Role of β-Lactam Hydrolysis in the Mechanism of Resistance of a β-Lactamase-Constitutive *Enterobacter cloacae* Strain to Expanded-Spectrum β-Lactams

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Enterobacter cloacae strains producing chromosomally mediated β-lactamase constitutively show high degrees of resistance to most of the third-generation β-lactams. It has been proposed that this resistance is due to the nonhydrolytic binding or trapping of β-lactams by the enzyme. We found that the outer membrane of *E. cloacae* strain 55M indeed had permeability to cefazolin about 14-fold lower than that of *Escherichia coli*, and that the number of β-lactamase molecules produced by this constitutive mutant was exceptionally large (2 × 10^5 per cell). These conditions are expected to produce a low degree of resistance, but could not explain the high resistance level of the mutant. We showed that the β-lactamase of this strain hydrolyzed third-generation β-lactams at measurable rates. Although the V_{max} for these compounds was less than 0.01% of that for cefazolin, the enzyme could hydrolyze them at rates comparable to the rate for cefazolin when the substrate concentration was near 0.1 µM, a concentration thought to be physiologically relevant for the inhibition of cell growth, because of the exceptionally high affinity of the enzyme to many third-generation compounds. Calculations based on kinetic parameters of the enzyme, outer membrane permeability, and affinity toward penicillin-binding proteins succeeded in predicting the MICs for several third-generation β-lactams. The data suggest that hydrolysis may be more important than nonhydrolytic binding for the expression of the resistant phenotype, and that studies on the susceptibility of β-lactams to β-lactamases should be carried out at physiologically relevant, very low concentrations of the drug, rather than the customary very high concentrations, such as 100 µM.

In recent years we have witnessed the isolation, both from clinical sources and in the laboratory, of strains of gram-negative bacteria, especially *Enterobacter cloacae*, that are resistant to a number of the third-generation β-lactams (15). These strains usually show the following characteristics (15): (i) they produce, in a constitutive manner, the chromosomally determined β-lactamase that is produced inducibly in the wild-type cells; and (ii) the spectrum of the third-generation β-lactams to which they become resistant includes many agents that their β-lactamase does not seem to hydrolyze with measurable rates.

To explain this puzzling observation, it has been proposed that the periplasmic β-lactamase produces resistance by tightly combining with the third-generation β-lactams without hydrolyzing them (15, 18–20). This concept of trapping or nonhydrolytic barrier has been challenged on the ground that, for this mechanism to work, the total number of β-lactamase molecules per milliliter of culture must be larger than the number of β-lactam molecules in the same volume (17). However, this argument fails to take into account the presence of an outer membrane barrier and the fact that, during any given time period, only a very small fraction of the β-lactam molecules present in the medium passes through this barrier and contacts the periplasmic β-lactamase. In this study we have examined, in a quantitative way, the extent of this barrier, the number of β-lactamase molecules available for trapping, and the possibility of slow, but significant, rates of hydrolysis of the third-generation agents by the β-lactamase to determine whether the resistance is truly caused by a trapping mechanism.

MATERIALS AND METHODS

Bacterial strains. *E. cloacae* 55W and its β-lactamase-constitutive mutant 55M were obtained from Christine C. Sanders (5). They were grown in L broth (10 g of Bacto-tryptone [Difco Laboratories], 10 g of Bacto yeast extract [Difco], and 5 g of NaCl per liter) at 37°C with aeration by shaking and were harvested when the Klett reading (red filter) reached 100 (about 0.26 mg [dry weight] per ml).

Chemicals. The β-lactams used were donated by the following companies: cefoxitin and imipenem (Merck Sharpe & Dohme), azthreonam (E. R. Squibb & Sons), ceftriaxone (Hoffmann-La Roche Inc.), cefotaxime (Hoechst-Roussel), ceftazidime (Glaxo), ceftizoxime (Fujisawa), and cefoperazone (Pfizer Inc.). Cefazolin was obtained from Sigma Chemical Co.

Determination of MIC. One drop of a suspension containing 10^5 cells per ml was deposited on the surface of L-agar plates containing serial twofold dilutions of β-lactams, and the growth was examined after overnight incubation at 37°C. Growth of fewer than 30 colonies was scored as negative.

Purification of β-lactamase by high-pressure liquid chromatography-gel filtration. Strain 55M was grown in minimal medium 63 (2) and was subjected to osmotic shock as described previously (14). Concentrated osmotic shock supernatant was injected into a TSK 3000SW column (6.5 by 600 mm, Beckman Instruments, Inc.) connected to a Perkin-Elmer LC-75 variable-wavelength detector and a Beckman model 110A pump, and gel filtration was performed with 0.1 M Na$_2$SO$_4$–0.01 M sodium phosphate buffer (pH 7.0) pumped at a flow rate of 1.0 ml min^{-1}. The effluent was monitored at 280 nm. Fractions were collected every 30 s and were analyzed for protein composition by sodium dodecyl sulfate

TABLE 1. Catalytic properties of the *E. cloacae* 55 M β-lactamase and their effect on resistance

β-Lactam	V_{max} (nmol mg^{-1} per s)	K_m (μM)	K_i (μM)	V at 0.1 μM (molecules cell^{-1} per s)	Permeability coefficient[a] (nm s^{-1})	C_o producing C_p of 0.1 μM[a] (μg/ml)	MIC (μg/ml)
Cefazolin	2300 (100)[b]	2080		34,600	35	114	>250
Cefoperazone	2.7 (0.12)	6.5[c]	2.1[d]	12,800	7	295	125
Ceftazidime	0.05 (0.002)	3.8[c]	2.6[d]	430	5	12	125
Ceftriaxone	ND[e]	ND	0.03[f]				250
Cefoxitin	0.06 (0.002)	<0.05[g]	0.023[h]	14,000	20	76	125
Ceftizoxime	0.008 (0.0004)	0.3[g]	0.12	620	15	4	125
Cefotaxime	0.023 (0.001)	<0.05[g]	0.003[i]	5,620	9	73	250
Azthreonam	ND	ND	0.012[j]				25

[a] See the text for methods of calculation.
[b] Values within parentheses indicate relative rates, with the rate for cefazolin set at 100.
[c] These values are similar to 16 and 6.6 μM for cefoperazone and ceftazidime, respectively, reported for *E. cloacae* P99 enzyme (1).
[d] These values are similar to 9 and 3 μM for cefoperazone and ceftazidime, respectively, reported for *E. cloacae* P99 enzyme (1).
[e] ND, Not determined.
[f] A fairly close value of 0.05 to 0.09 μM has been reported (19).
[g] These K_m values were approximated from the time course of complete hydrolysis of 1 μM substrates. When higher concentrations of substrates were used, higher apparent K_m values in the range of 2.5 to 5 μM were suggested from Lineweaver-Burk plots, as described in text.
[h] Reported values are 0.12 to 1.25 μM (19) for various strains and 0.5 μM for GN7471 (10).
[i] Reported values are 0.04 to 0.07 μM (19), 0.05 μM (10), and 0.035 μM (1).
[j] K_i of 0.012 μM has been reported (1).

(SDS)-polyacrylamide gel electrophoresis (see below) and for β-lactamase activity with cefazolin.

Assay of permeability. The permeability assay was carried out by the method of Zimmermann and Rosselet (22) by measuring the rate of hydrolysis of a β-lactam, usually cefazolin, by intact cells as well as by sonic extracts of these cells, and calculating the permeability of the outer membrane on the basis of the K_m (2.08 mM for cefazolin in strain 55M) and V_{max} of the enzyme (13). The hydrolysis rates by intact cells were corrected for the contribution by extracellular enzyme, but this correction amounted to less than 0.5% of the intact cell rates.

Assay of β-lactamase. The β-lactamase assay was carried out spectrophotometrically by following the decrease of the absorption peak around 260 nm in 10 mM potassium phosphate buffer (pH 7). The exact wavelengths and the molar differential absorbance values used were those listed in reference 11 for cefazolin, cefotaxime, ceftizoxime, cefoperazone, and cefoxitin. As reported by Bush et al. (1), hydrolysis of azthreonam produces maximum absorbance changes around 318 nm, but the magnitude of the change was too small to be useful for our assay. Seeberg et al. (17) recommend 272 nm for ceftazidime, but we found that the optical density changes were minimal at this wavelength and that they were maximal at 257 nm; we used the latter wavelength for assay. For ceftriaxone, Seeberg et al. (17) recommend 240 nm, and we confirmed that the maximal absorbance change occurred at 236 nm. However, the absorbance increased, rather than decreased, upon hydrolysis, and the extent of change was so small that it was not useful for the assay of the enzymatic hydrolysis of this compound. To detect the very slow hydrolysis of some of the agents accurately, we used spectrophotometers (Beckman DU-7 and Hitachi model 220) that did not show much noise even when the full scale was expanded to cover the range of 0.01 optical density unit. The assay was run at room temperature, and the results were recorded usually for 10 min with the activity determined from the initial slope of the tracing. For determination of K_i by Dixon plots, rates of hydrolysis of 100 μM cefazolin were determined in the presence of various concentrations of inhibitors. In these experiments 0.5 nM

enzyme was preincubated with the inhibitor for 2 min before the addition of cefazolin. For determination of the approximate K_m values in the submicromolar range, we used continuous tracing of the process of complete hydrolysis of 1 μM substrate by the enzyme (usually 20 nM), as described below.

Other methods. Protein was determined by the Lowry et al. method (9), and SDS-polyacrylamide gel electrophoresis was carried out with the slab version of the Laemmli method (7). Scanning of the stained gel was performed with a Quick Scan gel scanner from Helena Laboratories.

RESULTS

MIC values for various β-lactams. We could confirm that the β-lactamase-constitutive strain, 55M, was highly resistant to most of the third-generation β-lactams (Table 1). The strain remained very susceptible to imipenem (MIC, <0.5 μg ml^{-1}; data not shown).

Number of β-lactamase molecules per cell. When the cells of the constitutive mutant 55M and its inducible parent strain 55W were solubilized by heating at 100°C for 2 min in the Laemmli sample buffer (7) containing SDS and mercaptoethanol, and the solubilized samples were analyzed by SDS-polyacrylamide slab gel electrophoresis, the constitutive strain was seen to contain a prominent band with an apparent molecular weight of 42,000 which was absent in strain 55W (Fig. 1).

To confirm the identification of this band as the β-lactamase, cells of strain 55M harvested at the midexponential phase were exposed to osmotic shock by the method of Nossal and Heppel (14). The supernatant after the shock contained more than 95% of the β-lactamase activity present in the original cells and contained the 42,000-dalton protein as the most prominent component observed upon SDS-polyacrylamide gel analysis. When the supernatant was concentrated by ultrafiltration with a Millipore CX ultrafilter and was fractionated by high-pressure gel filtration (see above), the peak fraction containing β-lactamase activity was found to contain mostly the 42,000-dalton protein (Fig. 1).

When the amount of the 42,000-dalton band was quantitated by scanning the Coomassie blue-stained gel of the

whole cell proteins from strain 55M, it corresponded to 3.7% of the total cellular protein. Assuming that there are 2×10^9 cells per mg (dry weight), and that 70% of the dry weight is protein, this means that there are 1.9×10^5 β-lactamase molecules per cell of the constitutive strain, 55M.

Permeability of E. cloacae outer membrane to cefazolin. When exponential-phase, washed cells of strain 55M grown in L broth were incubated in the presence of 1 mM cefazolin and the rate of hydrolysis was determined spectrophotometrically with a cell of 1-mm optical path (13), the rate was only 30 nmol per mg (dry weight) per min, in comparison with the rate obtained with the sonic extract of these cells, 44,200 nmol per mg (dry weight) per min in the presence of the same cefazolin concentration. From the K_m of the periplasmic β-lactamase for cefazolin (see above) and the area of the cell surface per milligram (dry weight) (13), we calculated that the permeability coefficient of the outer membrane of this strain to cefazolin was 3.5×10^{-6} cm s^{-1}, 14 times less than the permeability of OmpF-containing *Escherichia coli* cells to this compound (13).

Activity of E. cloacae β-lactamase against various β-lactams. The kinetics of hydrolysis of several β-lactams was determined by using osmotic shock fluid from *E. cloacae* 55M as the enzyme source. All of the third-generation compounds we could test with good sensitivity were hydrolyzed with low, but measurable, rates (Table 1). We could usually observe a finite K_m value in the neighborhood of several micromolar by using substrate concentration range of 2 to 50 μM. However, we do not believe that these values reflected the true K_m values for the third-generation compounds except for cefoperazone and ceftazidime. First, the
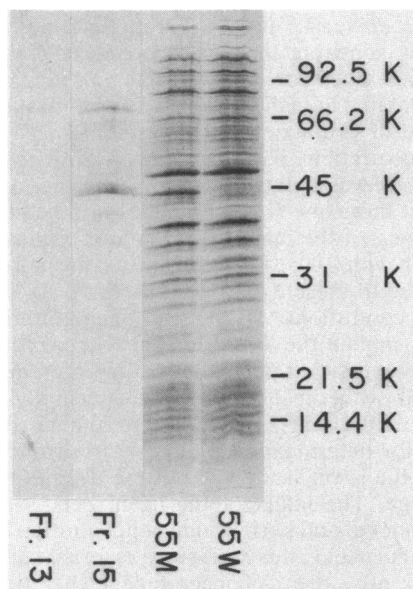

FIG. 1. SDS-polyacrylamide slab gel electrophoresis of fractionated osmotic shock fluids. Osmotic shock supernatant from strain 55M was applied to a high-pressure liquid chromatograph gel filtration column as described in the text, and the fractions were analyzed by slab gel electrophoresis. Only fraction 15, which contained strong β-lactamase activity, and fraction 13, devoid of the activity, are shown here. Fraction 15 is seen to contain mostly a 42,000-dalton protein, although it is contaminated by the trailing edge of a 75,000-dalton protein peak, some of which is seen in fraction 13. The two lanes on the right show the protein composition of whole cells of 55M and 55W; the only difference seen is the presence and absence of the 42,000-dalton protein.
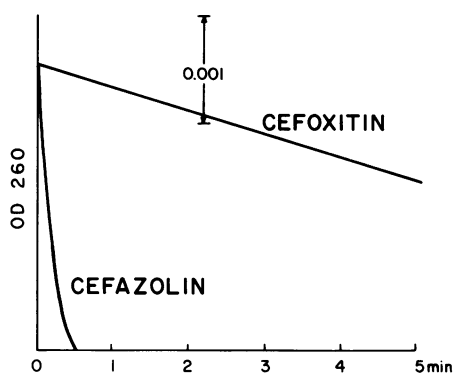
K_i values obtained by measuring the rate of hydrolysis of 100 μM cefazolin in the presence of these compounds were orders of magnitude lower than the observed K_m values, with the exception of ceftazidime and cefoperazone. Second, the rate of hydrolysis of low concentrations of these compounds other than cefoperazone and ceftazidime (for example, a 1 μM solution) was constant until very shortly before the substrate became exhausted, a result showing that the true K_m was far lower than 1 μM. Third, with cefotaxime, it was clear that the hydrolysis had two components, one with very low K_m and the other with the previously measured 4.8 μM K_m, and that the first process became inhibited at higher (i.e., >2 μM) substrate concentrations, because the hydrolysis rate was about twice as high with 1 μM substrate as with 2.5 μM substrate. Because of these observations, we determined the true K_m values by following the kinetics of hydrolysis of 1 μM solutions of β-lactams to completion and by reading the concentration at which the rate became one-half of the initial rate. For example, if the optical density decreased from 0.188 to 0.180 during the course of hydrolysis, and if the slope became one-half of the initial slope at the optical density of 0.181, the K_m was $1 \times (0.181 - 0.180)/(0.188 - 0.180) = 0.125$ μM. This procedure produced K_m values that were usually only slightly higher than K_i values for the respective β-lactams (Table 1), and which we believe represent the true K_m values. We do not know what produces the high K_m components observed. It may be that substrate inhibition at these relatively high substrate concentrations produced a bend in the Lineweaver-Burk plot, or that another enzyme exists in our preparation because we used a crude osmotic shock fluid for the reaction. In any case we do not believe that the high K_m components are relevant in the physiology of bacteria living in the presence of β-lactam, as described below.

V_{max} values obtained for the third-generation agents were low, but significant, in all cases. We believe that past studies that found these agents to be non-hydrolyzable failed to recognize this fact because of the lack of sensitivity of the methods employed. Indeed, in addition to cefoperazone, which is generally recognized as hydrolyzable, slow hydrolysis of cefoxitin (10, 16) as well as cefotaxime and ceftazidime (1) has been reported previously. The very low levels of V_{max} for the third-generation compounds may nevertheless be quite significant in a physiological context, as described fully below. But we can already calculate, by using the V_{max} and K_m values in Table 1, that, although there will be a more than 1,000-fold difference in the hydrolysis rates of cefazolin and cefoxitin at 0.1 mM substrate concentration, the difference will be reduced to about 20-fold at 1 μM and to only about 2-fold at 0.1 μM. Indeed, the assay of cefoxitin and cefazolin hydrolysis rates at 1 μM concentration with the same amount of enzyme showed a difference of 26-fold, close to the predicted value (Fig. 2).

DISCUSSION

It has been proposed by several laboratories that the tight binding of non-hydrolyzable β-lactams by periplasmic β-lactamase molecules, i.e., trapping of β-lactams, could create resistance to third-generation agents seen in β-lactamase-constitutive mutants of *E. cloacae* (see above). Furthermore, Seeberg et al. (17) ruled out the possibility that other changes accompanying the production of the periplasmic β-lactamase are the true cause of the resistance. However, for the trapping mechanism to work, the organism has to possess a very large number of β-lactamase molecules

FIG. 2. Hydrolysis of cefazolin and cefoxitin at 1 μM substrate concentration. Osmotic shock fluid corresponding to 1.2×10^7 cells was added to 1-ml solutions containing 1 μM substrate and 10 mM potassium phosphate buffer, pH 7.0, and the hydrolysis of the substrate was followed by observing optical density (OD) at 260 nm at room temperature in a Beckman DU-7 spectrophotometer. The curves were smoothed out to eliminate the effect of noise.

and at the same time allow only a very small number of β-lactam molecules to trickle into the periplasm. To our knowledge, quantitative estimation of neither of these parameters has been reported previously.

In this study we determined these parameters by using one such constitutive mutant, *E. cloacae* 55M. By identifying the β-lactamase band on SDS-polyacrylamide slab gel and quantitating this band on gels of whole cell proteins, we could determine that the strain produced a very large amount of this enzyme, corresponding to 3.7% of the total cellular protein, or about 2×10^5 molecules per cell. This number is similar to the number of another very abundant periplasmic protein of *E. coli*, maltose-binding protein, which is reported to be present in 4×10^5 copies per cell (4). If the cells are growing with a generation time of 20 min, a cell should be synthesizing, on an average, $(2 \times 10^5)/(20 \times 60) = 167$ molecules of new β-lactamase every second.

The outer membrane permeability to cefazolin was low, estimated to be about 7% of the permeability coefficient previously determined in *E. coli* K-12 (5×10^{-5} cm/s [13]). Since most of the third-generation β-lactams, with the notable exception of imipenem, have permeability coefficients lower than 20% of that of cefazolin (21), and since the major porin of *E. cloacae* appears to have a pore size slightly narrower than the *E. coli* porins (6, 12), it is reasonable to assume that the permeability coefficients of the *E. cloacae* for them would be lower than $5 \times 10^{-5} \times 0.07 \times 0.2 = 7 \times 10^{-7}$ cm s^{-1} (see also reference 13). The maximal rate of influx of such agents across the outer membrane is $v = P \times A \times C_o$, following Fick's first law of diffusion, where P, A, and C_o denote the permeability coefficient, area of the membrane per unit weight of cells, and the external concentration of the β-lactam, respectively. Using the value of 132 cm^2 mg^{-1} for A (13), and assuming $C_o = 0.4 \times 10^{-8}$ mol cm^{-3} or about 2 μg ml^{-1} with a β-lactam molecular weight of 500, $v = 7 \times 10^{-7}$ (cm s^{-1}) $\times$ 132 (cm^2 mg^{-1}) $\times$ 0.4 $\times$ 10^{-8} (mol cm^{-3}) $\times$ 6.023 $\times$ 10^{23} (molecules mol^{-1})/[2 $\times$ 10^9 (cells mg^{-1})] = 111 molecules per cell per s. Since, as we have seen, the cells are making new β-lactamases at a slightly higher rate, practically all the β-lactams slowly flowing into the periplasm can be trapped by this binding process, and thus in theory the mechanism should be able to produce a low level of resistance.

In practice, however, we have some serious problems.

First, strain 55M is resistant to concentrations of 100 μg ml^{-1} or more of some of the third-generation antibiotics (Table 1). Under these conditions, the rate of influx of the drug (v) is at least 50 times higher than that calculated above (due to the higher C_o), and the production of new β-lactamase molecules will not be able to catch up with this rate. Second, Seeberg et al. (17) transferred the gene for the *E. cloacae* β-lactamase into *E. coli* K-12 and found that the β-lactamase-producing *E. coli* strain was resistant to a fairly high concentration of cefotaxime, although the MIC (64 μg ml^{-1}) was lower than that in the original *E. cloacae* donor (>128 μg ml^{-1}). This is even more difficult to explain by trapping alone, because *E. coli* K-12 has a much higher outer membrane permeability (13). Finally, the cells will be growing much more slowly (and therefore much fewer molecules of new β-lactamase will be produced per unit of time) than we assumed above in some in vitro situations, and certainly in the body of the host. Thus it becomes difficult to explain why these β-lactamase-producing strains could develop resistance in host tissues, yet they have been isolated from clinical cases in which therapy with the third-generation β-lactams was not very effective (15).

We believe that, at least in the strain we studied, hydrolysis by the enzyme produces a much more important contribution than binding alone. By using spectrophotometers that exhibit very low noise and carry out computer averaging of signals over a short period of time, we could perform a very sensitive assay and found that all the third-generation agents whose hydrolysis could be followed spectrophotometrically with reasonable sensitivity were indeed hydrolyzed with slow, but significant, rates by the 55M enzyme. Although the assay with 2 to 100 μM substrate concentrations showed apparent K_m values that were rather high, for example, 4.8 μM for cefotaxime, an assay with 1 μM or less substrate usually showed the presence of an activity with much lower K_m (Table 1), which apparently became inhibited at higher substrate concentrations, at least in the case of cefotaxime. (This inhibition by higher substrate concentrations is very common with *E. cloacae* β-lactamase, as was pointed out by Seeberg et al. [17].) We believe that these low K_m components are the manifestation of the activity of the major β-lactamase, because K_i values determined with 100 μM cefazolin as the substrate were usually close to these K_m values (Table 1).

The concentrations of the third-generation β-lactams needed to inhibit the binding of [^{14}C]benzylpenicillin to functionally important penicillin-binding proteins (i.e., penicillin-binding proteins 1 through 3) are generally in the range of less than 0.1 μM (3). Therefore the most important processes for determining the level of resistance are those that affect the periplasmic level of the drug in this concentration range. The influx of the agent occurs at the rates discussed above, and periplasmic concentration of the agent has little influence on this rate, since external concentrations of the drug are orders of magnitude higher than the periplasmic one with resistant strains. The important parameter is then the rate of removal, either by binding or hydrolysis, of the β-lactam from the periplasmic space. The central observation we made is that the rate of hydrolysis of third-generation compounds at these low concentrations is quite significant in relation to the rate of influx of these agents. At 0.1 μM concentration, cefoxitin is expected to be hydrolyzed at a rate corresponding to 37% of that of cefazolin (Table 1). This occurs because of the very low K_m for cefoxitin, more than 40,000 times lower than that for cefazolin, despite the exceedingly low V_{max} for cefoxitin.

It is true that V_{max} values are very low for most third-generation β-lactams. Thus V_{max} values for cefotaxime have been reported to be less than 0.01% of that of cephazolin (10) and less than 0.003% of that of cephaloridine (1), and laboratories performing assays at an arbitrarily chosen, very high concentration of the substrate (often 100 μM) reported rates of less than 0.04 to 0.15% (17) or less than 1% (19). These striking figures apparently misled some workers to believe that third-generation β-lactams were non-hydrolyzable by most β-lactamases. We could indeed confirm the low V_{max} values (Table 1). However, at pharmacologically relevant concentrations that are likely to prevail in the periplasm, the rates of hydrolysis of many third-generation agents are surprisingly rapid, thanks to their exceptionally low K_m values.

Do the K_m and V_{max} values observed explain fully the resistance of the constitutive mutant strain? We can combine Fick's first law, which governs the influx of β-lactams across the outer membrane, with the Michaelis-Menten equation, which governs their enzymatic hydrolysis in the periplasmic space, by the method of Zimmermann and Rosselet (22). Combining these equations and solving for C_o results in the following formula: $C_o = C_p + (C_P \times V_{max})/[P \times A \times (K_m + C_p)]$, where C_o, P, and A have previously defined meaning, and C_p stands for the periplasmic concentration of the β-lactam. We do not know the values of P in $E.$ cloacae except for cefazolin, but we assumed that the $E.$ cloacae porin could behave like the OmpF porin of $E.$ coli, and we calculated the values of P by multiplying the P for cefazolin with the ratio, (P for third-generation cephalosporin)/(P for cefazolin), obtained with the OmpF porin (21). These expected P values are shown in Table 1. Solving the equation above with $C_p = 0.1$ μM (i.e., the periplasmic concentration at which peptidoglycan-synthesizing enzymes begin to be inhibited significantly) gives C_o values that are not so different from the actual MIC observed (considering the many assumptions involved) for cefoperazone, cefoxitin, and cefotaxime (Table 1). On the other hand, our predicted values are not in agreement with the observed high resistance to ceftazidime and ceftizoxime. With the former compound, a recent report suggests that concentrations close to 0.3 μM, rather than 0.1 μM, are needed to inhibit the penicillin-binding proteins of $E.$ coli (M. J. M. Hitchcock, personal communication). If this factor is taken into account, the theoretically predicted MIC for ceftazidime will become nearly 50 μg ml⁻¹, a better agreement with the observed value. However, we have no explanations at present for the resistance to ceftizoxime. The resistance to cefazolin was somewhat higher than expected. This could be due to the more rapid hydrolysis of this drug in the outer regions of periplasm, where the drug concentration might be higher.

When the C_o is close to the MIC and the C_p is close to the concentration at which penicillin-binding proteins are somewhat inhibited, the enzyme is calculated to be hydrolyzing thousands of molecules of β-lactams per cell per second (Table 1); this is much greater than the binding by the newly synthesized enzyme molecules, calculated above as 167 molecules per cell per s for a rapidly growing culture. However, we emphasize that the hydrolysis rates observed would not be enough to produce the level of resistance without the low permeability of the outer membrane to many of the third-generation agents. If we substitute 5×10^{-4} cm s⁻¹ for P, a permeability coefficient typical for rapidly penetrating, zwitterionic agents (11, 21), the formula above gives us a C_o value of only 0.8 μM or 0.36 μg ml⁻¹ for

cefoxitin; the cells cannot become resistant if the outer membrane has high permeability to the agents. This consideration explains very well the observation that the β-lactamase-constitutive $E.$ cloacae strains are always still susceptible to imipenem (15), because imipenem has the highest rate of penetration among the β-lactam compounds tested by us (21). The low general permeability of $E.$ cloacae outer membrane and the low intrinsic penetration rates of most of the third generation β-lactams other than imipenem (21) do indeed play crucial roles in creating significant levels of resistance in these strains. The interplay between the low penetration rates and the low, but significant, rate of removal from the periplasmic space emphasizes the need to determine the hydrolysis rates of β-lactams at a physiologically relevant range (0.1 to 1 μM) of concentrations, rather than in the customary range of about 100 μM, which may and indeed did give us completely misleading information.

The possibility of rapid hydrolysis of low concentrations of cefotaxime by $Pseudomonas$ $aeruginosa$ enzyme has been discussed earlier by Livermore (8). Although Livermore did not attempt to determine the hydrolysis rates at low substrate concentrations, and his arguments are essentially qualitative, his conclusion agrees very well with ours and suggests that hydrolysis may play an important role also in the resistance of $P.$ aeruginosa to many third-generation cephalosporins.

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