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Kinetic and structural consequences of the leaving group in substrates of a class C β-lactamase

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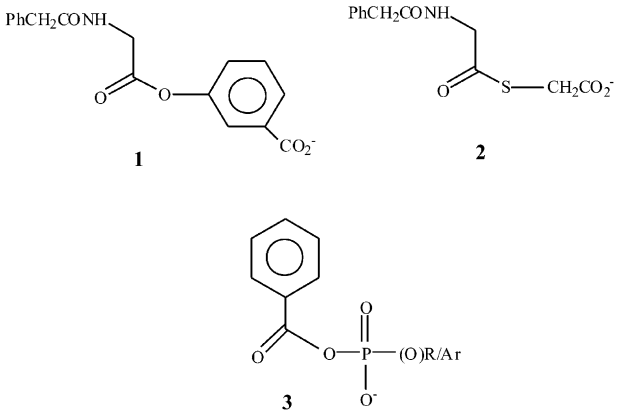
Abstract—The class C β-lactamase of *Enterobacter cloacae* P99 is known to catalyze the hydrolysis of certain acyclic (thio)esters. Previous experiments have employed thioglycolate, *m*-hydroxybenzoate, and phenylphosphate leaving groups. The relative effectiveness of these leaving groups has now been quantitatively assessed by employment of a series of compounds with common acyl groups, and found to rank in the order phenylphosphate > *m*-hydroxybenzoate > thioglycolate. Structural models suggest that these leaving groups interact during acylation principally with Tyr 150, Lys 315, and Thr 316 of the β-lactamase active site. The positions of the leaving group carboxylates in these models is compared with those in published crystal structures of complexes of class C β-lactamases with β-lactams. The particular effectiveness of the acyl phosphate indicates the positions of two oxyanions that strongly interact with the active site. This information should be useful in the design of inhibitors of class C β-lactamases.

1. Introduction

β-Lactamases provide bacteria with resistance to β-lactam antibiotics because of their ability to catalyse hydrolysis of the β-lactam ring of the antibiotic molecules. This catalysis is achieved by means of a double displacement mechanism and an intermediate acyl-enzyme (Scheme 1). In class C β-lactamases, the active site nucleophile is a serine hydroxyl group.[1]

These enzymes also catalyze the hydrolysis of a variety of acyclic substrates by the same mechanism. For example, the depsipeptide **1** and the thiodepsipeptide **2** are substrates of the class C β-lactamase of *Enterobacter cloacae* P99.[2,3] More recently, aroyl phosph(on)ates such as **3**, were identified as substrates of the same enzyme.[4,5] The latter compounds rapidly acylate the enzyme to form stable acyl-enzymes that hydrolyse only slowly; compounds **3** are therefore inhibitory substrates. The ability of **3** to acylate the enzyme was suggested to derive in large part from the properties of the leaving group—a phosph(on)ate dianion;[4,5] these probably include its relatively low basisity and its interaction with the active site of the enzyme.



The placement of leaving group oxyanions [carboxylate or phosph(on)ate oxygen atoms] of the substrates **1**–**3** in the active site of the enzyme could be very different because of their (potentially) different distances from the carbonyl carbon of the scissile ester bond. In this paper we compare the behavior of the three different leaving groups of **1**–**3** on the kinetics of turnover of substrates bearing them and discuss their occupancy of the active site. The distribution of negative charges

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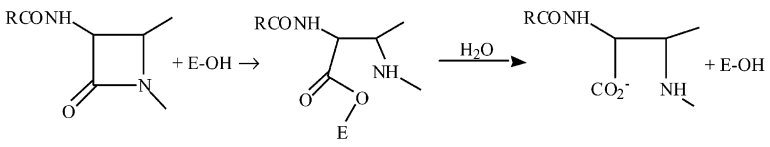
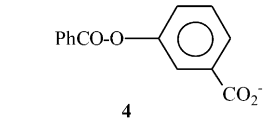


Scheme 1.

on a ligand at the active site of β-lactam-recognizing enzymes is an important issue in inhibitor design. The substrates primarily chosen for comparison in this work are the (thio)depsipeptides **4**–**7**. The 4-biphenyl compounds were chosen since the earlier work referred to above showed that this acyl group interacted more strongly than benzoyl with the P99 β-lactamase active site.[5] Results from compounds **8** and **9**, which were studied previously,[5,6] have been included for comparison.



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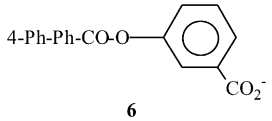

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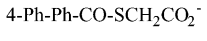

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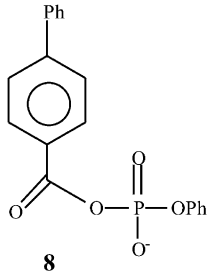

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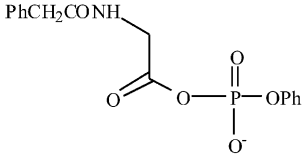

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2. Results and discussion

Disappearance of the esters **4**–**7** in aqueous buffer was catalyzed by the P99 β-lactamase. Absorption spectral changes accompanying these reactions were in accord with the supposition that the reaction observed was the expected ester hydrolysis. This expectation was confirmed in one case, that of **7**, from ^{1}H NMR observations of the reaction carried out in 50 mM $NaHCO_3/^2H_2O$. Under these conditions, the enzyme-catalyzed disappearance of **7** was correlated with the appearance of a singlet resonance at 3.16 ppm; this was found to be identical to that of the anticipated hydrolysis product thioglycolate. The spontaneous hydrolysis of **7** was negligible under the conditions of this experiment. Thus, it is clear that the P99 β-lactamase catalyzes the hydrolysis of the esters **4**–**7**.

Steady state kinetic parameters for the hydrolysis of **4**–**7** by the enzyme are presented in Table 1. These compounds were found to be poor substrates. Values of the steady state parameters for analogous compounds bearing the specific phenylacetylglycyl side chain, **1** and **2**, and for the phosphate analogues **3** (**OPh**), **8** and **9**, are also given in Table 1. These data are discussed below in terms of Scheme 2 which has been solidly established for turnover of acyclic substrates by the P99 β-lactamase.[2,3]

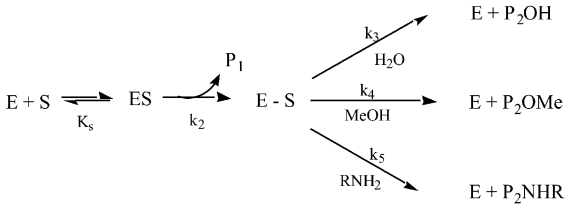
In this scheme, ES represents a noncovalent intermediate, E-S the covalent acyl–enzyme intermediate, P_1, the initially released product, that is, the leaving group from S, and P_2OH, P_2OMe and P_2NHR represent the products of hydrolysis, methanolysis, and aminolysis, respectively, of the acyl enzyme. There is prior evidence for a more complex aminolysis pathway involving ternary ES_2 complexes[3] but Scheme 2 is adequate for the present discussion.

The first significant conclusion from the data of Table 1 is that the m-hydroxybenzoate derivatives are more effective than the thioglycolates. This is seen in the k_{cat}/K_m values of **4** versus **5** and **6** versus **7**. It is also seen with the phenylacetylglycyl side chain in the comparison of **1** with **2**. Compounds with the *m*-hydroxybenzoate leaving group thus acylate the enzyme more rapidly than do those with the thioglycolate leaving group. Since the reactivities of acyl derivatives of *m*-hydroxybenzoic acid and thioglycolic acid with oxygen nucleophiles are comparable (S. A. Adediran and R. F. Pratt, unpublished), this difference in reactivity with the β-lactamase probably reflects more catalytically beneficial interactions of the former leaving group with the enzyme than the latter. Previous experiments showed that the *m*-hydroxybenzoate leaving group was more effective than its ortho and para analogues.[2]

Table 1. Steady state rate constants for (thio)depsipeptide hydrolysis

Substrate	k_{cat} (s^{-1})	K_m (mM)	$k_{cat}/K_m \times 10^3$ ($s^{-1}M^{-1}$)
4	0.0067±0.0003	0.090±0.012	0.074
5	0.0049±0.0014	1.4±0.9	0.0035
3(OPh)[a]	0.017	2.8×10^{-3}	6.07
6	0.017±0.001	0.029±0.006	0.586
7	0.0096±0.012	0.50±0.20	0.0192
8[a]	0.023	0.33×10^{-3}	68.3
1[b]	125	0.23	543
2[b]	54	8	6.71
9[c]	90	0.09	1000

[a] From ref 5.
[b] From ref 20.
[c] From ref 6.



Scheme 2.

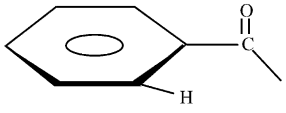
The quantitative difference between the *m*-hydroxybenzoate and thioglycolate leaving groups is not reflected in the k_{cat} parameter which most likely represents the deacylation rate constant k_3 (see references 2 and 3 and the discussion below), but rather in the K_m parameter, which is therefore smaller for the *m*-hydroxybenzoate derivatives **4** and **6** than for the thioglycolates **5** and **7**.

A second conclusion from Table 1 is that the biphenyl side chain (acyl group) of **6** and **7** produces a better (k_{cat}/K_m) substrate than does the phenyl side chain of **4** and **5**, respectively. As above, this result indicates more rapid acylation by the biphenyl carboxyl derivatives although, in this case, deacylation (k_{cat}) is also somewhat faster. A similar effect of the biphenyl side chain is observed in the acyl phosphates **3**(**OPh**) and **8**.[5]

Finally, and most striking, is the difference between the acyl phosphates and the m-hydroxybenzoates viz. **3**(**OPh**) versus **4** and **8** versus **6**. The k_{cat}/K_m values of the former compounds are some 100-fold larger than those of the latter. Although the pK_a of the conjugate acid of the phosphate leaving group is lower than that of the *m*-hydroxybenzoate (and thioglycolate), this factor is generally not dominant in the acylation of the P99 β-lactamase by acyclic substrates.[3,4] Thus, noncovalent interactions between the phosphate leaving group and the enzyme active site must be much more effective than those of the *m*-hydroxybenzoate and thioglycolate leaving group.

Methanolysis and aminolysis studies were performed to assess the access of alternative nucleophiles to an aroyl–enzyme intermediate and to determine the nature of the rate-determining step.[3] These experiments were performed with **6**, the best of the new substrates. The disappearance of **6** (0.3 mM, 10 Km) from solution in the presence of the β-lactamase was accelerated by methanol in a linear fashion (data not shown) and from the slope of this line a partition ratio, k_4/k_3 (Scheme 1), of 23.2 ± 1.4 was obtained.[3] These data suggest that methanol is able to attack the acyl–enzyme derived from **6** and that deacylation of the acyl–enzyme is rate-determining under conditions of substrate saturation. Comparison of this ratio with that for methanolysis of the phenylacetylglycyl-enzyme intermediate, for which a value of 27 was obtained,[3] suggests that methanol has comparable access to the acyl group of the aroyl–enzyme as to that of the phenylacetylglycyl-enzyme.

The aminolysis results were perhaps more interesting. At substrate concentrations well above the K_m value (0.3 mM), D-alanine, at concentrations up to 200 mM, did not affect the rate of turnover of **6**. D-Phenylalanine (to 48 mM) did increase the rate, but only slightly. Numerical analysis of this data[3] yielded an aminolysis partition ratio, k_5/k_3 (Scheme 1) of 39 for D-phenylalanine, barely larger than that of methanol (the absolute value of k_5 was 1.49 $s^{-1}M^{-1}$). In contrast, values of k_5/k_3 and k_5 for **1** with D-phenylalanine were 8×10^4 and 1.8×10^5 $s^{-1}M^{-1}$, respectively.[7] D-Phenylalanine therefore is a much more powerful nucleophile against the acyl–enzyme derived from **1** than against that from **6**. This must reflect either the positive effect of the phenylacetylglycyl side chain on the efficiency of the active site as a catalyst of deacylation, and/or the negative steric and/or electronic effect of the aroyl side chain. Both hydrolysis and aminolysis are therefore adversely affected by the aroyl group but aminolysis is affected more. This may point to a steric component of the effect, as in **10**, where the aroyl group is held in a conformation which could inhibit nucleophilic attack on the carbonyl group by a bulky nucleophile. The slow deacylation of **4**–**8** indicates their potential as leads to inhibitors—the currently known effective β-lactamase inhibitors are inhibitory substrates/suicide substrates.[8]



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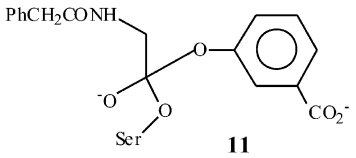
The interactions of the leaving groups described above with the β-lactamase active site were investigated by means of structural models constructed as described in the Experimental. In order to investigate the interactions of the *m*-hydroxybenzoate leaving group with the active site, a model of the tetrahedral intermediate **11** was built. Conformational searches, employing molecular dynamics simulations, produced the two well-populated structures shown in Figure 1. These structures did not interconvert on a 200 psec time scale but interaction energy calculations suggested that structure (b) of the Figure was the more stable by 31 kcal/mol (where the absolute E_{int} value of (b) was −512 kcal/mol).



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The general orientation of the substrate in the two structures is similar. The side chain (phenylacetylglycyl) and oxyanion (O^-) disposition, the latter with respect to the oxyanion hole (Ser 64 NH, Ser 318 NH), seems more solid in (b). The structures differ principally however in the conformation of the leaving group and therefore in the identity of the enzyme functional groups interacting with it. The leaving group carboxylate in (b) interacts directly with the hydroxyl groups of Tyr 150 and Thr 316, and the ammonium ion of Lys 315. In structure (a) however, these interactions are with Arg 349 and Ser 318.

It is an instructive exercise to compare the situation illustrated in Figure 1 with those of other substrates, to the extent that such details are currently available.

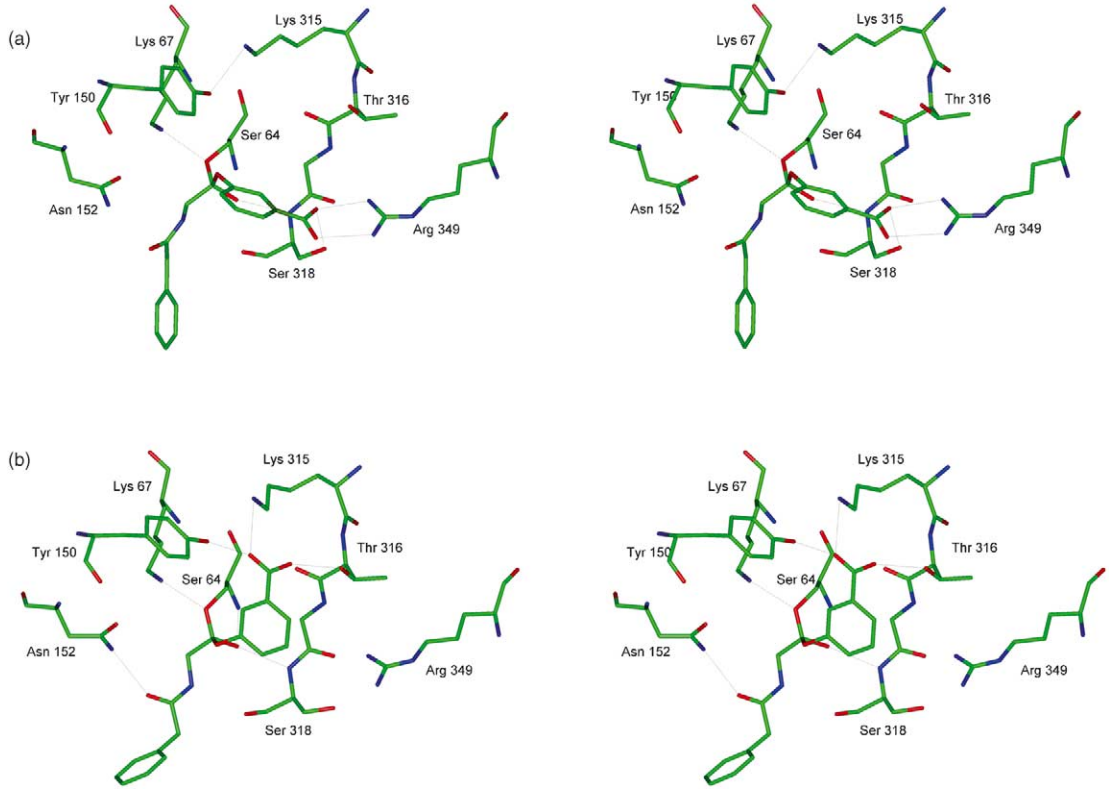


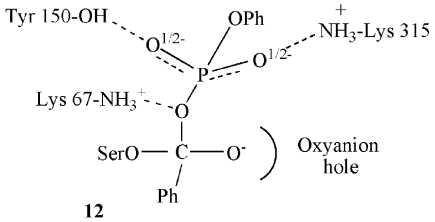
Figure 1. Stereoviews of the energy-minimized alternative conformations of the tetrahedral intermediate **11**. See text for details.

Models of peptide substrates bound to the P99 β-lactamase active site[7] resemble structure (b) where the carboxylate of the amino acid leaving group interacts with Thr 316 and Lys 315. This conformation would also likely suit the thioglycolate of **2**, **5** and **7**.

The crystal structures of several covalent complexes of β-lactams with another class C β-lactamase, the *E. coli* amp C enzyme, are available from the laboratory of Shoichet.[9–13] This enzyme is very similar in active site structure to the P99 enzyme but, notably, does have Ala at position 318 rather than Ser, and thus lacks one potential hydrogen-bond donor for a carboxylate group. Although there is considerable variability in the amino acid side chains interacting with the ligand carboxylates in these acyl–enzymes, they do tend to more resemble structure (a), where interactions with Arg 349, Asn 346, Thr 316, and Asn 343 dominate, rather than (b). In many cases, however, because of the fewer bonds and thus a generally shorter distance between the carboxylate and the acyl carbon at the reaction center of the intermediate, the interaction with the above-mentioned functional groups is not direct but extended via water molecules. In particular, all interactions with Arg 349 observed in these structures to date are indirect, via water. It is likely that this orientation of the carboxylate is required to keep the heterocyclic ring from blocking water attack of the acyl group and thus deacylation.[14] With acyclic substrates, this conformational restriction would not be present and thus perhaps the carboxylate orientation in (b) is preferred.

The crystal structure of an inactive mutant of the *E. coli* amp C enzyme with an intact β-lactam is also available.[15] This shows the carboxylate oriented more similarly to structure (b) with direct interactions with Tyr 150 and Thr 316, and water mediated interactions with Asn 346 and Arg 349. Modeling of the noncovalent complexes has also highlighted these residues.[16]

Models of the acyl–enzyme attained on reaction of the P99 β-lactamase with the acyl-phosphate **3**(**OPh**) suggest facile interaction of the negatively charged phosphoryl oxygen atoms with Tyr 150 and Lys 315, as in **12**.[6]



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The discussion above suggests that the orientation of the leaving group carboxylate group on interaction of the P99 β-lactamase with acyclic substrates, such as **1**–**9**, and intact bicyclic β-lactams may resemble that in structure (b). It is interesting to note that the leaving group of peptide substrates also favors such an orientation in the *Streptomyces* R61 DD-peptidase, a bacterial transpeptidase with tertiary structure and active site very similar to that of class C β-lactamases.[7,17] The inability of the DD-peptidase to employ residues such as Arg 349 and

Arg 346, which are available in the β-lactamase to orient the heterocyclic ring of a bicyclic β-lactam away from the acyl group and thus allow deacylation, may be a significant factor in the inhibition of this enzyme by β-lactams.

The principal conclusion from the experiments described above is that the phenylphosphate leaving group is much more effective than the m-hydroxybenzoate and thioglycolate groups in promoting acylation of the P99 β-lactamase. This probably arises from the optimal placing of negatively charged oxygens in the active site to interact with Tyr 150 and Lys 315. Tyr 150 may be able to assist departure of the leaving group by hydrogen-bonding/proton transfer to the non-bridgehead phosphoryl oxygen as well as it can by proton donation to the bridgehead (departing) oxygen. Design of inhibitors, both covalent and non-covalent, for class C β-lactamases, should take into account the optimal positioning of anions in the active site.

3. Experimental

The *Enterobacter cloacae* P99 β-lactamase was purchased from the Centre for Applied Microbiology and Research, Porton Down, Wilts., UK, and used as received.

3.1. 4-Phenylbenzoylthioglycolic acid (7)

4-Phenylbenzoic acid (Aldrich; 3.63 g, 18.3 mmol) was dissolved in dry tetrahydrofuran (80 mL) with stirring under a nitrogen atmosphere and cooled to 0 °C. Solid carbonyl diimidazole (Aldrich, 3.0 g., 18.5 mmol) was added and the mixture stirred for 1 h. Mercaptoacetic acid (Aldrich 1.28 mL, 18.3 mmol), dissolved in tetrahydrofuran (15 mL), was added and the reaction mixture stirred at 0 °C for four days. After rotary evaporation of the solvent, the residue was taken up into ethyl acetate (100 mL) and washed with 10% citric acid (2×100 mL) and water (2×100 mL). After drying over $MgSO_4$, the solvent was removed by rotary evaporation and the product recrystallized from 4/1 benzene/ethyl acetate in 40% recovered yield. Melting point 170–172 °C. ^{1}H NMR ($^2H_6$-DMSO) δ 3.93 (s, 2H, CH_2), 7.4–7.6 (m, 3H, ArH), 7.7–7.8 (m, 2H, ArH), 7.89 (d, J = 8.5 Hz, 2H, ArH), 8.02 (d, J = 8.5 Hz, 2H, ArH).

The benzoyl analogue, **5**, was prepared in the same way in 54% yield after recrystallization from 4/1 benzene/ethyl acetate. Melting point 94–96 °C. ^{1}H NMR ($^2H_6$-DMSO) δ 3.89 (s, 2H, CH_2), 7.59 (t, J = 7.5 Hz, 2H, ArH), 7.72 (t, J = 7.5 Hz, 1H, ArH), 7.95 (d, J = 7.5 Hz, 2H, ArH).

3.2. 3-[4′-phenylbenzoyloxy]benzoic acid (6)

4-Phenylbenzoic acid was condensed with benzyl 3-hydroxybenzoate in the presence of carbonyl diimidazole as described above. After recrystallization from cyclohexane the benzyl ester of **6** was obtained in 57% yield. Deprotection was then performed by hydrogenation (2 atm) over 10% Pd/C in ethanol. The product, **6**, was recrystallized from 4/1 benzene/ethyl acetate. Melting point 196–198 °C. ^{1}H NMR ($^2H_6$-DMSO) δ 7.5–7.7 (m, 6H, ArH), 7.8–7.9 (m, 3H, ArH), 7.95 (d, J = 8 Hz, 2H, ArH), 8.26 (d, J = 8 Hz, 2H, ArH).

The benzoyl analogue, **4**, was prepared in the same way and recrystallized from 3/2 ethyl acetate/hexane. Melting point 188–190 °C. ^{1}H NMR ($^2H_6$-DMSO) δ 7.6 (m, 4H, ArH), 7.76 (t, J = 7 Hz, 1H, ArH), 7.80 (s, 1H, ArH), 7.88 (d, J = 7 Hz, 1H, ArH), 8.14 (d, J = 7 Hz, 2H, ArH).

3.3. Analytical and kinetics methods

All kinetics experiments were performed at 25 °C in 20 mM MOPS buffer, pH 7.5. The concentrations of stock solutions of the enzyme were obtained spectrophotometrically. Reactions of **4**–**7** were monitored spectrophotometrically at 318 nm ($\Delta\varepsilon = -8130\ M^{-1}\ cm^{-1}$), 318 nm ($\Delta\varepsilon = -225\ M^{-1}\ cm^{-1}$), 312 nm ($\Delta\varepsilon = -3450\ M^{-1}\ cm^{-1}$), and 346 nm ($\Delta\varepsilon = -166\ M^{-1}\ cm^{-1}$), respectively. The accessible concentrations of **4**–**7** were limited by solubility such that the highest concentrations of each in the kinetics runs were 0.7 mM, 5.0 mM, 0.8 mM, and 5.0 mM, respectively. Steady state kinetics parameters were obtained from initial rates by non-linear least squares fitting of the data to the Michaelis–Menten equation. Enzyme-catalyzed methanolysis experiments were performed as described previously;[3] methanol concentrations were varied from zero to 2.5 M. Substrate aminolysis kinetics were also conducted as described in a previous paper;[7] D-alanine and D-phenylalanine were employed with concentrations ranging up to 200 mM and 48 mM, respectively.

The structures of Figure 1 were derived from computational models of enzyme–substrate complexes that were set up essentially as previously described[5,7] and run on an SGI Octane 2 computer with INSIGHT II (MSI, San Diego, CA). The starting point was the crystal structure of the P99 β-lactamase with a phosphonate inhibitor covalently attached to the active site serine residue (PDB file 1 bls[18]). This was transformed into the acylation tetrahedral intermediate **11** by means of the builder module of INSIGHT II. In this model, Lys 67 and Lys 315 were cationic, Tyr 150 was neutral, and the tetrahedral intermediate **11** was dianionic. A variety of initial conformations of the ligand were explored by manual positioning followed by molecular dynamics. Two general conformations seemed to dominate (see text and Fig. 1). Those were not interconvertable in 200 psec dynamics runs (where the entire protein together with solvating water molecules were unrestricted). Typical snapshots of each of these conformations were selected for energy minimization. The minimized structures were then used to calculate interaction energies, E_{int},[19] between ligand and protein. The residues included in these calculations were Ser 64, Lys 67, Tyr 150, Asn 152, Lys 315, Thr 316, Gly 317, and Thr 318, the residues shown to best discriminate between tetrahedral ligands,[20] and also Arg 349 which was found to interact with the carboxylate of the leaving group (see text).

Acknowledgements

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