The chromosome-encoded class C β-lactamase CHE-1 produced by Enterobacter cloacae exhibits a lower sensitivity to avibactam than the P99 enzyme from which it is derived by a 6-residue deletion in the H-10 helix. In the present study, we investigated the sensitivity of CHE-1 to two other β-lactamase inhibitors: LK-157 (or Lek 157), a tricyclic β-lactam, and BAL29880, a bridged monobactam. With both compounds, the second-order rate constants for inactivation were significantly lower for CHE-1, which can thus be considered an inactivator-resistant mutant of P99. However, the second-order rate constant for the inactivation by BAL29880 probably remains adequate for a rather rapid reaction with CHE-1 in the absence of protection by the substrate.

Bacterial strains and vectors. The pBK-CMV phagemid vector was a gift from Trenon Hospital (Paris, France). pGEM-T Easy vector (Novagen, Inc., Madison, WI) was used to clone the PCR products. The expression plasmid PET26b(+) (Novagen, Inc.) was used for the construction of the T7-based expression vector and for recombinant experiments. Escherichia coli BL21 (DE3)(pLysS) was used as the host strain in cloning experiments.

Construction of the cloning vector. To allow the removal of the signal peptide, the NdeI and HindIII restriction sites were introduced into blaCHE-1, after the signal peptide nucleotide sequence. These sites were generated by PCR. The primers were 5′-CCCATATGATGAAAAAATCCTTTGC-3′ (NdeI) and 5′-CCAGGCTTTACTGATGCCGCTC-3′ (HindIII) (the newly introduced restriction sites are underlined).

PCR conditions were as follows: incubation at 95°C for 1 min, followed by 40 cycles of amplification (denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 68°C for 2 min), using Pfu polymerase (Promega). The PCR product (1,200 bp) was purified using a miniprep kit and amplified using Taq polymerase for 30 min at 72°C. The PCR product was cloned into the pGEM-T Easy vector to obtain the recombinant plasmid CHE-1-C1, which was used to transform E. coli DH5α competent cells. The nucleotide sequences of the PCR-generated fragments were verified in order to rule out the presence of unwanted mutations. The CHE-1-C1 plasmid was digested with the NdeI and HindIII restriction enzymes, and PET26b(+)(Novagen, Inc.) was digested with the same restriction enzymes in order to subclone into the PET26b(+) vector. The new plasmid, CHE-1-P1, was introduced into E. coli BL21(DE3) (pLysS) competent cells for production.

β-Lactamase production and purification. CHE-1 expression was induced in 1.5 liters of brain heart infusion (BHI) medium at 37°C using 1 mM IPTG. After 4 h, the culture was centrifuged and the bacterial pellet was resuspended in 40 ml of 15 mM caccodylate, pH 6, containing 1 M NaCl (buffer A). After two freeze-thaw cycles, bacteria were disrupted by three sonication cycles for 3 s each at 10 W. Cell debris were removed by centrifugation at 20,000 × g and 4°C for 1 h. The supernatant was dialyzed with 20 mM Tris–HCl, pH 8, against 1 M NaCl (buffer B). These dialysates were dialyzed against buffer B and stored at −80°C.

Materials and methods. Antibiotics and other chemicals. Nitrocefin (Δε = +17,500 M⁻¹ cm⁻¹) was purchased from Unipath Oxoid (Basingstoke, United Kingdom), and BAL29880 (Basilea Pharmaceutica International Ltd., Basel, Switzerland) and LK157 (LEK Pharmaceuticals, Ljubljana, Slovenia) were provided by the respective companies. Isopropyl-β-D-thiogalactopyranoside (IPTG) and kanamycin were purchased, respectively, from Eurogenotech (Liège, Belgium) and Merck (Darmstadt, Germany).
overnight at 4°C against 10 liters of buffer A. After filtration through a 0.22-μm membrane (Millipore), the crude extract (conductivity of 1,700 μS/cm) was loaded onto an SP-FF column (1.6 by 20 cm; Pharmacia, Sweden) previously equilibrated with buffer A. The column was washed with buffer A until the A280 of the effluent was <0.1, and the enzyme was eluted using a linear salt gradient from 0 to 0.5 M NaCl in buffer A over 15 column volumes, at a flow rate of 2 ml/min. The active fractions were pooled and dialyzed overnight at 4°C against 10 mM Na phosphate buffer, pH 6.8 (buffer B). The protein solution (conductivity of 1.25 μS/cm) was loaded onto a hydroxyapatite Econo-Pac CHT II column (1 by 5 ml; Bio-Rad) previously equilibrated with buffer B, at 1 ml/min. The elution was performed with a linear gradient by using 400 mM Na phosphate buffer, pH 6.5. The fractions that exhibited β-lactamase activity were pooled (30 ml), and the final concentration was 0.65 mg/ml. The enzyme preparation was stored at −20°C in buffer B.

N-terminal sequencing. The N-terminal amino acid sequence of CHE-1 was determined with the help of an automated Procise protein sequencing system connected to a Macintosh 650 computer (Perkin-Elmer Corp., Wellesley, MA).

Mass spectrometry. The molecular mass of the CHE-1 enzyme was determined by electrospray ionization-quadrupole time of flight mass spectrometry (ESI-Q-TOF MS). Sodium ions were largely removed by three cycles of concentration/dilution in 25 mM ammonium acetate buffer by filtration and centrifugation, using an Amicon system (Amicon Ultra-15; Millipore) with a molecular mass cutoff of 30,000 Da. The analysis was performed under acidic conditions in the presence of 0.5% formic acid, final concentration (pH 3.0).

Kinetic parameters. The steady-state kinetic parameters of CHE-1 for nitrocefin were determined as follows. The hydrolysis of the antibiotic was monitored by recording the absorbance variation at 482 nm in 50 mM sodium phosphate buffer (pH 7.0) in a total volume of 500 μl at 37°C. Measurements were made with an Uvikon XL spectrophotometer. Bovine serum albumin was added at a final concentration of 10 μg/ml in order to prevent the denaturation of the enzyme. The kinetic parameters (Kcat and kcat) were determined by analysis of the complete hydrolysis time courses, as described by De Meester et al. (16), or by measuring initial rates and using the Hanes linearization of the Henri-Michaelis equation.

The interactions of LK157 and BAL29880 with the CHE-1 β-lactamase were studied at 30°C in 50 mM sodium phosphate, pH 7.0, containing 50 mM NaCl and 0.1 mg/ml of bovine serum albumin. The enzyme (20 to 100 ng) was incubated for about 5 min at 30°C with 100 μM nitrocefin as the reporter substrate and inactivator concentrations varying from 0 to 125 μM for LK157 or 0 to 30 μM for BAL29880. The absorbance was recorded at 482 nm for a period of 5 min. The values of the pseudo-first-order rate constants (ki) were determined according to the reactor substrate method (16) and were plotted as a function of the inactivator concentrations.

The interactions of BAL29880 and LK157 with the P99 β-lactamase were monitored at 30°C in 100 mM HEPES buffer, pH 7.5, containing 0.2 M NaCl and 0.1 mg/ml of bovine serum albumin. The enzyme (20 to 100 ng) was added to a 250 μM solution (BAL29880) or a 234 μM solution (LK157) of the reporter substrate (cephalosporin C), containing 0 to 0.5 μM BAL29880 or 0 to 1.2 μM LK157. The absorbance at 260 nm was recorded for a period of 80 to 120 s. The k1 values were calculated and plotted as described above.

The inactivation results were analyzed on the basis of the three-step model E + X→EX→EX→E + P, where X is the inactivator, E the enzyme, EX a noncovalent complex, and EX* the acylenzyme. K is the dissociation constant of EX, and k1 and k3 are first-order rate constants.

Note that, as far as class C β-lactamases are concerned, the reactivation (k2) step has been described for the interaction between LK157 and the Enterobacter cloacae 908R enzyme (10) but not for the interaction between BAL29880 and the FOX-4, CMY-2, or CMY-32 enzyme (14, 15). In the case of LK157 and 908R, this reactivation is very slow (k4 = 4 × 10^{-3} s^{-1}, half-life [t1/2] = 290 min) and does not significantly influence the properties of the inactivator. Nevertheless, we preferred to analyze the results on the basis of the complete model.

The pseudo-first-order rate constant for the inactivation is determined by k3 = k4 + k(X)/K + [X]), where K′ = K(Km + [R])/Km and [R] are, respectively, the Km and concentration of the reporter substrate. The ratio (K′ + [R])/K′ is referred to as the correction factor in the calculation of k3/K. When [X] is much smaller than K′, k3 = k4 + k(X)/K′ + [X] simplifies to k3 = k4 + k(X)K′, and if k4 is small, the line k3 versus [X] extrapolates at or near the origin.

RESULTS

Purification and characterization of the enzyme. The purification yield was estimated at 65%. SDS-gel electrophoresis and mass spectrometry indicated a degree of purity higher than 95%. The N-terminal sequencing of the mature form of the CHE-1 β-lactamase was H28,N-TPVSE, identical to those of the P99 and 908R enzymes (12).

The molecular mass of the CHE-1 enzyme as determined by ESI–Q-TOF MS was 38,708 ± 4 Da (expected, 38,705 Da) under denaturing conditions.

Kinetic results. The Kcat and Kcat values of the CHE-1 enzyme for nitrocefin were, respectively, 9.4 ± 1 μM and 17 ± 1 s^{-1}.

Figures 1 to 3 show the influence of the inactivator concentrations on the k3 values for the CHE-1–BAL29880 (Fig. 1), P99-BAL29880 (Fig. 2), and CHE-1–LK157 (Fig. 3) interactions. In all cases, the k3 values vary with the concentration of the inactivators in a linear way and extrapolate very close to the origin for zero concentration. These results show that, in all cases, [X] is much smaller than K′ and k3 = k4 + k(X)/K′ applies. Similarly, k3 is much smaller than k4[X]/K′, so that k3 = k4[X]/K′.

The slopes of the lines give the values of k4/K′, i.e., 253 ± 2 M^{-1} s^{-1} for CHE-1–BAL29880, 41,000 ± 2,000 M^{-1} s^{-1} for P99-BAL29880, and 23 ± 0.4 M^{-1} s^{-1} for CHE-1–LK157. Similarly, for the P99-LK157 interaction, the slope of the line was 20,600 ± 1,000 M^{-1} s^{-1} (not shown).

In the case of P99, the Km value for the reporter substrate (cephalosporin C) is 400 μM (17), so that the correction factors are 1.62 (BAL29880) and 1.58 (LK157) and the k4/K values are, respectively, 66,000 ± 3,000 M^{-1} s^{-1} for BAL29880 and 32,500 ± 1,600 M^{-1} s^{-1} for LK157.

With the CHE-1 enzyme, the nitrocefin concentration (100 μM) is much larger than the Km value so that a large correction factor (11.6 ± 2) must be introduced and the second-order rate constant (k3/K) values are 2,900 ± 300 M^{-1} s^{-1} and 270 ± 30 M^{-1} s^{-1} for BAL29880 and LK157, respectively.

As mentioned above, the lines extrapolate very close to the
were similar was not surprising. Note that for the E. cloacae 908R β-lactamase the reactivation rate constant with LK-157 was 0.4 × 10⁻⁴ s⁻¹ and the k₂/K value was 36,000 M⁻¹ s⁻¹ (10). Since the kinetic properties of the 908R and P99 enzymes are consistently very similar (18), the fact that the kₐ/K values for the interactions between each of the enzymes and LK157 (32,500 M⁻¹ s⁻¹) for P99 versus 36,000 M⁻¹ s⁻¹ for 908R) were similar was not surprising.

DISCUSSION

The purified CHE-1 enzyme exhibits the expected molecular mass and is significantly more resistant to both inactivators than both the P99 and 908R β-lactamases. The latter enzymes can be considered equivalent; they differ by only four mutations that do not significantly affect the enzyme activity (18, 19). Interestingly, two of the mutations present in 908R (I16V and A88P) are also present in CHE-1, a result which confirms that these mutations have little influence on the properties of the enzyme. Table 1 highlights the differences among the three sequences. The mutations at positions 16, 88, 242, 299, and 342 are situated far away from the active site, and it is very unlikely that they would modify the kinetic properties of the enzymes. The substitution of L with V at position 132 of 908R is very conservative and also unlikely to result in significant influence on the enzymatic properties.

In contrast, the deletion at residues 289 to 294, which is close to the active site, is specific to CHE-1 and is very probably responsible for the decreased sensitivity to inactivators. It is very interesting to note that avibactam (8), LK157, and BAL29880 exhibit very different structures and that the CHE-1 inactivation rates by these compounds decrease in all cases compared to rates of inactivation of the “parent” enzymes P99 and 908R (10- to 30-fold for avibactam, 14-fold for BAL29880, and more than 100-fold for LK157).

Among the three inactivators discussed above, BAL29880 exhibits the highest k_s/K value with the CHE-1 β-lactamase (2,900 M⁻¹ s⁻¹). This value is only about 4-fold lower than the equivalent acylation rate constants of the CMY-2, CMY-32, and FOX-4 class C β-lactamases (15, 16), so BAL29880 can still be considered a fair inactivator of CHE-1. Indeed, if one assumes a 5 μM (2 μg/ml) concentration of BAL29880 in the periplasm, the inactivation half time of CHE-1 would still be about 46 s, which should be adequate for a rather rapid inactivation. However, it should be kept in mind that a substrate with a very low K_m value affords some degree of protection, as reported above for nitrocefin.

In conclusion, the class C β-lactamase CHE-1 can be considered an “inactivator-resistant” mutant of P99, and this resistance is very probably due to the deletion of the hexapeptide at positions 289 to 294. If one considers the homogeneity of class C enzymes from both structural and functional points of view, the probability that the same deletion in other class C enzymes will result in similarly increased resistance to inactivators is rather high. This is not a very encouraging perspective, and one can only hope that the CHE-1 mutant remains exceptional. However, the positive result of the present study is the fact that BAL29880 retains a fair activity against CHE-1. Although the k_s/K value is decreased 20-fold compared to that with P99, it is only 3- to 4-fold lower than those with FOX-4, CMY-2, and CMY-32. With strains producing these enzymes, the addition of BAL29880 at 4 μg/ml (about 10 μM) restores the activity of β-lactamase-sensitive compounds (14, 15). The situation might not be very different with strains producing modified enzymes similar to CHE-1.

ACKNOWLEDGMENTS

We thank Malcolm Page (Basilea Pharmaceutica International Ltd., Basel, Switzerland) and Tom Solmajer (National Institute of Chemistry, Ljubljana, Slovenia) for providing BAL29880 and LK157, respectively.

FUNDING INFORMATION

This study was supported by an FP6 European program (CCE COBRA, conv 503335), in the framework of which Adriana Fernea was the recipient of a predoctoral fellowship.

REFERENCES


Table 1

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<th>Enzyme</th>
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<tr>
<td>P99</td>
<td>I A L N S K V A L A A T</td>
</tr>
<tr>
<td>908R</td>
<td>V P V N S K V A L A V T</td>
</tr>
<tr>
<td>CHE-1</td>
<td>V P L K — — — — — — A K</td>
</tr>
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</table>

*The nonconsensus residues are in boldface type. The dashes indicate deletion of the hexapeptide.

FIG 2 Variation of k_s versus [BAL29880] for P99.

FIG 3 Variation of k_s versus [LK157] for CHE-1.

TABLE 1 Differences between the sequences of CHE-1, P99, and 908R

<table>
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