Effect of Divalent Metal Cations on the Dimerization of OXA-10 and -14 Class D β-Lactamases from *Pseudomonas aeruginosa*

Franck Danel,*,‡ Mark Paetzel,§ Natalie C. J. Strynadka,§ and Malcolm G. P. Page‡

Pharmaceuticals Division, F. Hoffmann-La Roche Ltd., CH-4070 Basel, Switzerland, and Department of Biochemistry and Molecular Biology, University of British Columbia, 2146 Health Sciences Mall, Vancouver, British Columbia V6T 1Z3, Canada

Received November 10, 2000; Revised Manuscript Received May 24, 2001

ABSTRACT: The factors influencing the oligomerization state of OXA-10 and OXA-14 class D β-lactamases in solution have been investigated. Both enzymes were found to exist as an equilibrium mixture of a monomer and dimer, with a K_d close to 40 μM. The dimeric form was stabilized by divalent metal cations. The ability of different metal ions to stabilize the dimer was in the following order: $Cd^{2+} > Cu^{2+} > Zn^{2+} > Co^{2+} > Ni^{2+} > Mn^{2+} > Ca^{2+} > Mg^{2+}$. The apparent K_ds describing the binding of Zn^{2+} and Cd^{2+} cations to the OXA-10 dimer were 7.8 and 5.7 μM, respectively. The metal ions had a profound effect on the thermal stability of the protein complex observed by differential scanning calorimetry. The enzyme showed a sharp transition with a T_m of 58.7 °C in the absence of divalent cations, and an equally sharp transition with a T_m of 78.4 °C in the presence of a saturating concentration of the divalent cation. The thermal transition observed at intermediate concentrations of divalent metal ions was rather broad and lies between these two extremes of temperature. The equilibrium between the monomer and dimer is dependent on pH, and the optimum for the formation of the dimer shifted from pH 6.0 in the absence of divalent cations to pH 7.5 at saturating concentrations. The β-lactamase activity increased approximately 2-fold in the presence of saturating concentrations of zinc and cadmium ions. Reaction with β-lactams caused a shift in the equilibrium toward monomer formation, and thus an apparent inactivation, but the divalent cations protected against this effect.

β-Lactams have been the most heavily used antibiotics for the past 50 years (*1*). Many mechanisms of resistance have been developed by bacteria during this period, including efflux pumps, decreased levels of expression of porins, and modification of the PBPs[1] (penicillin binding proteins, the targets of the β-lactam antibiotics), but the most prevalent mechanism is the expression of β-lactamases (*2*). These enzymes inactivate the antibiotics by hydrolyzing the β-lactam ring, rendering the antibiotic inactive against PBPs. Four molecular classes of β-lactamases exist, A - D (*3* - *5*). The class A, C, and D β-lactamases are serine hydrolyses, while the class B enzymes are metalloenzymes. The number of known β-lactamases has increased rapidly in the past few years with an evolution in the spectrum of activity (penicillinase, extended-spectrum β-lactamase, and carbapenemase) that has kept pace with the introduction of new classes of β-lactams (*6* - *10*). Thus, there has been considerable interest in elucidating the molecular mechanisms of these enzymes to facilitate the design of mechanism-based inhibitors that could be used to rescue the susceptible antibiotics (*11*).

Among the serine β-lactamases, the class A and class C enzymes have been extensively studied, whereas relatively little is known about the class D enzymes (*11*, *12*). The first structure of a class D enzyme, OXA-10 (PSE-2) β-lactamase, has only recently been determined (*13*, *14*). Unlike the known enzymes from classes A and C, OXA-10 β-lactamase was found to be a dimer, both in the crystal structure and in solution (*14*). The monomer subunit of OXA-10 is organized like the other classes of serine β-lactamases comprising two domains lying on either side of a β-sheet that forms the core of the molecule. The OXA-10 enzyme differs significantly from either class A or class C enzymes in the ω loop region, close to the active site. In OXA-10, this loop runs in the opposite direction, and is more compact and shorter than in the class A enzymes. Also, this loop does not contain any acidic residue, equivalent to glutamate 166 of the class A enzymes, that would act as a general base to activate a water molecule for attack on the acyl - enzyme intermediate. The primary sequence showed that two elements (S-X-X-K and K/R-T/S-G) of the three identified in the active site in the class D enzyme were also a common motif among all other serine β-lactamases. The third element (S-X-V) lacks the polar asparagine residue that is highly conserved in class A (*15*) and essential for the activity of class C β-lactamases (*16*). The differences between the class D enzyme and the

* To whom correspondence should be addressed. Phone: +41-61-6880537. Fax: +

enzymes from other classes indicate the different origin of this class and suggest that this class operates a new mechanism for hydrolysis of the β-lactam ring (*14*). Kinetic analysis, site-directed mutagenesis, and further crystallographic analysis of complexes with inhibitors or substrate analogues are needed to be able to propose a mechanism.

OXA-10 β-lactamase is closely related to several ESBL variants that only differ by one or two amino acids, e.g., OXA-11 (Asn143Ser and Gly157Asp), OXA-14 (Gly157Asp), OXA-16 (Ala124Thr and Gly157Asp), and OXA-17 (Asn75Ser) (*17* - *21*). A single amino acid change is sufficient to give significant differences in properties. For example, the time course of hydrolysis of some β-lactams by OXA-10 β-lactamase follows a typical progress curve with many β-lactams, but some show more complex curves starting with a hyperstoichiometric burst of hydrolysis that decays to a slower steady-state rate. In contrast, the OXA-14 enzyme has exhibited such complex kinetics with every β-lactam tested so far (*18*, *23*). It has been suggested that the burst kinetics could be due to interconversion between monomer and dimer forms that have different specific activities, possibly stimulated by reaction with β-lactams (*24*). In this context, the observation that two cobalt ions were located at the interface between the two monomers in the crystal structure (*14*) suggested that divalent cations might also play a role in the kinetics of these enzymes. The cobalt ions are bound by the side chains of one glutamate and one histidine from one of the subunits and by the side chain of one glutamate from the other molecule. Two water molecules are also involved in the binding in such a way that the cation has 6-fold coordination in octahedral geometry (Figure 1). The involvement of ionizable residues in the binding of the cations that appear to be important for dimer formation clearly suggests that the pH will also be an important factor in determining the overall activity of the enzyme.

In this study, we have investigated the factors affecting the interconversion between the monomer and dimer forms of OXA-10 and OXA-14. This is important for the proper interpretation of the kinetic studies of these enzymes because the kinetic properties depend strongly on the state of the enzyme. We show that the complex kinetics observed with both enzymes are entirely consistent with the monomer - dimer model and that Zn^{2+} is the ion most likely to promote the association under physiological conditions.

EXPERIMENTAL PROCEDURES

Materials. OXA-10 and -14 β-lactamases were purified by anion and cation exchange as previously described (*18*, *20*, *21*). Antimicrobials that were tested included ceftazidime (Glaxo-Wellcome, Stevenage, Hertfordshire, U.K.), ceftriaxone (Roche, Basel, Switzerland), ampicillin sodium, carbenicillin disodium, cephaloridine, cephalothin, cloxacillin, methicillin, oxacillin, and penicillin G (Sigma, St. Louis, MO), aztreonam (Bristol-Myers Squibb AG, Baar, Switzerland), and Meropenem (Zeneca, Basiglio, Italy).

Analytical Centrifugation and Size-Exclusion Chromatography. Sedimentation equilibrium experiments were performed in 0.1 M phosphate buffer (pH 7.0) using a Beckman Optima XL-A analytical ultracentrifuge with radial scanning at 280 nm. The experimental conditions and data interpretation were as previously described (*14*).

Size-exclusion chromatography was performed using a Superdex 200 PC 3.2/30 or Superdex 75 PC 3.2/30 gel filtration column (Pharmacia, Dubendorf, Switzerland) connected to a HPLC system (Jasco-Omnilab, Mettmenstetten, Switzerland) at room temperature. The column was equilibrated by running at least 3 times the column volume of buffer through the system prior to loading. Five microliters of the protein solution was injected onto the column, and the sample was eluted at a flow rate of 0.1 mL/min. The protein elution was monitored by fluorescence at 330 nm after excitation at 280 nm (Jasco FP920 intelligent fluorescence detector) and by absorption at 280 nm (MD 1510, Jasco). The size-exclusion chromatography columns were calibrated using molar mass standards (Bio-Rad, Hercules, CA). The fluorescence and the absorption were calibrated against known concentrations of OXA-10 β-lactamase determined by quantitative amino acid analysis. The value of the maximum absorption or fluorescence of the peak was used for the determination of enzyme concentration and retention volume (*25*).

We assume that the change in the molar mass with enzyme concentration is due to a monomer - dimer equilibrium:

The observed molar mass (M_{OBS}) corresponds to the molar mass intermediate between the molar mass of the monomer (M_M) and that of the dimer (M_D) depending of the ratio of the concentration of the enzyme in the dimeric (E_D) and monomeric (E_M) form to the total concentration of the enzyme ($E_{TOTfr306:}$.

By incorporation of E_D from eq 2 into K_d and rearrangement of this equation as a quadratic below:

This equation possesses only one possible solution ($E_M \geq 0$):

By incorporation of eq 4 into eq 3

eq 5 was used for fitting the results with M_M, M_D, and K_d as variables using the Grafit program (*26*). We assumed that M_{OBS} corresponds to the apparent molar mass (M_{APP}) determined by the calibrated gel filtration column. We also assumed that the M_{APP} of the dimer deduced from eq 5 should not be obviously twice the one of the monomer, so both molar masses were considered as separate variables. The percentage of dimer was calculated as follows from eq 6:

Calorimetry. The enzyme for differential scanning calorimetry (DSC) was dialyzed against the appropriate buffer and filtered through a 0.2 μm pore filter. All sample and reference solutions were degassed for 15 min under vacuum prior to loading. The thermal unfolding was performed using a VP-DSC microcalorimeter (MicroCal, Inc., Northampton, MA). The scanning rate was 1 °C/min from 18 to 100 °C with equilibration for 20 min at 18 °C. The unfolding parameters were characterized using the program Origin supplied by MicroCal. T_m was defined as the temperature with the maximum heat capacity change.

Isothermal titration calorimetry (ITC) was performed with a MCS titration calorimeter (MicroCal) at 25 °C. The enzyme was first dialyzed overnight, against 500 times its volume of 0.1 M Tris-H_2SO_4 (pH 7.0) and 0.1 M K_2SO_4 with 2 mM EDTA. The enzyme was then dialyzed extensively against 0.1 M Tris-H_2SO_4 (pH 7.0) and 0.1 M K_2SO_4 (four changes against 500 times the enzyme volume) to eliminate the EDTA. The sample was filtered (0.2 μm pore filter) and degassed for 15 min prior to starting the experiment. During the titration experiment, the enzyme was stirred at 400 rpm in a 1.4 mL cell at 25 °C, and an injection series (1 ↜2 μ

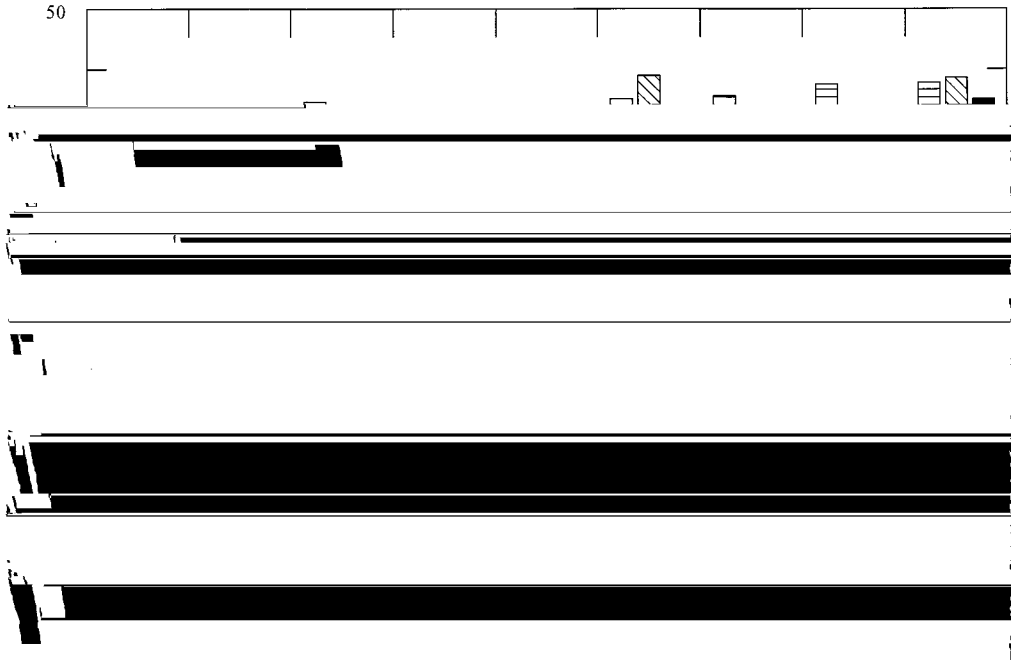

FIGURE 3: Effect of three concentrations of metal on the dimerization of 6 nM OXA-14 β-lactamase: (striped) 10, (cross-hatched) 0.5, and (black) 0.05 mM.

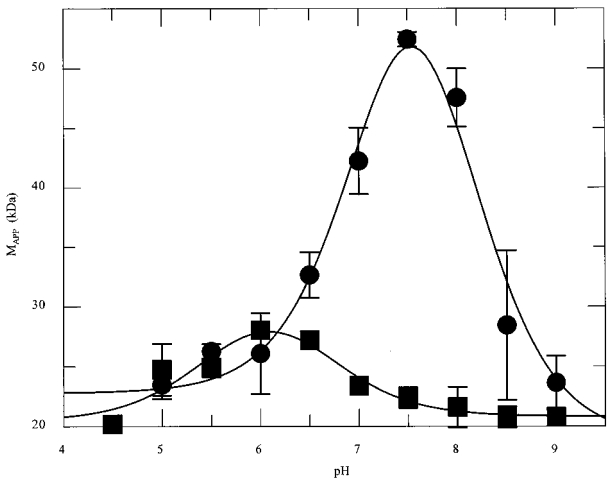

FIGURE 4: Effect of pH on the apparent molar mass of 6 nM OXA-10 β-lactamase with and without 0.1 mM $ZnCl_2$: (■) without Zn^{2+} and (●) with 0.1 mM $ZnCl_2$.

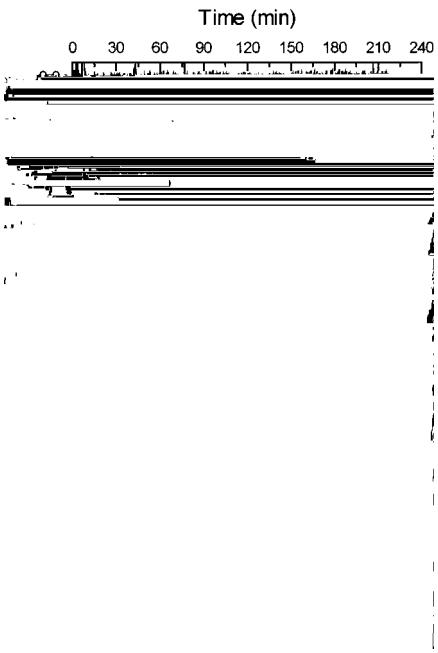

FIGURE 5: Thermogram from an ITC experiment for the binding of $ZnCl_2$

Effect of the Metal Ions on the Thermal Stability of OXA-10 β-Lactamase. The thermogram of OXA-10 in 0.1 M phosphate buffer (pH 6.5) with 0.1 M K_2SO_4 gave a sharp peak corresponding to the unfolding, with a T_m of 58.8 °C. The same experiments in 0.1 M Tris-H_2SO_4 (pH 7.0) with 0.1 M K_2SO_4 gave a T_m of 57.4 °C. The pH of the last buffer is more sensitive to the temperature than phosphate buffer, decreasing nearly 1 pH unit with the increase of the temperature from 20 to 57 °C (*35*). The difference in stability may thus only reflect the instability of the enzyme at low pH. The addition of 1 mM EDTA to the enzyme did not modify this value, whereas the addition the 0.5 mM $ZnCl_2$ greatly increased the stability (T_m) 71.2 °C). Performing the experiment in 50 mM PIPES (pH 7.8) with 0.1 M K_2-SO_4 gave a T_m of 58.7 °C, and the addition of metal salts also increased the stability (see Table 3). The effect of metals on the thermal stability of the enzyme showed that the enzyme has three different unfolding states depending of the concentration of metal ions. The effect of Zn^{2+} on OXA-10 (Figure 6) represents a typical behavior observed for this enzyme with the different metals. At low concentrations, or without added metal salt, a sharp peak (type A) with a T_m

close to 58.7 °C was obtained. At high concentrations, a sharp peak (type C) was again observed around 78 - 79.5 °C that corresponded to an increase in T_m of close to 20 °C in comparison to peak A. At intermediate concentrations, a broad peak was always observed in the temperature range between 60 and 75 °C. This featureless peak (type B) with a T_m at a temperature higher than that observed with the enzyme alone seems to correspond to a transition or equilibrium between the type A and type C conformations.

At pH 6.5, 7.0, or 7.8, the transition peak did not appear during a second scan after the solution was heated to just after the end of the first transition. These results indicate that the unfolding transition was not reversible. NaCl did not show any effect up to 0.1 M; however, an increase in stability is observed for a concentration of 1 M, and this is also observed with a high concentration of K_2SO_4 (Table 3).

Effect of β-Lactams on the Apparent Molar Mass. Ampicillin, carbenicillin, ceftazidime, and Meropenem caused a decrease in the M_{APP} at a high concentration of OXA-10 or -14 β-lactamase (1.2 μM in 0.1 M Tris-H_2SO_4 with 0.3 M K_2SO_4) in the absence of divalent cations in the elution buffer (see Table 4). We interpret this as a shift in the monomer - dimer equilibrium toward the monomer. OXA-14 was generally less affected than OXA-10 by β-lactams. For both enzymes, the carbapenem Meropenem was the most potent in converting the dimeric form of the enzyme to the monomeric form. The M_{APP} of OXA-14 was more affected by ceftazidime than by the penicillins that were tested, whereas OXA-10 was strongly affected by the two penicillins.

The M_{APP} at low concentrations of OXA-14 stabilized in dimeric form by Zn^{2+} (6 nM enzyme in 0.1 M Tris-H_2SO_4 with 0.3 M K_2SO_4 with 0.1 mM $ZnSO_4$) was not significantly affected by any of the β-lactams. With OXA-10, only Meropenem and carbenicillin evolved significant reaction under these conditions (Table 4).

Effect of Divalent Cations on β-Lactamase Activity. Figure 7 shows the effect of some divalent cations on the hydrolysis of nitrocefin by OXA-10 and -14. Cd^{2+}, Zn^{2+}, and Ni^{2+} have the most potent effect and increase the activity 2-fold. These

(Figure 1). Cu^{2+} can use this coordination but in a distorted structure, which might explain the problems with enzyme stability observed in the presence of this ion. The metals can be classified as forming hard or soft acid cations in terms of Lewis-base theory (*37*). Hard acid metal cations tend to be small, with high positive charge, and do not easily possess unshared valence electrons. Soft acid metal cations tend to be large, with low positive charge, and readily polarizable with unshared electrons that are easily removed or are in orbitals that are readily distorted. The hard acid metal cations are usually held by electrostatic forces with oxygen as the preferred ligand. Soft acid metal cations bind by forming partially covalent bonds (e.g., π bonds) with nitrogen or sulfur, forming a strong complex. Ca^{2+}, Mg^{2+}, and Mn^{2+} are typical hard acid cations; Ni^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+} are borderline, and Cd^{2+} is a typical soft acid metal cation. Clearly, OXA-10 has a preference for soft, or borderline, acid metal cations in comparison to the hard acid cations.

The K_d observed for the dimeric form of the OXA-10

2-fold. A very good correlation was observed between the ability of the cations to stabilize the dimer at low concentrations and the ones that were able to keep the high activity. The higher activity for OXA-10 and OXA-14 β-lactamase can be explained by the fact that the dimer was more active against nitrocefin than the monomer. OXA-10 has previously been reported to give burst kinetics for some β-lactams (including ampicillin, cephaloridine, carbenicillin, oxacillin, and cloxacillin), while for OXA-14, all 17 β-lactams that were tested gave biphasic curves (*18*, *22*). The burst kinetics have been attributed to interconversion between a more active dimer and a less active monomer. We are able to confirm that the (metal-complexed) dimer is the more active species. Further, we have shown that reaction with β-lactams does indeed have a strong effect on the monomer - dimer equilibrium (Table 4), in particular, shifting the position toward the less active monomer. It remains to be seen whether dimer - monomer interconversion is an integral part of the catalytic mechanism or only a consequence of conformational changes accompanying some of the reaction steps.

OXA-10 β-lactamase is found predominately in *P. aeruginosa* (*34*, *38*), only once appearing in Enterobacteriaceae (*39*). *P. aeruginosa* is found in hospitals but also in drain and sewage water where the concentration of metals can be high. In humans, *P. aeruginosa* can cause serious infections in burns, wounds, eyes, skin, and the respiratory and urinary tracts. The septicemia occur through the overspill from other loci or after surgery, and the mortality rate is high. With its periplasmic location, the β-lactamase will be situated at a metal ion concentration similar that of its environment. In this context, the concentration of metals in the plasma can be taken as an example, but the concentration of metals in the human body can vary with the environment and in some cases with age. The concentrations of divalent metal ions such as Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, and Ni^{2+} in plasma are 1000, 3000, 20, 15, 2, 0.8, 0.02, and <0.005 μM, respectively (*40* - *42*). The only metals that have the affinity in the correct concentration range to bind to OXA-10 are Zn^{2+}, Cu^{2+}, and Ca^{2+}, and of these, Zn^{2+} seems to be the most likely to be involved. The concentration of OXA-10 β-lactamase in *P. aeruginosa* PU21 (pMLH51) periplasmic space was estimated to be 2 - 8 μM. Plasmid pMLH51 was not an overproducer, but it was found in a clinical isolate and transferred to *P. aeruginosa* PU21 by transconjugation (*17*, *39*). Under this condition, the concentration of enzyme determined here very likely corresponds to a concentration of β-lactamase found in a clinical isolate. This concentration was very close to the value previously estimated for OXA-14 β-lactamase (8 μM) in *P. aeruginosa* (*24*), but much lower than the concentration of 0.4 - 0.9 mM reported in *Serratia marcescens* for another class of β-lactamases (*43*). At the estimated concentration, the enzyme is already mainly in the dimeric form (see Figure 2) and the metal should have only a small effect on the dimerization, but it may protect the dimer from conversion to the monomer in the presence of β-lactams.

In conclusion, OXA-10 and OXA-14 are dimers at high concentrations (such as in stock solutions) but are monomers at the concentration at which assays are usually performed. The burst kinetics, which can now be clearly attributed to a shift in the monomer - dimer equilibrium after dilution of the enzyme into the reaction mixture, are something of an artifact of the experimental design. The kinetics of β-lactam hydrolysis are complicated, and a detailed study of the kinetic properties of these enzymes taking into account all the various species (monomer, monomer - metal, dimer, and dimer -

15. Matagne, A., and Frere, J. M. (1995) *Biochim. Biophys. Acta 1246*, 109 - 127.
16. Dubus, A., Normark, S., Kania, M., and Page, M. G. (1995) *Biochemistry 34*, 7757 - 7764.
17. Hall, L. M., Livermore, D. M., Gur, D., Akova, M., and Akalin, H. E. (1993) *Antimicrob. Agents Chemother. 37*, 1637 - 1644.
18. Danel, F., Hall, L. M., Gur, D., and Livermore, D. M. (1995) *Antimicrob. Agents Chemother. 39*, 1881 - 1884.
19. Danel, F., Hall, L. M., Gur, D., and Livermore, D. M. (1997) *Antimicrob. Agents Chemother. 41*, 785 - 790.
20. Danel, F., Hall, L. M., Gur, D., and Livermore, D. M. (1998) *Antimicrob. Agents Chemother. 42*, 3117 - 3122.
21. Danel, F., Hall, L. M., Duke, B., Gur, D., and Livermore, D. M. (1999) *Antimicrob. Agents Chemother. 43*, 1362 - 1366.
22. Ledent, P., and Frère, J. M. (1993) *Biochem. J. 295*