Site-directed Mutagenesis of Glutamate 166 in Two β-Lactamases

KINETIC AND MOLECULAR MODELING STUDIES*

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The catalytic pathway of class A β-lactamases involves an acyl-enzyme intermediate where the substrate is ester-linked to the Ser-70 residue. Glu-166 and Lys-73 have been proposed as candidates for the role of general base in the activation of the serine OH group. The replacement of Glu-166 by an asparagine in the TEM-1 and by a histidine in the Streptomyces albus G β-lactamases yielded enzymes forming stable acyl-enzymes with β-lactam antibiotics. Although acylation of the modified proteins by benzylpenicillin remained relatively fast, it was significantly impaired when compared to that observed with the wild-type enzyme. Moreover, the E166N substitution resulted in a spectacular modification of the catalytic water molecule that is covalently bound to the active-site serine residue, characterizing the acylation and deacylation steps.

DD-peptidases and most β-lactamases, belong to the superfamily of active-site serine penicillin-recognizing enzymes (1). The interaction between these proteins and β-lactams involves the formation of an acyl-enzyme (E-S) intermediate where the antibiotic is covalently bound to the active-site serine residue,

\[
\begin{align*}
E + S & \rightarrow ES \\
& \rightarrow ES^* \\
& \rightarrow E + P
\end{align*}
\]

(Eq. 1)

In contrast to DD-peptidases, β-lactamases efficiently catalyze the deacylation step (high \(k_3\) value) which regenerates the active enzyme and releases a biologically inactive product (P). Serine β-lactamases are divided into three classes A, C, and D on the basis of their primary structures. Tertiary structures of various enzymes belonging to classes A and C have been solved by X-ray crystallography underlining similarities in the folds of all these proteins (2–6). Moreover, several conserved residues were identified, some of which appear to be essential for catalysis (7).

The mechanism by which serine β-lactamases hydrolyze penicillins and cephalosporins has received a lot of attention and, for the class A enzymes the identity of the residue involved in the activation of the active serine (Ser-70 in the ABL numbering system (8)) has been subject of controversy. Both Lys-73 (9) and Glu-166 (10) have been proposed as potential candidates for this essential role. By contrast, the function of Glu-166 in activating the hydrolytic water molecule during the deacylation step is unanimously recognized. According to Adachi et al. (11) and Strynadka et al. (9), accumulation of an acyl-enzyme during the interaction between the TEM-1 Glu-166 → Asn mutant (E166N) and benzylpenicillin suggested that the mutation affected only the deacylation step in a severe manner. In contrast, kinetic studies of the Glu-166 → Ala mutant of the Bacillus licheniformis β-lactamase (12) and of the Glu-166 → Asp mutant of the Bacillus cereus I β-lactamase (13) showed that accumulation of the acyl-enzyme could result from simultaneous but different decreases of the kinetic parameters characterizing the acylation and deacylation steps.

The present paper reports the analysis of the kinetic properties of the TEM-1 E166N mutant. Methods were developed to monitor the rapid formation of the acyl-enzyme. One of the most striking results was a sharp modification of the substrate profile of the enzyme. To explain these results, the interactions between different β-lactams and the E166N mutant were analyzed by molecular modeling. These confirmed the role of the catalytic water molecule in the mechanism and underlined the importance of the Ω-loop residues in the specificity profile. Similar kinetic data were obtained with the E166H mutant of the Streptomyces albus G class A β-lactamase.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**

Escherichia coli TG1 strain (Δlac-pro), Sup E, thi, hsdD5/F′traD36, proA− B−, lacIq, lacZΔM15) was used for routine transformation, DNA preparation and production of the TEM-1 β-lactamase and as a host strain for M13 phage growth. The Streptomyces lividans TK24 strain (14) was used for the production of the Streptomyces albus G β-lactamase.

Plasmid pAD25 (15) encodes both the tetracycline resistance and an isopropyl β-D-thiogalactopyranoside-inducible TEM-1 β-lactamase. It was used both for the mutagenesis procedure and the expression of the WT and mutant enzymes.

The gene encoding the S. albus G β-lactamase was cloned into the M13tg131 phage for mutagenesis. The mutant gene was entirely sequenced, recloned into the pPJ702 Streptomyces plasmid (a gift from Dr. J. Altenbuchner, Universität-Regensburg, Germany), and expressed in S. lividans.

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1 The abbreviation used is: WT, wild type.
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Nucleic Acid Techniques

Sequencing was performed by the method of Sanger et al. (16) using the Sequenase sequencing kit (U.S. Biochemical Corp.). T4 DNA ligase and kinase were purchased from Boehringer Mannheim GmbH. Oligonucleotides were purchased from Eurogentec (Liège, Belgium). Specific Streptomyces DNA manipulations, such as protoplast preparation, transformation, and plasmid extraction, were based on Hopwood et al. (17).

Mutagenesis

The E166N TEM-1 β-lactamase mutant was obtained by inverse polymerase chain reaction mutagenesis (18) with the following oligonucleotides: 5′-AAGCCGATCCATAGTCCGCG-3′; 5′-GTTAATGGTGCACCATGCA-3′. The latter carried the two mismatched bases designed to mutate the GAA (Glu) codon into AAC (Asn).

Amplification was performed with the Vent DNA polymerase (Boehringer-Mannheim) in the buffer supplied by the manufacturer to which MgSO₄ was added to a final concentration of 4 mM; 30 cycles (60 s at 94 °C, 60 s at 56 °C, 270 s at 72 °C) were performed on the reaction mixture. The polymerase chain reaction product was subsequently treated as described by Lakaye et al. (19) and used to transform E. coli TG1.

For the E166H mutant of the S. albus G β-lactamase, mutagenesis was performed as described by Eckstein et al. (20). The oligonucleotide used to replace the GAC (Glu) codon by CAC (His) was 5′-CTGACCTGCGACCGGATGAC-3′.

Chemicals

Benzylenicillin was from Rhône Poulenc (Paris, France), cephalaxine and cepazidime were from Glaxo Group Research (Greenford, Middlesex, United Kingdom), cefoxaxime from Hoechst-Roussel (Romainville, France). Imipenem, moxalactam, timenticin, and cepfizoxin from Merck, Sharp and Dohme Research Laboratories ( Rahway, NJ). Cephalosporin C and cephaloridine from Eli Lilly and Co. (Indianapolis, IN). All these compounds were kindly given by the respective companies.

[14C]Cefuroxime (54 mCi/mmol) was purchased from Amersham International.

Isopropyl β-D-thiogalactopyranoside and tetracycline were purchased from Sigma. Fluoresceyl-6-aminopenicillanic acid was prepared as described by Lakaye et al. (21).

Expression and Purification of Mutant β-lactamases

The E166H mutant of the S. albus G β-lactamase was isolated from 3 liters of culture supernatant and purified as described by Brannigan et al. (22) with an additional chromatographic step on a Superdex 75 HR 10/30 column in 25 mM sodium phosphate buffer, pH 7.

For the production of the E166N TEM-1 mutant, E. coli TG1 was grown at 30 °C in 15 liters of M9 minimal medium supplemented with 50 μg/ml tetracycline. When the A₅₀₀ value reached 0.6, 0.1 mM isopropyl β-D-thiogalactopyranoside was added and the culture was further incubated for 3 h. Cells were collected, suspended in a 50 mM Tris-HCl buffer, pH 7, containing 27% sucrose (w/v) and lysed by lysozyme (1 mg/ml final) as described by Dubus et al. (15). Cell debris were discarded by centrifugation and the supernatant dialyzed against 10 mM Tris-HCl, pH 7.5. The enzyme was purified as described by Vanhove et al. (23). The fractions containing the enzyme were identified by substrate assays of fluoresceyl-6-aminopenicillanic acid-labeled enzyme to electrophoresis on a 15% polyacrylamide gel in the presence of SDS (24).

The purity of the different enzyme preparations was verified by Coomasie Blue staining of SDS-polyacrylamide electrophoresis gels. The TEM-1 mutant was purified with [14C]cephalosporin C and found to be more than 95% pure. The E166H mutant of S. albus G was only 50% pure and remained contaminated by a single, low molecular mass protein which did not interfere with the kinetic measurements.

Determination of the Kinetic Parameters

Due to the very low k₂ values observed with these two mutants the determination of their kinetic parameters rested on methods similar to those used for penicillin-binding proteins rather than for regular β-lactamases. Unless otherwise mentioned, all the kinetic assays were performed at 30 °C in 50 mM sodium phosphate buffer, pH 7.

Determination of k₃/K Values

Pseudo-first order reactions are only obtained when the β-lactam concentration is at least five times greater than that of enzyme. Due to the high k₂/K values, these conditions where only fulfilled when the experiments were performed in a stopped-flow apparatus. The β-lactam concentration was also decreased close to that of enzyme and analysis performed according to the general second order equation. Finally after the direct determination of the k₂/K values for cefoxitin and cephalosporin C, those for the other compounds were determined by a competition method.

Direct Determination with Manual Mixing (Method A)

The enzyme solution was added with cefoxitin, cephalosporin C, cepoxaxime, moxalactam, or cefazidime. The total volume was 0.5 ml and the final enzyme concentration 16 μM. The antibiotic concentration was always slightly higher than that of the enzyme. Decrease of the A₅₀₀ value resulting from the opening of the β-lactam ring was monitored with the help of an Uvikon spectrophotometer connected to a COPAM PC85C microcomputer. The data were fitted to Equation 2 using the Enzfitter program (Biosoft, Cambridge, United Kingdom (25)) yielding the value of the second-order rate constant k, i.e. k₂/Kₐ, where K = (k₋₁ + k/κ)k₋₁. Under conditions of similar initial enzyme and substrate concentration, the general Equation 2 prevails.

\[
\ln \left[ \frac{(E_0 - x)}{S_0 - x} \right] = \ln \left( \frac{E_0}{S_0} \right) + \left( \frac{E_0}{S_0} \right) \cdot k \cdot t \quad \text{(Eq. 2)}
\]

where S₀ and E₀ are the initial concentrations of substrate and enzyme and x the concentration of E (or S) transformed at time t.

Under our experimental conditions (S₀ > E₀) concentrations could be replaced by absorbance values as in Equation 3, leading to Equation 4.

\[
A_0 - A/\left( \frac{S_0}{E_0} \right)(A_0 - A_0 - A_0 + A) = \left( \frac{1}{S_0/E_0} \right) \cdot \exp \left( \frac{A_0 - A}{A_0} \right) \cdot \left( 1 - \frac{S_0/E_0}{\Delta t} \right) \quad \text{(Eq. 3)}
\]

Stopped-flow Experiments (Method B)

The enzyme (16 μM) was mixed with increasing concentrations of cefoxitin with the help of a Biologic Stopped-flow apparatus (Grenoble, France). The S₀/E₀ ratio varied from 1.2 to 25. For data analysis, Equation 2 was adapted to the stopped-flow recording conditions. Alternatively, for the highest S₀/E₀ ratios the pseudo-first order approximation was used, yielding identical results.

Competition Method (Method C)

The enzyme (0.9 μM) was mixed with a mixture of [14C]-benzylenicillin (B) and another antibiotic (I) at concentrations ranging from 2.5 × 10⁻⁶ to 1.0 × 10⁻⁴ M. After 5 min, the reaction was stopped and the protein precipitated, isolated, and the radioactivity determined as described by Martin and Waley (26). Since both antibiotic concentrations were larger than that of the enzyme and the incubation time was much shorter than the half-life of both acyl-enzymes (see below), the respective quantities of labeled (E-B⁻) formed with [14C]-benzylenicillin and unlabeled (E-I⁻) formed with the other antibiotic acy-enzymes were given by Equation 5 as shown by Frère et al. (27).

\[
[\text{E}^{1+}]^{\left[\text{E}^{1-}B^{+}\right]} = \left( k_0/K_0 \right) \cdot \left[ k/\text{K}_{0B} \right] \quad \text{(Eq. 5)}
\]

The value of [E⁻] was estimated as \([\text{E}^{1-}B^{+}] - [\text{E}^{1-}B^{0}]\), where \([\text{E}^{1-}B^{0}]\) was the quantity of acyl-enzyme formed with [14C]-benzylenicillin in the absence of competing substrate.

Direct Binding of a Labeled Antibiotic (Method D)

With the E166H mutant of the S. albus G enzyme the second-order rate constant for acylation (k₂/K) was obtained by monitoring acyl-enzyme formation as a function of time (method D). The enzyme was incubated with an excess of [14C]-benzylenicillin (S₀ ≫ E₀) and after increasing periods of time, the reaction was stopped by addition of 1% SDS. The labeled acyl-enzyme was isolated by SDS-polyacrylamide gel electrophoresis and quantified by fluorography (24).

Determination of k₃

Labeled Antibiotic—The protein (1.6 μM) was reacted with an excess of [14C]-benzylenicillin (10⁻⁴ M) and the excess of free antibiotic eliminated by addition of 600 ng of the wide-type enzyme. The mixture was incubated at 30 °C and after increasing periods of time, the residual acyl-enzyme quantified after precipitation as above (26).

Unlabeled Antibiotics—After formation of the acyl-enzyme in 50 mM
The **pH Dependence of the Enzyme Activity**

The following pH range was explored: acetic acid/sodium acetate, pH 4–5.5, sodium phosphate, pH 6–8, Tris-HCl, pH 8.5–9. All the buffers were 50 mM. The acyl-enzyme was then left to decay during different time periods. 1 mM EDTA was added to inactivate the Zn-β-lactamase and the regenerated active-serine enzyme was quantified by saturation methods (28).

**Thermal Denaturation Curves**

Temperature-induced unfolding was monitored by recording the intrinsic fluorescence of the TEM-1 E166N mutant and wild-type enzymes with various β-lactams.

**RESULTS**

Production and Purification—About 16 mg of the TEM-1 E166N mutant were obtained per liter of culture. The protein was purified to 95% homogeneity with a yield of 45%. For the S. albus G mutant, the culture and purification yields were 20 mg/liter and 25%, respectively; as stated above, the final preparation was only 50% pure. Attempts to eliminate the low molecular mass contaminant by various chromatographic procedures, including molecular sieve filtration, remained unsuccessful.

Kinetic Properties—The values of the rate constants obtained for the TEM-1 E166N mutant are reported in Table I. As expected and in agreement with the observations of Adachi et al. (11) and Strynadka et al. (9), the rates of deacylation ($k_d$) were exceedingly low.

Different strategies were utilized to determine the second-order rate constant ($k_s/K_p$), characteristic of the acylation rate. Rapid acylation could not be monitored by the usual methods. With [14C]benzylpenicillin, the reaction was so rapid that it could not be followed by manual mixing methods. In consequence, the first experiments were performed with compounds exhibiting lower acylation rates and whose modifications upon acyl-enzyme formation could be directly monitored by spectroscopic methods. The method A allowed the determination of the $k_s/K_p$ values for the TEM-1 E166N mutant and wild-type enzymes with various β-lactams.

**Molecular Modeling**

Models of the mutant enzymes were obtained from the x-ray structures of the wild-type proteins (5) and the local conformational space of the mutated amino acid was searched by a minimum perturbation approach (31).

The system was solvated by cubes of standard “Monte Carlo” water molecules and the positions of these molecules after minimization were compared to those in the crystal structures. The geometry of the protein was optimized with the AMBER 4.1 set of programs (32) using a distance-dependent dielectric constant.

The β-lactam molecules were docked in the modeled active sites with the β-lactam amide oxygen atom oriented into the oxyanion-hole formed by the main chain nitrogen atoms of Ser-70 and Ala-237. Hydrogen bonds appeared to be formed between the C7 side chain amide group and the Nα amide nitrogen atom of Asn-132 and the main chain oxygen atom of Ala-237. The energy of the Michaelis complexes thus obtained was minimized using AMBER (32).

**Table I**

| Compound            | $k_{ac} \times 10^9$ s$^{-1}$ | $k_{ac} \times 10^9$ s$^{-1}$ | Mutant/WT | $k_0$ WT | $k_0$ E166N |
|---------------------|-------------------------------|-------------------------------|------------|-----------|-------------|
| Benzylpenicillin    | 84 × 10⁹                     | 450 × 10⁷                     | 0.005      | 1500      | 5.2 × 10⁻⁶  |
| Cephalosporin C     | 90 × 10⁹                     | 2 × 10⁷                       | 0.022      | >36       | 0.71 × 10⁻⁶ |
| Cephaloridine       | 2.24 × 10⁵                   | 15 × 10⁴                     | 0.007      | >1500     | 0.76 × 10⁻⁵ |
| Cefoxitin           | 6 × 10³                      | 4.5 × 10³                     | 750        | 4 × 10⁻³  | —           |
| Moxalactam          | 3 × 10⁸                      | 3.5 × 10¹                     | 219        | —         | —           |
| Temocillin          | 500(C)                       |                               | >1500      | —         | —           |
| Cefotaxime          | 1.5 × 10⁸                    | 2.5 × 10⁵                     | 1.7        | >9        | 0.91 × 10⁻⁶ |
| Cefuroxime          | 6 × 10⁴                      | 6 × 10⁴                       | 1          | >6        | 0.63 × 10⁻⁵ |
| Cefazidime          | 70                           | 400                           | 5.7        | >0.34     | 2.1 × 10⁻⁶ |
| Imipenem            | 2 × 10³                      | 30 × 10⁶                      | 15         | >2 × 10⁻² | —           |

$^a$ Not determined.

$^b$ No hydrolysis detected (3).
The $k_{2}/K$ values for the mutant were determined by direct binding of a labeled antibiotic (method D). For the WT values see the footnote to Table I.

| pH  | E166H $k_{2}/K$ | WT $k_{2}/K$ | E166H $k_2$ | WT $k_2$ |
|-----|----------------|--------------|-------------|----------|
| 5   | 6,000          | 1,950 × 10^3 | 1.8 × 10^{-3} | >3,800  |
| 7   | 10,000         | 3,000 × 10^3 | 3.9 × 10^{-3} | >3,400  |
| 9   | 32,000         | 2,300 × 10^3 | 4.5 × 10^{-4} | >2,800  |

was only utilized with cefoxitin. Here the antibiotic concentrations were as high as 400 μM. An average $k_{2}/K$ value of 2200 ± 440 m^{-1} s^{-1} was found, in fair agreement with that obtained in the manual mixing experiments (4500 ± 130 m^{-1} s^{-1}). The values for the other compounds were obtained by the competition method C. First, competitions between [14C]benzylpenicillin and cephalosporin C or cefoxitin were performed, allowing the computation of the $k_{2}/K$ value for the [14C]-labeled compound. Subsequently, the values for all the other β-lactams were derived from competition experiments with [14C]benzylpenicillin.

For good substrates of the wild-type enzyme (benzylpenicillin and cephaloridine), the E166N mutation resulted in a decrease of the acylation rates by 2–3 orders of magnitude. This was, however, much less drastic than for the $k_3$ value. The most striking results concerned the modification of the substrate profile.

In contrast to what occurred with the good substrates, the $k_{2}/K$ for the poorest WT substrates (i.e. temocillin, cefotaxim, and moxalactam which bear a methoxy side chain on the α-face of the β-lactam ring) were increased by several orders of magnitude. With the oximinocephems, the $k_{2}/K$ value was not much affected. In consequence the $k_{2}/K$ values of the mutant only spanned 3 (from 400 to 400 × 10^3) versus 7 orders of magnitude for the wild-type protein.

With benzylpenicillin and the S. albus G E166H mutant, similar results were obtained, involving a significant decrease of $k_{2}/K$ accompanied by a much more drastic decrease of $k_3$ (Table II).

**Effect of the pH on Catalytic Parameters**—The pH dependence of the acylation rate of the E166N mutant by cefoxitin was studied using method A (see Fig. 1). The pH dependence of the $k_{2}/K$ value could be fitted to Equation 6 with $pK_a$ and $pK_b$ values of 6.0 and 8.5, respectively. With the wild-type enzyme $pK_b$ values of 7.2 and 9 were found. The mutation thus resulted in a shift of the curve to lower pH values, with a more significant effect to the acid limb. The $k_3$ constant was not significantly influenced by the pH value (not shown).

The $k_{2}/K$ value of the E166H mutant of the S. albus G β-lactamase (Table II) exhibited a new pH dependence which seemed to indicate that the base form of the His-166 side chain was adequately oriented to actively participate in the acylation step. By contrast, the $k_3$ value at pH 5 was higher than at pH 7 or 9. This suggested that the His-166 residue of the mutant enzyme was not involved in the deacylation step as a general base catalyst.

**Stability of the E166N TEM-1 Mutant**—The melting temperature ($T_m$) and the enthalpy ($\Delta H_m$) of unfolding obtained by fitting the experimental curves to Equation 7 were: 51.1 ± 0.2 °C and 112 ± 7 kcal/mol °C, 43 ± 1.4 °C and 87 ± 5 kcal/mol °C for the wild-type and the E166N mutant proteins, respectively. This result highlighted a significant destabilization of the mutant which was corroborated by the proteolysis experiments in which the pseudo-first order rate constants ($k_i$) characterizing trypsin-mediated inactivation were 0.29 min^{-1} and 0.012 min^{-1} for the TEM-1 E166N and WT proteins (33), respectively.

**Models of the Mutant Enzymes**—The strong hydrogen bonds found between the carboxylic group of Glu-166 and the side chain nitrogen atoms of Lys-73 and Asn-170 of TEM-1 disappeared in the E166N mutant, allowing free rotation of these residues. In the most stable structure obtained by conformational analysis, the Asn-170 acyl group was rotated by 180° around the C_{p}-C_{α} bond with respect to the orientation found in the WT enzyme. A similar rotation was observed in the structure of the E166A mutant of the B. licheniformis β-lactamase (34). There was no change in the position of Lys-73 relative to its WT position. All calculations were performed considering both the protonated and unprotonated states of Lys-73 and yielded similar results.

The position of the “catalytic” water molecule in the mutant was found to be the same whether it was generated by a Monte Carlo simulation or minimized from the position found in the WT structure. The water hydrogen atoms were oriented toward the Asn-166 and Asn-170 O_{α}s, respectively. By comparison with the WT structure, it was shifted by 1.3 Å away from Ser-70 with which it no longer formed a H-bond (Fig. 2).

In the E166H mutant, the plane of the imidazole side chain was rotated by 75° with respect to that formed by the glutamate carboxylate in the WT enzyme. The catalytic water molecule slightly (0.8 Å) moved to the bottom of the cavity away from its initial position and was thus further from Ser-70. The lengths of the hydrogen bonds were 3.12 and 2.85 Å with an unprotonated and protonated Lys-73 residue, respectively, as previously shown in the model of the E166D mutant of the S. albus G enzyme (35). The N170 side chain was more outward-oriented due to a 30° rotation around the C_{p}-C_{α} bond.

**Modeled Interactions of the E166N Mutant with β-Lactam Substrates**—Three cephalosporins: cephaloridine, cefuroxime, and cefoxitin, respectively, good, bad, and very bad substrates of the WT TEM-1 but of similar properties with the mutant were chosen as models for the study of the specificity of the E166N mutant. Benzylpenicillin was also studied (not shown),

**FIG. 1.** pH dependence of the second-order acylation rate constant ($k_{2}/K$) of the TEM-1 E166N and wild-type enzymes. ● WT; ○ E166N mutant. Above pH 10.5, spontaneous hydrolysis of the antibiotic did not allow precise measurements. S.D. values were below 20%. The solid line was obtained by fitting data to Equation 6. Fitting with $k_{bond} = 0$ lead a somewhat poorer fit in the low pH range. It was, however, not possible to be certain that this was statistically meaningful.
DISCUSSION

Both mutant proteins were more poorly produced than the WT enzymes. This might be related to the relative instability of the mutant proteins which increases their sensitivity to proteases as shown for the TEM-1 E166N mutant. With this protein, utilization of a minimal medium and a lower temperature significantly improved the production yield. This instability might due to an increased mobility of the Ω-loop as shown for various other TEM-1 mutants (36, 37).

On the basis of a preliminary study of the TEM-1 E166N mutant, Adachi et al. (11) concluded that the deacylation rate was severely decreased while the acylation reaction was unimpaired. Since the first conclusion was in disagreement with the data of Gibson et al. (13) and Leung et al. (38), however, obtained with different Glu-166 mutants of another enzyme, a careful kinetic study of the TEM-1 E166N protein was performed. As expected and with all substrates, our results indicated extremely large decreases of the $k_a$ value, a $10^9$ factor for benzylpenicillin. This result underlined the pivotal role of the Glu-166 in the deacylation step, a feature on which a general consensus has been reached. By contrast, with the good substrates, the $k_a/K^*$ value, which characterizes the efficiency of acylation and corresponds to the $k_{cat}/K_m$ of the WT enzyme, was decreased by 2 or 3 orders of magnitude indicating a non-negligible, although less pivotal role of Glu-166 in the acylation step. This was, however, not true for all substrates since the acylation rates by poorer substrates were unchanged or even slightly increased and for the very bad substrates, cefoxitin and other compounds exhibiting a methoxy group on the α-face, acylation was significantly facilitated.

Consequently, the “activity” profile of the enzyme was deeply modified, with a spectacular leveling effect. According to Strynadka et al. (9), the position of the Ω-loop was not strongly modified in the mutant. The situation was the same with the β-lactamase E166A mutant but quite different with the TEM-1 E166Y (39) and S. aureus D179N (40) proteins where the loop was disordered. Here the most striking structural modifications were the displacement of the water molecule W1 away from the active site Ser-70 side chain and the disappearance of several strong hydrogen bonds. It should be noted that this water molecule has been hypothesized to serve as a relay in the activation of the Ser-70 hydroxyl group by the Glu-166 side chain acting as a general base in the acylation process. However, an alternative mechanism has been proposed by Strynadka et al. (9), where the general base would be the unprotonated side chain of Lys-73 and the Glu-166 carboxylate in the acylation and deacylation steps, respectively. Although it would explain the properties of E166X mutants which would catalyze the acylation reaction with the same efficiency as the wild-type enzyme but would fail to deacylate, this hypothesis also implies a significantly decreased $pK_a$ value for the alkylammonium group of the lysine 73 side chain in the wild-type enzyme. A recent NMR titration of the Lys-73 residue in the TEM-1 protein failed to supply evidence for such unusual $pK_a$ value (41). Other Glu-166 mutants have been prepared with other enzymes (12, 38, 42). Whenever detailed kinetic studies were performed, both acylation and deacylation rates appeared to be decreased by the mutation, although deacylation was sometimes much more severely impaired. Moreover, the K73R mutant of the B. cereus 569/H β-lactamase I (13) was significantly more active than its E166D counterpart. The drastic substrate profile modifications observed in this present study suggest that the acylation of the mutant might rely on an alternative mechanism. One can hypothesize that, as proposed by Lamotte-Brasseur et al. (10) and Matagne et al. (43), the very low acylation rates of the WT enzyme by cefoxitin, moxalactam, and temocillin are due to a displacement of W1 by the methoxy side chain of the substrates. With the mutant, the Lys-73 side chain might replace Glu-166 as the general base as in acetoacetate decarboxylase (44) and as suggested by Strynadka et al. (9) for the WT β-lactamase. The disappearance of the negative Glu-166 charge would explain a decrease of the Lys-73 $pK_a$ when compared to the WT enzyme.

This alternative acylation mechanism would be significantly less efficient for good substrates but, since it would not involve W1, the reaction rates with cefoxitin and similar compounds would be increased. Finally, the unchanged acylation rates with oxacillin and cephalosporins would result from the same negative factor as with the good substrates compensated by a positive effect due to the increased mobility of the omega loop as observed for example with the Arg-164 mutants of the TEM-1 enzyme (37).

Note that an unprotonated Lys-73 may not be necessary to act as a proton relay. The softness of the electronic atmosphere of the nitrogen atom allows an easy adaptation to environmen-
It is finally interesting to note that the acylation rates observed with the E166N mutant are of the same order of magnitude as those measured with several penicillin-binding proteins. The *Actinomadura R39* (45) DD-peptidase and *B. licheniformis PBP1* (46) contain all the residues constituting the conserved elements of the class A $\beta$-lactamases with the exception of the $\Omega$-loop and, in consequence, have no equivalent of Glu-166 as shown by the three-dimensional structures of the homologous *Streptococcus pneumoniae PBP2x* (47) and *Streptomyces K15 DD-peptidase.*

The results obtained with the *S. albus G* enzyme generally support these conclusions. In this case, the $k_3$ value is also strongly decreased and this residue does not appear to act as a general base in the deacylation reaction, as indicated by the absence of significant variation of $k_3$ between pH 5 and 9. By contrast, the $k_2/K_9$ value significantly increases with increasing pH values and, although a real titration curve is not observed, this might suggest that the base form of this residue might participate in the acylation reaction.

These results underline a major role of the W1-Glu-166 pair in the enzymatic acylation-deacylation process and indicate that although Glu-166 is involved in both acylation and deacylation reactions in class A $\beta$-lactamases, these two reactions are not necessarily “mirror” images of each other since the
disappearance of Glu-166 affects the two steps in a different way. A careful kinetic analysis of mutant enzymes is thus a prerequisite to meaningful mechanistic conclusions.

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