Ser-Gly box, as we have just seen and a glycine, found in all four carbenicillinases at position 240, where there is usually an aspartic or a glutamic acid for the TEM and SHV enzymes. The low affinity of K234R can be caused both by a steric hindrance and by an electrostatic repulsion between the lateral chain of carbenicillin or ticarcillin with the acidic residue at position 240. We are just testing this hypothesis by creating the single mutation E240G on the TEM-1 enzyme and on the K234R mutant.

The effects of the mutations on $k_{cat}$ can be correlated with a modification in transition state binding. The difference between the activation free energy of the native and the mutant enzymes can be calculated using the following equation (38).

$$ \Delta G_{act} = -RT \ln \left( \frac{k_{cat}/K_m \text{mut}}{k_{cat}/K_m \text{wt}} \right) $$

Comparison of the ratios $k_{cat}/K_m$ for wild type and mutants for benzylpenicillin gives the apparent contribution of the side chain of the different amino acids to the binding energy of the enzyme transition state complexes. The transition state energy increased by 1.4 kcal/mol for K234R and 4.6 kcal/mol for K234T. The first value fits with a deletion of a side chain that forms a good hydrogen bond with an uncharged group, while the second value fits with a deletion of a side chain that forms an ionic bond (38, 39). According to the crystallographic data for the B. licheniformis β-lactamase (5), we postulate that in the first case, the mutant K234R lost the H-bond between serine 130 and lysine 234 which plays a role to maintain the geometry of the active site, but the arginine maintains the electrostatic environment for the substrate binding. In the second case, both the aforementioned hydrogen bond and the ionic interaction of the basic group of the enzyme with the carboxylate of the substrate are inexistent.

The variation of $k_{cat}$ values over the pH range was unexpected, since both lysine and arginine are protonated at the pH where the measures of kinetic constants were done. Such a paradoxical result was also obtained by Ellerby et al. (13) for their K234E mutant. Moreover Anderson and Pratt (40) have shown that $K_s$ and the rate constants $k_2$ and $k_3$ vary differently according to pH. The modification of the $K_s$ value clearly suggests that some of or all of these parameters, $k_2$, $k_3$, $k_{cat}$, are perturbed by the replacement of lysine to arginine or lysine to threonine. One can only tentatively conclude that the observed behavior results from several perturbations of the different existing bonds in the active site, although lysine 234 does not seem to be directly implied in the proton transfer during hydrolysis (since $pK_a$ remains unmodified). This assumption is supported by the variations of the activation free energy.

In conclusion, for a broad spectrum β-lactamase like TEM-1, single amino acid substitutions reveal the complexity of the interaction between the enzyme and its various substrates. Replacing the lysine with an arginine enhances transition state binding for carboxypenicillins, whereas it does not change that of cephalosporins and slightly decreases $k_{cat}$ for penicillins. At the same time, $K_m$ for cephalosporins is unaffected, whereas it is increased for penicillins, including carboxypenicillins. Thus, increasing the size of the charged side chain at position 234 affects both binding and catalysis for penicillins, while it does not affect cephalosporins. Removing the positive charge, on the other hand, results in drastic loss of activity toward all β-lactams, especially cephalosporins, affecting both $K_m$ and $k_{cat}$. The electrostatic environment provided by lysine 234 plays a major role in transition state stabilization, mainly as an electrostatic anchor between the basic group and the carboxylate of substrates.

Acknowledgments—We thank J. P. Samama, A. Petit, and M. Barthélémy for their support and helpful discussions.

REFERENCES

1. Ambler, R. P. (1980) *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 289, 321-331
2. Joris, B., Ghuysen, J.-M., Dive, G., Renard, A., Dideberg, O., Charlier, P., Frère, J.-M., Kelly, J. A., Boyington, J. C., Moews, P. C., and Knox, J. R. (1988) *Biochem. J.* 250, 313-324
3. Herzberg, O., and Moult, J. (1987) *Science* 236, 694-701
4. Dideberg, O., Charlier, P., Wery, J.-P., Debottay, P., Dusart, J., Épicaut, T., Frère, J.-M., and Ghuysen, J. M. (1987) *Biochem. J.* 245, 911-913
5. Moews, P. C., Knox, J. R., Dideberg, O., Charlier, P., and Frère, J. M. (1990) *Proteins Struct. Funct. Genet.* 7, 156-171
6. Fisher, J., Belasco, J. G., Kolas, S., and Knowles, J. R. (1980) *Biochemistry* 19, 2895-2901
7. Gibson, R. M., Christensen, M., and Waley, S. G. (1990) *Biochem. J.* 272, 613-619
8. Madgwick, P. J., and Waley, S. G. (1987) *Biochem. J.* 248, 657-662
9. Jacob, F., Joris, B., Lepage, S., Dusart, J., and Frère, J.-M. (1990) *Biochem. J.* 271, 399-406
10. Healey, W. J., Labghol, M. R., and Richards, J. H. (1989) *Proteins Struct. Funct. Genet.* 6, 275-283
11. Lenfant, F., Labia, R., and Masson, J. M. (1990) *Biochimie* (Paris) 72, 495-503
12. Collatz, E., Labia, R., and Gutmann, L. (1990) *Mol. Microbiol.* 4, 1615-1620
13. Ellerby, L. H., Eschbar, W. A., Fink, A. L., Mitchinson, C., and Wells, J. A. (1990) *Biochemistry* 29, 5797-5806
14. Hetlatsky, A., Couture, P., and Lévesque, R. C. (1990) *Antimicrob. Agents Chemother.* 34, 1725-1732
15. Normanly, J., Masson, J. M., Kleina, L. G., Abelson, J., and Miller, J. H. (1986) *Proc. Natl. Acad. Sci. U.S.A.* 83, 6548-6552
16. Kleina, L. G., Masson, J. M., Normanly, J., Abelson, J., and Miller, J. H. (1990) *J. Mol. Biol.* 213, 705-717
17. Normanly, J., Kleina, L. G., Masson, J. M., Abelson, J., and Miller, J. H. (1990) *J. Mol. Biol.* 213, 719-726
18. Kleina, L. G., and Miller, J. H. (1990) *J. Mol. Biol.* 212, 295-318
19. Declercq, N., Joyet, P., Gaillardin, C., and Masson, J. M. (1990) *J. Biol. Chem.* 265, 15481-15488
20. McClain, W. H., and Foss, K. (1988) *Science* 241, 1804-1807
21. Masson, J. M., and Miller, J. H. (1986) *Gene (Amst.)* 47, 179-183
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22. Chen, S. T., and Clowes, R. C (1984) *Nucleic Acids Res.* **12**, 3219–3234
23. Lenfant, F., Masson, J. M., Labia, R., Barthélémy, M., Péduzzi, J., Twary, K., and Sirot, J. (1988) *28th Interscience Conference on Antimicrobial and Chemotherapy*, Abstr. 482
24. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
25. Hanahan, D. (1985) in *DNA Cloning* (Glover, D. N., ed) Vol. 1, pp. 109–135, IRL Press, Oxford
26. Taylor, J. W., Ott, J., and Eckstein, F. (1985) *Nucleic Acids Res.* **13**, 8764–8785
27. Blanchin-Roland, S., and Masson, J.-M. (1989) *Protein Eng.* **2**, 473–480
28. Barthélémy, M., Guionie, M., and Labia, R. (1978) *Antimicrob. Agents Chemother.* **13**, 695–698
29. O’Callaghan, C. H., Morris, A., Kirby, S. M., and Shingler, A. H. (1972) *Antimicrob. Agents Chemother.* **1**, 283–288
30. Labia, R., Andrillon, J., and Le Goffic, F. (1973) *FEBS Lett.* **33**, 42–44
31. Labia, R., and Péduzzi, J. (1978) *Biochim. Biophys. Acta* **526**, 572–579
32. Fisher, J., Charnas, R. L., and Knowles, J. R. (1978) *Biochemistry* **17**, 2160–2164
33. Cohen, N. C. (1983) *J. Med. Chem.* **26**, 259–264
34. Laws, A. P., and Page, M. I. (1989) *J. Chem. Soc. Perkin Trans II* 1577–1581
35. Boissinot, M., and Levesque, R. C. (1990) *J. Biol. Chem.* **265**, 1229–1230
36. Campbell, J. I. A, Scahill, S., Gibson, T., and Ambler, R. P. (1989) *Biochem. J.* **260**, 803–812
37. Labia, R., Guionie, M., and Barthélémy, M. (1981) *J. Antimicrob. Chemother.* **7**, 49–56
38. Fersht, A. R., Shi, J. P., Knill-Jones, J., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye, M. M., and Wintner, G. (1985) *Nature* **314**, 235–238
39. Warshel, A., Naray-Szabo, G., Sussmann, F., and Hwang, J. K. (1989) *Biochemistry* **28**, 3629–3637
40. Anderson, E. G., and Pratt, R. F. (1983) *J. Biol. Chem.* **258**, 13120–13126