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Class C β-Lactamases Operate at the Diffusion Limit for Turnover of Their Preferred Cephalosporin Substrates

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It has been suggested that class C β-lactamases have evolved to carry out a metabolic reaction other than hydrolysis of β-lactam antibiotics. It is demonstrated in the present study that the class C β-lactamase from *Enterobacter cloacae* P99 has reached the diffusion limit in its ability to hydrolyze its preferred cephalosporin substrates. The increase in the solution viscosity by addition of a microviscogen (sucrose) caused the decline in the parameter k_{cat}/K_m for hydrolysis of cephaloridine and cephalosporin C (approximately 2.5-fold at a relative viscosity of 2.9). A similar increase in viscosity has no effect on the turnover rate of the poorer substrates cefepime and penicillin G. Addition of a macroviscogen (polyethylene glycol) to the reaction mixture did not change the rate of turnover for any of the substrates tested because in this case the viscogen would not interfere with the motion of small molecules, as was expected. Therefore, it would appear that the driving force behind the evolution of this class C β-lactamase and, in principle, other enzymes of this class is indeed the functional reaction of this enzyme as a drug resistance factor.

β-Lactamases are the primary cause of bacterial resistance to β-lactam antibiotics. These enzymes hydrolyze the β-lactam bonds of these antibacterial agents, whereby the activity of the drug is lost and the phenotypic expression of resistance is manifested. There are four classes of β-lactamases, of which class A enzymes are the most common group and class C enzymes are the second most common group (7, 8).

Literature from the late 1960s had suggested that certain β-lactamases might have additional metabolic functions besides hydrolysis of β-lactams (32, 34). The recent disclosure of an elaborate system for regulation and recycling of the peptidoglycan has revealed metabolic ties to induction of the class C β-lactamases from gram-negative organisms (19, 29, 30). These observations have prompted the assertion that, indeed, for the case of the class C β-lactamases an alternative metabolic function may have been at the roots of the evolution of these enzymes (26). We disclose herein evidence that evolution of class C β-lactamases has been driven solely by the need of the organisms that harbor them as a protective means against cephalosporin antibiotics.

Enzymes as biocatalysts evolve to perform the metabolic task for which they specialize. A measure of the catalytic competence of any enzyme is the kinetic parameters (k_{cat}, K_m, and k_{cat}/K_m) for the given reaction performed by the enzyme. The k_{cat}/K_m ratio has acquired a special place in these analyses since it can be considered a "bimolecular rate constant" for the reaction between the enzyme and the substrate, permitting direct comparison of different catalysts to one another. It has been noted that there exists an upper limit for this ratio in enzymatic reactions. According to theory, for the reaction of a large molecule (i.e., an enzyme) and a small molecule (a typical nonpolymeric substrate) this value approaches 10^8 to 10^9 M^{-1} s^{-1} (33, 35, 36). Once this limiting level for catalysis is reached for any enzyme, the actual chemical steps in the catalytic processes, that is, bond making and bond breaking, are considered to have reached "catalytic perfection" (1). That is, the steps that require covalent bond making and bond breaking, which typically are slow processes, are no longer limiting for such a perfect catalyst. On the contrary, diffusional steps, which are rapid, become the limiting steps in catalysis by such an enzyme. To put this differently, travel (diffusion) of the substrate into the active site of the enzyme or movement of the product away from the active site becomes the slow step in catalysis. Such a "perfect" enzyme can no longer improve its catalytic ability in the course of evolution from that point on and is said to be "diffusion controlled." The chances are that many critical metabolic enzymes have reached such a diffusion-controlled state, because the advantage that the rapid reaction provides for the organisms is selected in the course of evolution. However, few enzymes have specifically been shown to operate at such a level. The following are a few examples: triosephosphate isomerase (20), phosphorylase *b* (11), horseradish peroxidase (14), chymotrypsin (6), carbonic anhydrase (17, 31), invertase (27), acetylcholinesterase (2), adenosine deaminase (22), class A β-lactamase (16), and aminoglycoside 3′-phosphotransferase type III (25).

Of the four classes of β-lactamases (7, 8, 23), the class A β-lactamases (penicillinases) are the most common, and it is widely accepted that they have evolved to hydrolyze penicillins (10). This matter was put on firm ground by the demonstration of Hardy and Kirsch (16) that indeed the class A β-lactamase from *Bacillus cereus* (β-lactamase I) operates at the diffusion-controlled limit. As discussed earlier, it has been suggested in the literature that the chromosomal class C β-lactamases may have evolved to catalyze a reaction other than hydrolysis of β-lactam (4, 26, 32, 34, 38). This assertion can be tested, even if one does not know the nature of the alternative reaction. The rationale is as follows. These enzymes are known as cephalosporinases, and if one demonstrates that they catalyze hydrolysis of cephalosporins at the diffusion limit, then it is unlikely that their evolution may have been driven by a different reaction. Indeed, we have performed such an analysis, and it is clear that these enzymes have evolved to "perfection" for their reaction in hydrolysis of their preferred cephalosporin substrates, as will be detailed below.

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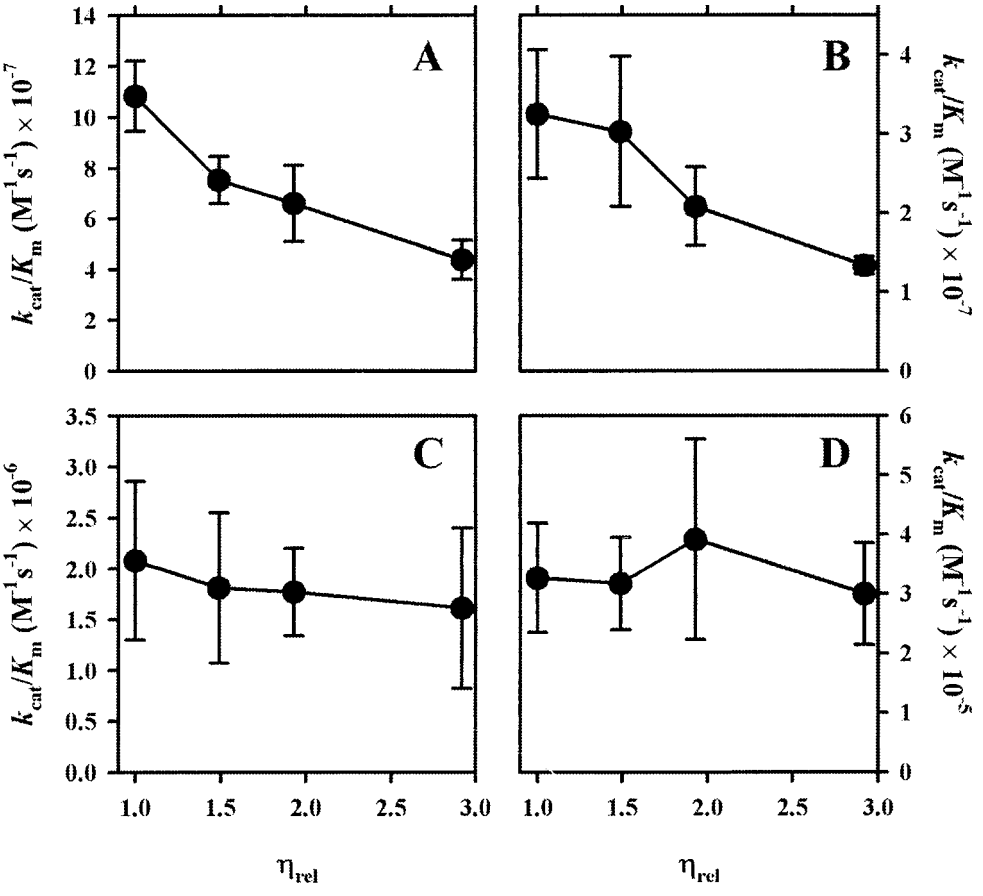

FIG. 1. Dependence of k_{cat}/K_m on relative viscosity (η_{rel}) of the solution for hydrolysis of cephaloridine (A), cephalosporin C (B), penicillin G (C), and cefepime (D) by the class C β-lactamase from *E. cloacae* P99.

MATERIALS AND METHODS

Cephaloridine, cephalosporin C, penicillin G, sucrose and polyethylene glycol (PEG) 8000 were purchased from Sigma Chemical Co. (St. Louis, Mo.). Cefepime was a gift from Bristol-Myers Squibb (Princeton, N.J.). Spectrophotometric studies were performed on a Hewlett-Packard 8453 diode array instrument. Nonlinear regression analysis was performed by the use of the program SigmaPlot (Jandel Scientific). Other calculations were performed with the Microsoft Excel software. The class C β-lactamase was purified from *Enterobacter cloacae* P99 by affinity chromatography (9). The purified enzyme was homogeneous, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The kinetic parameters for turnover (K_m and k_{cat}) of substrates were determined either from the Lineweaver-Burk plot or by nonlinear regression of the equation for Michaelis-Menten kinetics. Six to seven substrate concentrations were used for each kinetic determination, and the reported parameters were the averages for at least three independent measurements. All the experiments were carried out in 100 mM sodium phosphate (pH 7.0) at 20°C with the corresponding amount of viscogen added. The typical assay volume was 1.0 ml. The concentration ranges for various substrates were as follows: cephaloridine, 200 to 600 μM; cephalosporin C, 200 to 800 μM; penicillin G, 100 to 700 μM; cefepime, 10 to 150 μM. A portion of the enzyme was added to a solution of substrate to give a final enzyme concentration of 15 nM. Substrate hydrolysis was monitored at 290 nm for cephaloridine ($\Delta\varepsilon_{290}$ = 2,070 M^{-1} cm^{-1}), 280 nm for cephalosporin C ($\Delta\varepsilon_{280}$ = 2,390 M^{-1} cm^{-1}), 240 nm for penicillin G ($\Delta\varepsilon_{240}$ = 560 M^{-1} cm^{-1}), and 260 nm for cefepime ($\Delta\varepsilon_{260}$ = 750 M^{-1} cm^{-1}). The viscosity of the solution was controlled by the addition of the appropriate amounts of sucrose or PEG 8000 to the buffer. The relative viscosities (η_{rel}) of the solutions were determined from the reference data (37).

RESULTS AND DISCUSSION

The way to demonstrate that the rate of enzymatic reaction is controlled by the diffusion-controlled limit is to probe for the change in the rate of the reaction as a function of the viscosity of the solution. The more viscous the solution, the more difficult will be the diffusion of the molecules in and out of the active site of the enzyme, resulting in a decrease in the value of k_{cat}/K_m. Moreover, the K_m component should be influenced more than the k_{cat} component. We hasten to add that the decrease in the second-order rate constant on an increase in solution viscosity does not necessarily mean that the reaction is under diffusion control. The decrease in the rate could also be attributed to the decrease in the free energy of the unbound substrate. To prove that the reaction is indeed under the diffusion limit, a control experiment should be performed. In a control experiment one can use either a poor substrate for the given enzyme (16) or, if no poor substrate is available for the system, a sluggish mutant variant of the enzyme (5). In either case the rate for hydrolysis of a sluggish enzyme-substrate system should not undergo change upon the increase in solution viscosity.

The viscosity of the solution is commonly altered by the addition of viscogens such as sucrose, glycerol, Ficoll, or PEG. Although the presence of any of the four compounds in solution would increase the macroscopic viscosity of the solution, at the microscopic level their behaviors are quite different. According to theory, polymers such as Ficoll and PEG do not influence the rates of diffusion of small molecules (3, 28). On the other hand, small-molecule viscogens such as sucrose and glycerol not only will increase the macroscopic viscosity of the solution but also will slow down the diffusion of molecular particles in solution (18, 21). For this reason, sucrose and

TABLE 1. Kinetic parameters for turnover of cephaloridine by the class C β-lactamase from *E. cloacae* P99

η_{rel} (% viscogen)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
1 (0)	230 ± 20	2.1 ± 0.2	(1.08 ± 0.14) × 10^8
1.49 (13% sucrose)	310 ± 25	4.1 ± 0.4	(7.5 ± 0.9) × 10^7
1.93 (21.6% sucrose)	280 ± 40	4.3 ± 0.7	(6.60 ± 0.15) × 10^7
2.92 (28% sucrose)	345 ± 50	7.9 ± 0.9	(4.4 ± 0.7) × 10^7
2 (6.7% PEG)	285 ± 15	3.4 ± 0.2	(8.3 ± 0.7) × 10^7

TABLE 3. Kinetic parameters for turnover of penicillin G by the class C β-lactamase from *E. cloacae* P99

η_{rel} (% viscogen)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
1 (0)	22 ± 5	10 ± 3	(2.1 ± 0.8) × 10^6
1.49 (13% sucrose)	21 ± 4	11 ± 4	(1.8 ± 0.7) × 10^6
1.93 (21.6% sucrose)	21 ± 2	12 ± 3	(1.8 ± 0.4) × 10^6
2.92 (28% sucrose)	22 ± 6	14 ± 6	(1.6 ± 0.8) × 10^6

glycerol are called microviscogens, in contrast to macroviscogens, such as Ficoll and PEG.

β-Lactamases are typically efficient catalysts in hydrolysis of the β-lactam bonds of their preferred substrates. In many cases the k_{cat}/K_m values for the β-lactamase hydrolysis of a good substrate is in the range of 10^7 to 10^8 M^{-1} s^{-1}. That is also true for the AmpC family of β-lactamases, for which the k_{cat}/K_m for turnover of cephaloridine by several of the members is in the range of 10^7 to 10^8 M^{-1} s^{-1} (13).

We have investigated the hydrolysis rates for four selected β-lactam substrates for the *E. cloacae* P99 β-lactamase in the presence of viscogens. The substrate selection was made such that both good and poor substrates would be represented. Two cephalosporins, cephaloridine and cephalosporin C, are exceptionally good substrates for this β-lactamase. Penicillin G, which was used as a representative penicillin substrate, is not preferred by class C β-lactamases. Finally, cefepime is one of the worst cephalosporin substrates for the enzyme.

Analysis of the kinetic parameters (k_{cat}, K_m, and k_{cat}/K_m) revealed the following trends (Tables 1 to 4 and Fig. 1). As expected, addition of PEG (macroviscogen) did not influence appreciably the kinetic parameters for any of the substrates tested. However, in a manner similar to that for other enzymes that operate at the diffusion-controlled limit, the rate of hydrolysis of the good substrates for the *E. cloacae* P99 β-lactamase decreased proportionally with the increase in the relative viscosity in the presence of sucrose (microviscogen). The k_{cat}/K_m value decreased 2.5-fold in the case of cephaloridine and cephalosporin C (Fig. 1A and B, respectively). An important factor that affected the ratio k_{cat}/K_m for these substrates was the increase in K_m (Tables 1 and 2). The effect of viscosity on k_{cat} values was very small throughout the viscosity range, giving no trends as a function of increasing viscosity, as would be expected.

The situation is quite different for the poorer substrates. The k_{cat}/K_m value virtually did not change, when one considers the calculated standard deviations in each case for penicillin G (Fig. 1C; Table 3) and cefepime (Fig. 1D; Table 4), as would be expected. The effects on other kinetic parameters were also negligible, with no trends for the fluctuation being observed.

These observations confirm the hypothesis that the hydrolytic process for the good substrates for the *E. cloacae* P99 β-lactamase is diffusion controlled. In the case of moderate to poor substrates, the slow steps are at the bond-making and bond-breaking levels. Therefore, the diffusional ability in the presence of the microviscogen has minimal to no effect on the overall rate of turnover of the poorer substrates by the *E. cloacae* P99 class C β-lactamase.

The processing of murein (peptidoglycan) in gram-negative bacteria is elaborate, and it involves several gene products (19). The presence of some of the intermediates in this process induces the expression of the AmpC gene product, which encodes the class C β-lactamase of gram-negative bacteria. It is likely that the presence of such intermediates is a signal for expression of the resistance enzyme because, indeed, such degradation of peptidoglycan takes place as a consequence of the action of β-lactam drugs on the organism. Therefore, this may serve as a signal to upregulate the expression of the resistance enzyme to come to the rescue of the organism in distress.

It is actually tantalizing that it has been demonstrated that the AmpC gene products do perform other reactions such as hydrolysis of depsipeptides and amides (12, 15). These are taken as "vestigial reactions" for these enzymes, suggestive of their relationships to other proteins such as certain penicillin-binding proteins (PBPs). However, it is evident that true to the term "vestigial reaction," these atypical transformations for the AmpC enzyme are carried out at rates that approach those for some of the poorer β-lactam substrates for the class C β-lactamases (12, 15, 39, 40).

An enzyme would reach catalytic "perfection" only for the reaction that drives its evolution. On the basis of the results presented here, it would appear that that reaction for class C β-lactamases is hydrolysis of their preferred cephalosporin substrates. Previous findings argued the same for the evolution of class A β-lactamases in response to the challenge by penicillins (16). It would appear to be intuitive, in retrospect, that these enzymes should be chemically perfect for their resistance function, since this matter has a direct bearing on the ability of the bacteria to survive in the presence of the antibacterial agent.

Structural and kinetic considerations led Matagné et al. (24) to suggest recently that class C enzymes are "primitive" forms of β-lactamases. The results presented in this report are inconsistent with this characterization of class C enzymes. We have argued recently that the diversification of the two lines of PBPs that ultimately gave rise to classes A and C of β-lactamases was an early event in the evolution of PBPs (23). Further-

TABLE 2. Kinetic parameters for turnover of cephalosporin C by the class C β-lactamase from *E. cloacae* P99

η_{rel} (% viscogen)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
1 (0)	460 ± 15	14 ± 3	(3.25 ± 0.80) × 10^7
1.49 (13% sucrose)	790 ± 70	26 ± 8	(3.0 ± 0.9) × 10^7
1.93 (21.6% sucrose)	600 ± 35	29 ± 7	(2.1 ± 0.5) × 10^7
2.92 (28% sucrose)	670 ± 12	50 ± 4	(1.3 ± 0.1) × 10^7
2 (6.7% PEG)	420 ± 20	16 ± 4	(2.6 ± 0.7) × 10^7

TABLE 4. Kinetic parameters for turnover of cefepime by the class C β-lactamase from *E. cloacae* P99

η_{rel} (% viscogen)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
1 (0)	3.7 ± 0.2	11 ± 3	(3.3 ± 0.9) × 10^5
1.49 (13% sucrose)	8.4 ± 0.6	27 ± 6	(3.2 ± 0.7) × 10^5
1.93 (21.6% sucrose)	7.4 ± 1.3	19 ± 7	(3.9 ± 1.7) × 10^5
2.92 (28% sucrose)	3.3 ± 0.2	11 ± 3	(3.0 ± 0.8) × 10^5
2 (6.7% PEG)	3.1 ± 0.5	10 ± 3	(3.1 ± 1.0) × 10^5

more, the details of the mechanisms for the catalytic processes of the two classes of β-lactamases argue for independent and perhaps parallel evolutions for the two classes of enzymes. The results presented in this report shed further light on this process by showing that the evolutionary developments of both classes A and C of β-lactamases have been driven to catalytic perfection. Since class A β-lactamases prefer penicillins as substrates, whereas class C enzymes show better competence in turnover of cephalosporins, it is clear that evolution of each class of enzymes was advanced by those respective substrates. The structures of penicillins and cephalosporins are different, and they each provided a differential selection pressure for evolution of β-lactamases. This differential selection pressure is at the roots of the differences in evolution of classes A and C of β-lactamases, but what is significant in our opinion is the fact that each selection pressure was sufficient individually to drive the evolution of their respective enzymes to catalytic perfection.

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REFERENCES

1. **Albery, W. J., and J. R. Knowles.** 1976. Evolution of enzyme function and the development of catalytic efficiency. Biochemistry **15:**5631–5640.
2. **Bazelyansky, M., E. Robey, and J. F. Kirsch.** 1986. Fractional diffusion-limited component of reactions catalyzed by acetylcholiesterase. Biochemistry **25:**125–130.
3. **Biancheria, A., and G. J. Kegeles.** 1957. Diffusion measurements in aqueous solutions of different viscosity. J. Am. Chem. Soc. **79:**5908–5912.
4. **Bishop, R. E., and J. H. Wiener.** 1992. Coordinate regulation of murein peptidase activity and AmpC β-lactamase synthesis in *Escherichia coli*. FEBS Lett. **304:**103–108.
5. **Blacklow, S. C., R. T. Raines, W. A. Lim, P. D. Zamore, and J. R. Knowles.** 1988. Triosephosphate isomerase catalysis is diffusion controlled. Biochemistry **27:**1158–1167.
6. **Brouwer, A. C., and J. F. Kirsch.** 1982. Investigation of diffusion-limited rates of chymotrypsin reactions by viscosity variation. Biochemistry **21:**1302–1307.
7. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
8. **Bush, K., and S. Mobashery.** 1998. How β-lactamases have driven pharmaceutical discovery: from mechanistic knowledge to classical circumvention, p. 71–98. *In* B. P. Rosen and S. Mobashery (ed.), Resolving the antibiotic paradox: progress in understanding drug resistance and development of new antibiotics. Plenum Press, New York, N.Y.
9. **Cartwright, S. J., and S. G. Waley.** 1984. Purification of β-lactamases by affinity chromatography on phenylboronic acid-agarose. Biochem. J. **221:**505–512.
10. **Christensen, H., M. T. Martin, and S. G. Waley.** 1990. Beta-lactamases as fully efficient enzymes. Determination of all the rate constants in the acyl-enzyme mechanism. Biochem. J. **266:**853–861.
11. **Damjanovich, S., J. Bot, B. Somogyi, and J. Sumegi.** 1972. Effect of glycerol on some kinetic parameters of phosphorylase *b*. Biochim. Biophys. Acta **284:**345–348.
12. **Dryjanski, M., and R. F. Pratt.** 1995. Steady-state kinetics of the binding of β-lactams and penicilloates to the second binding site of the *Enterobacter cloacae* P99 β-lactamase. Biochemistry **34:**3561–3568.
13. **Dubus, A., P. Ledent, J. Lamotte-Brasseur, and J.-M. Frère.** 1996. The roles of residues Tyr150, Glu272, and His314 in class C beta-lactamases. Proteins **25:**473–485.
14. **Dunford, B. H., and W. D. Hewson.** 1977. Effect of mixed solvents on the formation of horseradish peroxidase compound I. The importance of diffusion-controlled reactions. Biochemistry **16:**2949–2957.
15. **Govardhan, C. P., and R. F. Pratt.** 1987. Kinetics and mechanism of the serine β-lactamase catalyzed hydrolysis of depsipeptides. Biochemistry **26:**3385–3395.
16. **Hardy, L. W., and J. F. Kirsch.** 1984. Diffusion-limited component of reactions catalyzed by *Bacillus cereus* β-lactamase I. Biochemistry **23:**1275–1282.
17. **Hasinoff, B. B.** 1984. Kinetics of carbonic anhydrase catalysis in solvents of increased viscosity: a partially diffusion-controlled reaction. Arch. Biochem. Biophys. **233:**676–681.
18. **Hasinoff, B. B., and S. B. Chisthi.** 1982. Viscosity dependence of the kinetics of the diffusion-controlled reaction of carbon monoxide and myoglobin. Biochemistry **21:**4275–4278.
19. **Jacobs, C., J.-M. Frère, and S. Normark.** 1997. Cytosolic intermediates for cell wall biosynthesis and degradation control inducible β-lactam resistance in gram-negative bacteria. Cell **88:**823–832.
20. **Knowles, J. R., and W. J. Albery.** 1977. Perfection in enzyme catalysis: the energetics of triosephosphate isomerase. Acc. Chem. Res. **10:**105–111.
21. **Kramers, H. A.** 1940. Brownian motion in a field of force and the diffusion model of chemical reactions. Physica (Amsterdam) **7:**284–304.
22. **Kurz, L. C., E. Weitkamp, and C. Frieden.** 1987. Adenosine deaminase: viscosity studies and the mechanism of binding of substrate and of ground- and transition-state analogue inhibitors. Biochemistry **26:**3027–3032.
23. **Massova, I., and S. Mobashery.** 1998. Kinship and diversification of bacterial penicillin-binding proteins and β-lactamases. Antimicrob. Agents Chemother. **42:**1–17.
24. **Matagné, A., A. Dubus, M. Galleni, and J.-M. Frère.** 1999. The beta-lactamase cycle: a tale of selective pressure and bacterial ingenuity. Nat. Prod. Rep. **16:**1–19.
25. **McKay, G. A., and G. D. Wright.** 1996. Catalytic mechanism of enterococcal kanamycin kinase (APH(3′)-IIIa): viscosity, thio, and solvent isotope effects support a Theorell-Chance mechanism. Biochemistry **35:**8680–8685.
26. **Medeiros, A. A.** 1997. Evolution and dissemination of β-lactamases accelerated by generation of β-lactam antibiotics. Clin. Infect. Dis. **24**(Suppl. 1)**:**S19–S45.
27. **Monsan, P., and D. Combes.** 1984. Effect of water activity on enzyme action and stability. Ann. N. Y. Acad. Sci. **434:**48–60.
28. **Muhr, A. H., and J. M. V. Blanshard.** 1982. Diffusion in gels. Polymer **23:**1012–1026.
29. **Normark, S.** 1995. β-Lactamase induction in gram-negative bacteria is intimately linked to peptidoglycan recycling. Microb. Drug Res. **1:**111–114.
30. **Olson, O., S. Bergstrom, and S. Normark.** 1982. Identification of a novel ampC β-lactamase promoter in a clinical isolate of *Escherichia coli*. EMBO J. **1:**1411–1416.
31. **Pocker, Y., and N. Yanjic.** 1987. Enzyme kinetics in solvents of increased viscosity. Dynamic aspects of carbonic anhydrase catalysis. Biochemistry **26:**2597–2606.
32. **Pollock, M. R.** 1967. Origin and function of penicillinase: a problem in biochemical evolution. Br. Med. J. **4:**71–77.
33. **Samson, R., and J. M. Deutch.** 1978. Diffusion-controlled reaction rate to a buried active site. J. Chem. Phys. **68:**285–290.
34. **Saz, A. K.** 1970. An introspective view of penicillinase. J. Cell. Physiol. **76:**397–404.
35. **Schurr, J. H., and J. Schmitz.** 1976. Orientation constrains and rotational diffusion in bimolecular solution kinetics. Simplification. J. Phys. Chem. **80:**1934–1936.
36. **Solc, K., and W. H. Stockmayer.** 1973. Kinetics of diffusion-controlled reaction between chemically asymmetric molecules. II. Approximate steady-state solution. Int. J. Chem. Kinet. **5:**733–752.
37. **Weast, R. C. (ed.).** 1981. Handbook of chemistry and physics, 61st ed., p. D-270, CRC Press, Inc., Boca Raton, Fla.
38. **Wise, E. M., and J. T. Park.** 1965. Penicillin: its basic site of action as an inhibitor of a peptide cross-linking reaction in cell wall mucopeptide synthesis. Proc. Natl. Acad. Sci. USA **54:**75–81.
39. **Xu, Y., and R. F. Pratt.** 1994. β-Lactam-recognizing enzymes exhibit different structural specificity in acyclic amide and ester substrates: a starting point in β-lactamase evolution? Bioorg. Med. Chem. Lett. **4:**2291–2296.
40. **Xu, Y., G. Soto, H. Adachi, M. P. G. van der Linden, W. Keck, and R. F. Pratt.** 1994. Relative specificities of a series of β-lactam-recognizing enzymes towards side-chain of penicillins and of acyclic thioldepsipeptides. Biochem. J. **302:**851–856.