Novel Mechanism of Hydrolysis of Therapeutic β-Lactams by *Stenotrophomonas maltophilia* L1 Metallo-β-lactamase*

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Stopped-flow tryptophan fluorescence under single turnover and pseudo-first-order conditions has been used to investigate the kinetic mechanism of β-lactam hydrolysis by the *Stenotrophomonas maltophilia* L1 metallo-β-lactamase. For the cephalosporin substrates nitrocefin and cefaclor and the carbapenem meropenem, a substantial quench of fluorescence is observed on association of substrate with enzyme. We have assigned this to a rearrangement event subsequent to formation of an initial collision complex. For the colorimetric compound nitrocefin, decay of this dark intermediate represents the overall rate-determining step for the reaction and is equivalent to decay of a previously observed state in which the β-lactam amide bond has already been cleaved. For both cefaclor and meropenem, the rate-determining step for hydrolysis is loss of a second, less quenched state, in which, however, the β-lactam amide bond remains intact. We suggest, therefore, that the mechanism of hydrolysis of nitrocefin by binuclear metallo-β-lactamases may be atypical and that cleavage of the β-lactam amide bond is the rate-determining step for breakdown of the majority of β-lactam substrates by the L1 enzyme.

Zinc-dependent or metallo-β-lactamases (1) are bacterial enzymes of considerable clinical and mechanistic interest. In the clinical context, their ability to hydrolyze the newer generation of carbapenem antibiotics (2), together with the potential for interspecies transfer arising from their presence on mobile genetic elements (3, 4), is a cause for increasing concern as carbapenem use becomes more widespread. The group includes both nonspecific enzymes (able to efficiently hydrolyze penicillin and cephalosporin antibiotics in addition to carbapenems) and specific enzymes (5) and may utilize one or two zinc ions for maximal activity (6–8). Although mechanistic analogies with zinc peptidases have been attempted (7), it is now clear that metallo-β-lactamases are members of a distinct superfamily of hydrolases that includes bacterial arylsulfatases and type II glyoxalases (8, 9). Despite increasing attention in recent years (10–12), understanding of metallo-β-lactamase mechanism remains incomplete, and there are to date no clinically effective inhibitors.

There is general agreement that metallo-β-lactamases hydrolyze substrate by a nucleophilic attack of zinc-bound water upon the carbonyl carbon of the scissile bond (Ref. 13 and references therein). This would be expected to lead to formation of a tetrahedral oxyanion intermediate that would decay to product via cleavage of the β-lactam amide bond and protonation of the amide nitrogen. Several models of such a reaction scheme have been postulated (7, 10, 14, 15); these vary primarily in the source of the proton required for product formation. Experimental evidence points to different conclusions for different enzymes: in the BCII enzyme from *Bacillus cereus*, a conserved aspartate in the active site appears to shuttle a proton from the oxyanion to the amide nitrogen, and the major intermediate is a dianionic species (10), whereas in CcrA from *Bacteroides fragilis*, the major intermediate in hydrolysis of the colorimetric β-lactam nitrocefin is the deprotonated product, and the rate-determining step is donation of a proton by a second zinc-bound water (11). This apparent mechanistic heterogeneity between different metallo-β-lactamases is also manifest in the results of attempts at inhibitor design (16–19), in which many compounds show a wide variation in potency against the different enzymes, highlighting the existence of subtle variations in active site structure within the family and thus need for detailed study of the individual proteins.*

*Stenotrophomonas maltophilia* (formerly *Pseudomonas maltophilia*) is an organism of increasing clinical significance through its action as an opportunistic nosocomial pathogen of immunocompromised individuals, such as transplant, cystic fibrosis, and cancer patients (20). Although associated primarily with respiratory infections, it has been identified in a wide variety of bacteremias and can be a significant cause of mortality. It is intrinsically resistant to a wide range of antibiotics and in clinical strains may possess additional high level multidrug resistance (21). Resistance of this organism to a wide range of β-lactam antibiotics is due primarily to expression of a pair of chromosomal β-lactamases, a class A active-site serine enzyme L2 (22), and a metallo-β-lactamase L1 (23). The L1 enzyme is the prototypical member of a distinct subclass (B3) of metallo-β-lactamases that diverge markedly in sequence from the better characterized enzymes, such as BCII and CcrA (24, 25). The structure of L1 (15) confirms that it shares the αββα sandwich fold characteristic of the superfamily and has a binuclear zinc site, but the structure also reveals an active site geometry that is considerably at variance with those of the BCII, CcrA, and *Pseudomonas aeruginosa* IMP-1 enzymes. L1 is unique among known metallo-β-lactamases in that it exists in solution as a compact tetramer whereas all the other enzymes appear to be monomeric. L1 hydrolyzes a wide range of penicillin, cephalosporin, and carbapenem substrates.

We have previously proposed (15) that L1 hydrolyzes substrate through a tetrahedral oxyanion intermediate; however, pre-steady-state kinetic experiments using nitrocefin as substrate (12) show that the major intermediate has the same characteristics as that observed for the CcrA enzyme (11). Nitrocefin (Fig. 1) differs from the majority of β-lactams in that it bears a dinitrostyryl substituent on the cephalosporin nucleus that may serve to selectively stabilize this state (26). Moreover, there are almost no reported pre-steady-state data...
for hydrolysis of therapeutic β-lactams by any metallo-β-lactamase (27, 28). Accordingly, as part of an ongoing investigation into the structure, mechanism, and regulation of the S. maltophilia L1 enzyme, we have sought to examine the hydrolysis by L1 of a range of β-lactam substrates using pre-steady-state kinetics in conjunction with UV absorbance and tryptophan fluorescence spectroscopy. Our results confirm the presence of the previously reported intermediate in nitrocefin hydrolysis, confirm that the rate-determining step in this process occurs after C–N bond cleavage, and show that tryptophan fluorescence of L1 is a suitable probe for investigating conformational changes of the enzyme during turnover of substrates. However, with the alternative substrates cefaclor (cephalosporin) and meropenem (carbapenem), we observe that the rate-determining step is coincident with loss of substrate UV absorbance. We therefore propose that this corresponds to opening of the C–N bond and thus that the major populated intermediate is not equivalent to that observed in the nitrocefin case. Breakdown of these substrates also involves additional processes prior to the rate-determining step that may be resolved by stopped-flow fluorescence experiments under pseudo-first-order conditions. We present here a kinetic mechanism for hydrolysis of these β-lactams by the L1 enzyme. This suggests that the mechanism by which L1 hydrolyzes nitrocefin may be atypical and that for the majority of β-lactam substrates, populated intermediates possess an intact β-lactam amide bond the cleavage of which corresponds with the rate-determining step for the reaction.

**EXPERIMENTAL PROCEDURES**

S. maltophilia L1 was expressed and purified as previously described (15, 29). Nitrocefin was purchased from Becton Dickinson. Cefaclor was purchased from Sigma. Meropenem was purchased from AstraZeneca. Substrate concentrations were determined from measured absorbance changes on complete hydrolysis using extinction coefficients and wavelength lengths given by Crowder et al. (29). All other reagents were obtained from Sigma or BDH. Steady-state experiments were performed on a PerkinElmer Life Sciences Lambda 2 UV-VIS spectrophotometer equipped with a thermostatted cell holder and circulating water bath to maintain temperature at 10 °C. Stopped-flow experiments were performed on an Applied Photophysics SX18MV apparatus equipped with a constant temperature circulating water bath. The path length of the observation cell was 0.2 cm, and both excitation and emission slits were maintained at 4 nm. Fluorescence data were collected using an excitation wavelength of 295 nm and a WG320 nm cut-on filter on the emission photomultiplier. The photomultiplier input was adjusted for each protein concentration to maintain a total signal change of 1V between protein in the absence of substrate and dark current readings. Data were recorded as photomultiplier output in volts. For absorbance experiments, data were recorded as absorbance units, with photomultiplier input first adjusted to give a reading of 0 for buffer only at each wavelength used. All experiments were performed in 50 mM sodium cacodylate buffer, pH 7.0, containing 100 μM zinc chloride. Individual transients were averaged and fitted using the nonlinear least-squares algorithms contained in the Applied Photophysics software or in the program Grafit (30). Single turnover progress curves were fitted using the numerical integration algorithm implemented in the FACSIMILE package (31). Residue numbers referred to under “Discussion” are given according to the proposed standard numbering scheme for metallo-β-lactamases (32).

**RESULTS**

We initially investigated the utility of tryptophan fluorescence as a probe for events during hydrolysis of the synthetic cephalosporin nitrocefin by the L1 enzyme. Nitrocefin (26) has been extensively used in mechanistic investigations of metallo-β-lactamase action (11, 12, 27), as both the loss of substrate and appearance of product are accompanied by substantial changes in absorbance ($\lambda_{\text{max}}$ ≈ 390 and 482 nm, respectively). It has further been shown that nitrocefin hydrolysis by L1 proceeds through a transiently populated intermediate with a strong absorbance at 665 nm, the breakdown of which coincides with appearance of product (12). Accordingly, single turnover experiments were performed in which 25 μM L1 was mixed with 25 μM nitrocefin at 25 °C, and the reaction was monitored using tryptophan fluorescence and absorbance at all three wavelengths. The results are shown in Fig. 2. Tryptophan fluorescence reports a biphasic transient in which a rapid and substantial quench of fluorescence is followed by a regain to baseline levels. The overall time course may be well described by fitting to two exponentials the rates of which (Table I) are broadly consistent with those reported both by monitoring absorbance at 390 nm (substrate) and at 665 nm (intermediate) when these traces are fitted to a biexponential decay. (The low amplitude regain of absorbance at 390 nm we ascribe to formation of product from intermediate. Such a signal would arise from the slight overlap of product with substrate spectra compared with the negligible overlap of substrate and intermediate.) The rate of regain of fluorescence (Table I, $k_2$) is further consistent with the rate of appearance of product (obtained by fitting a single exponential to the progress curve at 482 nm) and may therefore be confidently assigned to the rate-determining step of the reaction. We consider the possibility that a substantial proportion of the fluorescence signal arises from inner filter effects due to the absorbance of nitrocefin at 295 nm unlikely, as the change is from high to low to high fluorescence. Absorbance events recorded at 295 nm are equivalent to those at 390 nm (large loss followed by small regain; data not shown) and would in this case be expected to result in an increase in
intensity of exciting light and of fluorescence as the first event. We therefore conclude that tryptophan fluorescence is a valid method for study of nitrocefin hydrolysis by L1 and that it reports the same events (formation and decay of intermediate) as the absorbance signals at 390 and 665 nm.

Subsequently, we used fluorescence to investigate further the early events in nitrocefin hydrolysis under pseudo-first-order conditions. 0.5 μM L1 was mixed with nitrocefin concentrations between 10 and 100 μM, and the rate of approach to the steady state was recorded. These and subsequent experiments were performed at 10 °C in an attempt to extend the range of substrate concentrations over which these rates could be measured. All of the data were well described by a single exponential process. The results of this experiment are shown in Fig. 3. It is apparent that the concentration dependence of the rate of fluorescence quenching is nonlinear, although the rate of reaction at 100 μM substrate is approaching the limit of time resolution of the stopped-flow apparatus employed and precludes further data collection to accurately determine the rate at which this process ultimately saturates. Data were therefore fitted to a saturation equation of the following form,

\[ k_{obs} = (k_1[S]/[K + [S]]) + k_s \]

(Scheme 1)

where \( k_{obs} \) is the observed rate; \( k_1 \) and \( k_s \) are forward and reverse rate constants, respectively; and \( K \) is the dissociation constant for the preceding step. As this fit yielded a negative value for \( k_1/k_s \), we set it at 0, giving figures for \( k_1 \) of 1790 ± 215 s\(^{-1}\) and for \( K \) of 146 ± 26 μM.

We next obtained progress curves for nitrocefin hydrolysis under single turnover conditions at 10 °C. 20 μM L1 was mixed with 5 μM nitrocefin, and the reaction was monitored over 200 ms. The results are shown in Fig. 4a. The data were fitted to two exponential as above, giving a rate for turnover (regain of fluorescence) of 12.6 s\(^{-1}\). This data set was then reduced and imported into the FACSIMILE package for fitting to the reaction scheme,

\[ E + S \xrightarrow{k_1} ES \xrightarrow{k_2} EI_1 \xrightarrow{k_3} E + P \]

(Scheme 2)

in which the collision rate \( k_1 \) was set to \( 1 \times 10^{8} \) M\(^{-1}\) s\(^{-1}\), and experimentally determined values of \( K \), \( k_r \), and turnover (above) were used to define starting points for \( k_2 \), \( k_3 \), and \( k_s \), respectively. \( k_4 \) was set at 0. The fluorescence yields of the different states were varied such that \( EI_1 \) was assigned as the quenched state and \( E \) and \( ES \) to be of equal fluorescence. The results (Fig. 4a and Table II) demonstrate that a good fit to the data may be obtained from this simple reaction scheme, representing the minimum necessary to accommodate both the observations presented here and those reported by McManus-Munoz and Crowder (12). Varying different combinations of the different rate constants suggested that the dissociation rate constant for the ES complex (\( k_2 \)) is essentially undefined by the single turnover data and confirmed that generation of \( EI_1 \) from ES is irreversible (\( k_3 \rightarrow 0 \)). Thus, \( k_2 \) was fixed at 1.46 × 10\(^4\) s\(^{-1}\) and \( k_s \) at 0 s\(^{-1}\). Some movement in \( k_3 \) is required for optimal fitting; however, this rate is defined by only a relatively small number of points in the single turnover data set, and its definition by the pseudo-first-order data must be considered approximate, as it was impossible to measure this rate under fully saturating conditions. Our results using tryptophan fluorescence therefore confirm the minimal kinetic mechanism for L1-catalyzed nitrocefin hydrolysis reported by McManus-Munoz and Crowder (12) using absorbance measurements. Using our fitted values to calculate \( k_{cat} \) (\( k_{cat} = k_2 k_3/(k_3 + k_4) \) and \( K_m \) (\( K_m = (k_2 k_5 + k_3 k_4)/(k_1 k_3 + k_2 k_4) \)) yields values in good agreement both with the results of steady-state experiments at 10 °C and with previously reported values measured at 25 °C, where rate constants might be expected to be 1.5–2 times greater (see Table V).

The data obtained with nitrocefin thus suggest that tryptophan fluorescence of L1 could be a useful optical probe with which events in β-lactam hydrolysis might be followed. Accordingly, a range of additional substrates was tested under single turnover conditions in order to identify those for which the complete hydrolysis reaction could be followed. All substrates tested (azlocillin, benzylpenicillin, cefaclor, and meropenem) produced qualitatively similar progress curves in which a rapid quench of fluorescence was followed by a rate-determining regain. Unfortunately, however, for both penicillins, the quenching process occurred within the dead time of the stopped-flow even at 10 °C. For meropenem and cefaclor, however, both quenching and regain rates were accessible. Fig. 4b, c, and d, shows the results of single turnover experiments in which 20 μM L1 was mixed with 15 μM substrate at 10 °C, and the reaction was followed using both tryptophan fluorescence and the absorbance signal of the β-lactam amide bond. When the data are fitted (to a single exponential in the case of the absorbance and a double exponential for the fluorescence traces), it is apparent in both cases that the rate of regain of fluorescence (34 and 10 s\(^{-1}\) for meropenem and cefaclor, respectively) is the same as that reported by the loss of absorbance (32 and 9.1 s\(^{-1}\)). Because the major contributor to absorbance of β-lactams that lack aromatic substituents (such as cefaclor and meropenem) is the amide bond (26), we conclude that in these cases, the rate-determining step in hydrolysis is likely to be breakdown of the β-lactam bond.

In order to collect additional information about steps preceding this rate-determining step, the initial quenching of tryptophan fluorescence was examined under pseudo-first-order conditions as described above. At low substrate concentrations, the

| Data set          | \( k_1 \) | \( k_2 \) |
|-------------------|----------|----------|
| Fluorescence      | 101      | 62       |
| Absorbance 390 nm | 110      | 65       |
| Absorbance 665 nm | 122      | 58       |
| Absorbance 482 nm | 142      | 49       |

Fig. 3. Plot of observed rate constant (\( k_{obs} \), s\(^{-1}\)) against nitrocefin concentration (μM) for fluorescence quenching rate (appearance to steady-state) on hydrolysis by 0.5 μM S. maltophilia L1 under pseudo-first-order conditions at 10 °C. Solid line shows the fit to Scheme 1, with offset \( k_s \) set to 0.
data for cefaclor are well described by a single exponential. In the case of meropenem, the absorbance change at 295 nm on hydrolysis of substrate is not negligible, and data were accordingly fitted using a linear phase in addition to exponential terms. Control experiments demonstrated that the change in absorbance at 295 nm is linear and that there are no additional events that might give rise to spurious fluorescence signals (data not shown). However, as substrate concentration was increased, it was apparent in both cases that a single exponential (or single plus steady-state) no longer describes the observed curves and that the approach to the steady-state is in each case a biexponential process in which a rapid loss of fluorescence is followed by a smaller regain. Sample curves are shown in Fig. 5. These data indicate the presence of an additional step in the reaction that was not detected in hydrolysis of nitrocefin (where even at 100 μM substrate progress curves under pseudo-first-order conditions are well described by a single exponential process). Fig. 6 shows the concentration dependence of the observed rate constants for both phases (quenching and regain of fluorescence) for both substrates. It is apparent that all four data sets obey saturation kinetics and may be fitted to Scheme 1 (above). Although the deviation from linearity for the rates of fluorescence quenching is less marked than for the nitrocefin data (Fig. 3), the slopes of linear fits to these data sets are of the order of 10^6 M^{-1} s^{-1}, i.e. 2 orders of magnitude slower than would be expected for a diffusion-limited collision of enzyme and substrate (33). We therefore conclude that for these two substrates, the process reported by fluorescence quenching is also a two-step process in which an initial association event is followed by a slower rearrangement. Fitted parameters are given in Table III. Note that for the second cefaclor rate, Scheme 1 gives a negative value for k_r, which was accordingly set to 0, and the data were refitted without an offset for use in subsequent rounds of FACSIMILE fitting.

These results were then used in the FACSIMILE package in order to fit the complete single turnover reactions (Fig. 4 and Table IV). The minimal scheme required to accommodate all the above observations is shown below.

E + S \rightleftharpoons ES \rightleftharpoons ES^* \rightarrow EI_2 \rightarrow E + P

(Scheme 3)
Formation of a rearrangement complex ES* from an initial collision complex ES is reported by quenching of tryptophan fluorescence (k$_{q}$ and k$_{1}$, Table III), that of a second intermediate EI$_3$ by a small regain of fluorescence (k$_{q}$ and k$_{1}$, Table III), and the rate-determining step (breakdown of EI$_3$) by a return to ground state fluorescence (measured from fits to this phase of the single turnover curve). Hence, the fluorescence yields of free enzyme E andcollision complex ES are held to be equal, and those of ES* and EI$_3$ varied independently. (For the meropenem data set, an additional fluorescence term was initially included to account for inner filter effects arising from the difference in absorbance between substrate and product at 295 nm. However, this was found to be unnecessary for these data sets, in which protein concentration is 40-fold increased compared with that used for the pseudo-first-order data sets.) Experiments in which different combinations of rate constants were varied in the FACSIMILE model suggest that the single turnover data fail to define rate constants for formation and dissociation of the collision complex ES; thus, these were fixed at $1 \times 10^8$ M$^{-1}$ s$^{-1}$ and $7.1 \times 10^4$ s$^{-1}$ (cefaclor) or $2.7 \times 10^4$ s$^{-1}$ (meropenem), respectively. Additionally, the rate constants k$_7$ (for both substrates) and k$_5$ (for cefaclor) were considered to be defined by the rate-limiting regain of fluorescence in the single turnover data and by the fit to the rate of fluorescence increase under pseudo-first-order conditions, respectively. These were all therefore applied as additional constraints on the FACSIMILE fitting process (Table IV). Both single turnover data sets are well described by this model although the meropenem data require some adjustment of k$_5$ (formation of EI$_3$ from ES*) to obtain the best fit. The change in fluorescence associated with this process is small compared with those for the preceding and subsequent steps (Figs. 4 and 5), and the definition of this rate from single turnover experiments at 15 μM substrate must therefore be considered approximate. Hence, we conclude that Scheme 3 represents a minimal kinetic scheme for hydrolysis of cefaclor and meropenem by L1.

The values obtained from the FACSIMILE fit were used to calculate steady-state parameters using Scheme 3. The results are given in Table V and show reasonable agreement with published values (29, 34), especially given the likely poor definition of k$_5$ (above). It should also be noted that steady-state K$_m$ values for hydrolysis by L1 of a range of β-lactams have been demonstrated to be considerably tighter in cacodylate than in phosphate buffer (29).

**DISCUSSION**

Although recent work has led to significant advances (13), understanding of the mechanism of metallo-β-lactamases remains far from complete. In particular, there have been few reports (27, 28) of investigations into the kinetic mechanism of hydrolysis of clinically relevant antibiotics by any metallo-β-lactamase. Accordingly, we set out to investigate the use of the intrinsic tryptophan fluorescence of the S. maltophilia L1 enzyme as a possible optical probe in pre-steady-state kinetic investigation of the mechanism of hydrolysis of these substrates. Whereas L1 hydrolyzes a wide range of β-lactams, (5, 29), we find that the generally weak binding and consequently very rapid approach to the steady state precluded collection of useful data for many compounds even at reduced temperatures. Nevertheless, we were able to collect data that allowed evaluation of the kinetic mechanism for hydrolysis of the cephalosporin cefaclor and the carbapenem meropenem. We consider the establishment of such a mechanism for carbapenem hydrolysis to be particularly important, as the action of metallo-β-lactamases against this newest generation of therapeutic β-lactams is of the most immediate clinical relevance.

Our initial investigations of hydrolysis of the colorimetric β-lactam nitrocefin confirm the usefulness of the tryptophan fluorescence signal for monitoring the hydrolysis reaction. The large changes in fluorescence that accompany the different steps in the hydrolytic reaction permit the use of low enzyme concentrations without loss of signal and consequently experimental determination of individual rate constants. Furthermore, as hydrolysis of nitrocefin by L1 (12), CcrA (11, 35), or BCII (27) has been shown to involve intermediates with ab-

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**Table III**

| Parameter | Cefaclor | Meropenem |
|-----------|----------|-----------|
| k$_f$ (s$^{-1}$) | 1300 ± 160 | 1400 ± 200 |
| k$_F$ (s$^{-1}$) | 41 ± 20 | 55 ± 55 |
| K$_s$ (μM) | 710 ± 180 | 272 ± 112 |
| k$_{2f}$ (s$^{-1}$) | 150 ± 9 | 1110 ± 215 |
| k$_{2f}$ (μM) | 72 ± 19 | 244 ± 136 |

**Table IV**

| Parameter | Cefaclor | Meropenem |
|-----------|----------|-----------|
| k$_1$ (M$^{-1}$ s$^{-1}$) | 1 × 10$^6$ (fixed) | 1 × 10$^6$ (fixed) |
| k$_2$ (s$^{-1}$) | 7.10 × 10$^8$ (fixed) | 2.72 × 10$^8$ (fixed) |
| k$_3$ (s$^{-1}$) | 1.01 × 10$^9$ | 2.27 × 10$^9$ |
| k$_4$ (s$^{-1}$) | 0 | 0 |
| k$_5$ (s$^{-1}$) | 1.50 × 10$^6$ (fixed) | 2.78 × 10$^6$ |
| k$_6$ (s$^{-1}$) | 0 (fixed) | 0 |
| k$_7$ (s$^{-1}$) | 9.94 × 10$^9$ (fixed) | 3.20 × 10$^3$ (fixed) |

**Fig. 6.** Plot of observed rate constant (k$_{ob}$ s$^{-1}$) against cefaclor (A) and meropenem (B) concentration (μM) for fluorescence transients (approach to steady-state) on hydrolysis by 0.5 μM L1 under pseudo-first-order conditions at 10°C. In each case, open circles depict rates of quenching, and filled circles depict rates of regain of fluorescence. Solid lines show fits to the data as described in the text and given in Table III.
sorbedance spectra distinct from those of substrate or product, it is possible to assign changes in fluorescence to particular processes in this reaction pathway. Comparison with previously published work (12) suggests, therefore, that the observed quench in fluorescence corresponds to formation of enzyme-intermediate complex (EI1, Scheme 2) from an ES complex that is optically indistinguishable from free enzyme, and that the return to ground state fluorescence corresponds to the rate-determining step for the reaction. These steps correspond to the absorbance signals for loss of substrate/formation of intermediate and loss of intermediate/formation of product, respectively. A model in FACSIMILE containing only these states well describes fluorescence data obtained from a single turnover of nitrocefin by L1. The resulting rate constants are in reasonable agreement with those obtained by McManus-Munoz and Crowder (12) at 25 °C for $k_1$ and $k_2$, although our pseudo-first-order data suggest $k_2$ to be 2 orders of magnitude greater. Although the rapid quenching rates observed at high concentration precluded more accurate determination of this value, it is apparent from the shape of the curve that the initial binding step is relatively weak.

Our initial investigation of the fluorescence changes accompanying hydrolysis of alternative β-lactam substrates by L1 confirms that the major events are a rapid quench in fluorescence followed by a regain that reports the rate-determining step and the rate of which approximates to the steady-state $k_{cat}$. This appears to be true even for those substrates for which detailed experiments were impractical and suggests that similar structural rearrangements in the vicinity of one or more tryptophan residues are a common feature in the hydrolysis of many β-lactam substrates. Measurement of the rates of this quench over a wide range of substrate concentrations for cefaclor and for meropenem indicate that the dissociation constants $K_1$ for the respective collision complexes (ES, Scheme 3) are of similarly weak magnitude to those measured for nitrocefin (ES, Scheme 2). For all three substrates, the rates of the succeeding rearrangement that generates the quenched states EI1 (Scheme 2) or ES* (Scheme 3) are of similar order ($\sim 10^3$ s$^{-1}$). The respective processes generating these states are, however, fundamentally different: whereas for nitrocefin, the appearance of EI1 corresponds to loss of substrate absorbance, for both meropenem and cefaclor, the rate reported by β-lactam amide absorbance is equivalent to the rate-determining step in the fluorescence experiments. We therefore conclude that ES* is a state in which the β-lactam bond remains intact. Furthermore, for both of these substrates, an additional, low amplitude regain of fluorescence prior to the steady-state becomes evident at high substrate concentrations. This is indicative of an additional intermediate EI2, the major species in the steady state, which retains an intact β-lactam bond and the breakdown of which corresponds to the rate-determining step. These data therefore show that hydrolysis of these two diverse substrates proceeds through a common mechanism, the rate-determining step of which is hydrolysis of the β-lactam amide bond.

The data we present clearly show that when certain substrates are hydrolyzed by L1 the populated enzyme-bound intermediate state is one in which the β-lactam amide bond remains intact. This situation is in marked contrast to previous pre-steady-state studies using nitrocefin as substrate, in which the same intermediate has been identified, from the appearance of an intense UV absorbance at 665 nm, for reaction of both the L1 (12) and B. fragilis CcrA enzymes (11, 35). Wang et al. (11) convincingly show that this spectrum closely resembles that obtained by treating hydrolyzed nitrocefin with strong base under anhydrous conditions and therefore propose the populated intermediate to be deprotonated product and the rate-determining step to be protonation of the β-lactam amide nitrogen. In this model, the deprotonated intermediate may be stabilized by two factors: interaction with one of the two zinc ions in the active site and the presence on the cephalosporin nucleus of the dinitrostyryl substituent, which could accommodate additional electrons by formation of an extended conjugated system. The alternative substrates cefaclor and meropenem, along with the majority of therapeutic β-lactams, lack such a substituent at this position (Fig. 1). Consequently, a deprotonated product-like intermediate might be expected to be considerably less stable. Our data demonstrate that for L1, the kinetic mechanism observed with nitrocefin does indeed appear to be atypical. We suggest that in the majority of cases, for L1-catalyzed β-lactam hydrolysis, C–N bond cleavage, rather than protonation of product, is likely to be the rate-determining step.

These data therefore warrant some speculation regarding the likely nature of populated intermediates on a general reaction scheme for the L1 enzyme. It is clear that for all three substrates, the initial collision complex ES is of relatively low affinity and that hydrolysis requires a subsequent rearrangement of enzyme to a high affinity state, EI1, or ES*. This process is accompanied by a substantial reduction in tryptophan fluorescence and is common to all substrates tested. We therefore consider it likely that this is a relatively large-scale rearrangement of the active site region and does not reflect local interactions with specific tryptophan residues (38 and 53) in the active site that might be expected to show significant variation between the different substrates. Crystallographic and NMR investigations of the interactions of the CcrA and P. aeruginosa IMP-1 enzymes with non-β-lactam inhibitors (17, 36–38) show that in these cases, binding of inhibitor is facilitated by movement of a flexible flap, formed by residues 61–65, that closes over the active site and restricts access of bulk solvent. Deletion of these residues in CcrA severely disrupts the binding of substrate (39). Although these residues are not present in the L1 sequence, the active site of this enzyme is surrounded by extended loops, in particular that between residues 150 and 168, which is absent in the better characterized subclass B1 enzymes. Furthermore the extended N terminus of the L1 enzyme aborts the active site and may interact with substrate both directly, possibly through tyrosine 32, and indirectly through the interaction of tryptophan 38 with the zinc

### Table V

| Substrate   | Expt. $k_{cat}$ | Expt. $K_M$ | Calc. $k_{cat}$ | Calc. $K_M$ | Ref. $k_{cat}$ | Ref. $K_M$ |
|-------------|----------------|------------|----------------|------------|---------------|------------|
| Nitrocefin  | 4.7           | 3.0        | 11             | 2.2        | 41            | 4.0        |
| Cefaclor    | 20            | 8.7        | 28             | 3.7        | 45            | 10         |
| Meropenem   | 77            | 13         | 6.5            | 100        | 5.0           | 100        |

Steady-state parameters for hydrolysis of nitrocefin, cefaclor, and meropenem by S. maltophilia L1

Experimental values (expt.) are measured as described at 10 °C (see under Experimental Procedures). Calculated values (calc.) are derived from FACSIMILE fits as described (see under Results). Published values (Ref.) are taken from Ref. 29 (nitrocefin in 50 mM cacodylate buffer, pH 7.0; 100 μM ZnCl$_2$, cefaclor in 50 mM phosphate buffer, pH 7.0; 100 μM ZnCl$_2$, both at 25 °C) and Ref. 34 (meropenem in 30 mM cacodylate buffer, pH 6.5; 100 μM ZnCl$_2$ at 30 °C).
ligand histidine 118 (15). Recent data 3 indicate that point
mutations and larger deletions within the N-terminal region of
L1 affect both $k_a$ and $k_{cat}$ for a range of $\beta$-lactam substrates.
Accordingly, we suggest that ES* may correspond to a state
in which movement of such elements adjoining the active site enables
optimization of substrate binding. By way of contrast, the small
change in fluorescence when ES is generated from ES* suggests
that this second structural rearrangement may be far more subtle,
possibly corresponding to orientation of active site groups for catal-
ysis and stabilization of higher energy states. The persistence of
substrate amide absorbance in ES* confirms that this is a sub-
strate-like rather than product-like species, and we accordingly
consider it likely that ES* may correspond to a tetrahedral oxyanion.

We have thus demonstrated that for wild-type L1 enzyme, the
major populated intermediate observed on hydrolysis of nitrocefin is
unlikely to correspond to that which will be present during the
hydrolysis of therapeutic $\beta$-lactam antibiotics. We have further
shown that two additional states, ES* and EL*, are observed on
hydrolysis of cefaclor and of meropepen. We cannot, however, rule
out the possibility that similar states are present on the hydrolysis
pathway of nitrocefin but, as a result of the unusual stability of the
deprotonated intermediate, are less well populated. In particular,
the impracticality of measuring quenching rates for nitrocefin at
higher substrate concentrations may preclude observation of the
regain of fluorescence that would be indicative of an ES* state. A
tetrahedral species has been proposed to be important in hydrolysis
of nitrocefin by site-directed mutants of the CcrA enzyme (40),
where alterations at the conserved active-site aspartate 120 appear
to retard formation of the deprotonated intermediate sufficiently to
alter the rate-determining step in nitrocefin hydrolysis to C–N
bond cleavage. The rate-determining step for nitrocefin hydrolysis
has also been assigned as breaking of the amide bond in the case
of site-directed mutants of the CcrA enzyme, in which only one zinc
site is occupied (8). In this case, the deprotonated intermediate
could still be detected, albeit at greatly reduced levels. This further
suggests that C–N bond cleavage and protonation may still be
uncoupled for nitrocefin hydrolysis by CcrA in the presence of one
zinc ion only and supports the idea that the aromatic substituent of
nitrocefin is primarily responsible for stabilizing this state.
Unfortunately, our data do not permit any conclusions to be drawn as
to the existence of a deprotonated intermediate, and thus whether the
processes of amide bond cleavage and protonation are coupled, for
alternative $\beta$-lactams.

The data we present here underline the necessity of determining
the kinetic mechanism of hydrolysis for a number of different $\beta$-
lactams in order to obtain more complete insight into the means by
which metallo-$\beta$-lactamases achieve catalysis. Identification of
common states, such as those we describe here, populated during
reaction with various disparate substrates, will facilitate the design
of experiments, enabling their more detailed characterization and
pinpointing aspects that may be exploited in drug design. Our
results suggest that in the majority of cases populated reaction
intermediates will be tetrahedral at the $\beta$-lactam carbonyl carbon
and that mimics of such states could offer one route toward mechan-
ism-based metallo-$\beta$-lactamase inhibitors.

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Novel Mechanism of Hydrolysis of Therapeutic β-Lactams by Stenotrophomonas maltophilia L1 Metallo-β-lactamase
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