The Role of Tyrosine 150 in Catalysis of β-Lactam Hydrolysis by AmpC β-Lactamase from *Escherichia coli* Investigated by Site-Directed Mutagenesis

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ABSTRACT: The kinetics of β-lactam hydrolysis by wild-type AmpC β-lactamase from *Escherichia coli* and three mutant proteins created by substitution of tyrosine 150 have been examined. The catalytic efficiency was decreased 10- to 1000-fold according to the substrate and mutant being studied. The effect of the mutation was much stronger with rapidly hydrolyzed substrates (e.g., cephalothin) than it was with slowly hydrolyzed substrates (e.g., ceftriaxone). With the latter substrates, the mutagenesis had a much stronger effect on apparent affinity than it did on rates of catalysis. Indeed, the enzyme appeared to be more reactive toward certain of the slowly hydrolyzed substances (e.g., methicillin, aztreonam, and ceftriaxone). These observations were not compatible with an obligatory role of tyrosine 150 in catalysis. The analysis of the effects of the mutation on activity was complicated by the observation of at least two, kinetically distinct, forms of the enzymes. It appeared that mutation of tyrosine 150 influenced the kinetic properties of one state and that this residue is involved in the partitioning of the enzyme between the different reactive states.

β-Lactamases make a major contribution to the resistance of Gram-negative organisms such as *Escherichia coli* to β-lactam antibiotics (Richmond & Sykes, 1973). There is considerable interest in understanding the mechanism of the various enzymes in order to design more effective inhibitors. Of the several types of β-lactamases that are now known (Ambler, 1980; Bush, 1989a–c), three classes (A, C, and D) all have a serine residue that carries out the attack on the β-lactam ring. X-ray crystallography has shown that this serine residue is positioned at the end of an α-helix and is juxtaposed to two lysine residues in both class A and class C enzymes (Dideberg *et al.*, 1987; Oefner *et al.*, 1990; Moews *et al.*, 1990; Herzberg, 1991). In the class C enzymes, tyrosine 150 is located with its hydroxyl group between these two lysine residues and within hydrogen bonding distance of the active serine residue (Oefner *et al.*, 1990). It was suggested that, in this environment, the tyrosine residue would be kept as an anion at pH values well below the normal pK_a of tyrosine in aqueous solution. Thus, it was proposed that tyrosine 150 could play an integral part in the catalytic mechanism of class C enzymes by acting as a general base, first, by accepting a proton from serine 64 during the attack on the β-lactam ring to form a tetrahedral intermediate and, second, by donating a proton to the nitrogen expelled during collapse of the tetrahedral intermediate (Oefner *et al.*, 1990). It was also proposed that tyrosine 150 could play a similar, but less effective, role in activation of a water molecule for attack on the acyl intermediate. This hypothesis predicts an essential role for tyrosine 150 in catalysis of β-lactam hydrolysis in a similar way that the histidine residue of the catalytic triad of serine proteases is essential to peptide bond hydrolysis (Carter & Wells, 1988). Therefore, we undertook an investigation of the effects that mutagenesis of this residue had on the kinetics of hydrolysis. The results indicate that, while tyrosine is important for hydrolysis of good substrates (high k_{cat}/K_M), it is by no means essential for activity and only influences the rate of reaction with slow substrates through an effect on substrate recognition.

EXPERIMENTAL PROCEDURES

Mutagenesis. The strains of *Escherichia coli* K-12 used in this work were SNO3 (*ampA1*, *ampC8*, *pyrB*, *recA*, *rpsL*) (Normark & Burman, 1977) and FLO1 (*recA* derivative of *E. coli* MM294; Lindberg & Normark, 1987) for routine transformation and DNA preparation and TG1 [Δ(lac-pro), supE, thi, hsdD5/F'traD36, proA$^+$B$^+$, lacI^q, lacZΔM15] as a host strain for M13 phage growth. Bacteria were grown at 37 °C in Terrific Broth (Sambrook *et al.*, 1989) containing 15 μg/mL tetracycline. The plasmids used were derivatives of pBR322 and were constructed by standard DNA-manipulation techniques (Sambrook *et al.*, 1989). Growth of phage M13 and preparation of single-stranded DNA were performed as described by Messing (1983). The mutagenesis was an adaptation of the method described by Taylor *et al.* (1985), whereby T7 DNA polymerase (Sequenase) and T4 DNA polymerase were used for the first and second extensions, respectively. Sequencing was done according to the dideoxy chain-termination method of Sanger *et al.* (1977) using the Sequenase DNA sequencing kit (United States Biochemical). T4 DNA ligase and polymerase were purchased from Boehringer Mannheim GmBH, and restriction endonucleases were obtained from Gibco BRL, New England Labs, or Boehringer Mannheim and used in the buffer supplied by the producer. [^{35}S]dATPαS (10 mCi/mL) was purchased from Amersham Inc., and dCTPαS was a product of PL Biochemicals (Pharmacia); other dNTPs were from Boehringer Mannheim. Oligonucleotides were obtained from the Protein Chemistry Laboratory at the Department of Biochemistry and Molecular Biophysics, Washington University.

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Purification and Kinetic Measurements. The mutants were expressed in *E. coli* SNO3 and purified using ion-exchange chromatography on Pharmacia S-Sepharose followed by affinity chromatography on immobilized (aminophenyl)-boronate (Page, 1993). Control experiments showed that the host organism, without the plasmid, did not express an AmpC β-lactamase and that, although there was very weak activity with some substrates, this was associated with proteins with different size and isoelectric points to AmpC β-lactamase that were removed during chromatography. Kinetic measurements were performed as described elsewhere (Page, 1993) using either 50 mM sodium phosphate or 0.1 M Tris-HCl at pH 7.0 and 37 °C. Steady-state kinetic parameters were determined with at least three replicates for each concentration of substrate and with two or three separate batches of enzyme. The parameters were obtained by a direct weighted fit to the Michaelis–Menten equation using the program Grafit (Leatherbarrow, 1989). The pH dependence of activity was investigated using either 0.1 M sodium phosphate (pH 5.5–8.0) or 50 mM sodium acetate/50 mM Tris-HCl at pH values between 4 and 10.5. Within these limits there was no irreversible loss of enzyme activity after readjusting the pH to 7.0.

The extent of acylation was measured in the following manner. One microliter of enzyme (0.5–2 μM in sodium phosphate buffer, pH 7.0) was mixed with an equal volume of inhibitor solution. At an appropriate time after mixing, 1 mL of 100 μM nitrocefin solution was added and the mixture rapidly transferred to a spectrophotometer (within 15 s of mixing) to measure residual activity toward nitrocefin.

CD Spectroscopy. Measurements of the ultraviolet circular dichroic spectra of the wild-type and mutant enzymes were made in the laboratory of Dr. R. Virden, University of Newcastle-upon-Tyne, using a Jobin Yvon Dichrographe 6 CD spectrophotometer. All enzyme solutions were prepared in 20 mM potassium phosphate, pH 7.0.

RESULTS

(1) *Construction of Plasmids and Mutagenesis.* Plasmid pAD6 is a derivative of pNU35 (Grundström *et al.*, 1980) from which a 1438 base pair fragment has been deleted (Figure 1). The *ampC* gene encoded by this plasmid contains two up-promoter mutations that confer high levels of resistance to β-lactam antibiotics through an increased expression of the β-lactamase (Jaurin *et al.*, 1981). A 2150 base pair *Eco*RI/*Pvu*II fragment from pAD6 was cloned into the *Eco*RI/*Sca*I sites of pBR322, from which the unique *Hin*dIII restriction site had previously been deleted, to obtain plasmid pAD7 (Figure 1). The latter plasmid was used for the subsequent construction and expression of the mutant enzymes. A 420 base pair *Xho*I/*Hin*dIII fragment from pNU35 was ligated into phage M13mp18 (Yanisch-Perron *et al.*, 1985), and this construct provided the single-stranded DNA for site-directed mutagenesis. The TAT codon of Tyr150 was replaced by GAA (Glu), TTT (Phe), and TCC (Ser) using a 21-base and two 17-base synthetic oligonucleotides, respectively, with the mismatched bases situated at the center of the primer. The entire 420 base pair mutated insert was sequenced from phage M13 single-stranded DNA before subcloning it back into pAD7 to ensure that no secondary mutations were present.

(2) *Physical Properties of the Mutants.* (*A*) *Purification.* All the proteins were recovered in good yield (>75% of initial activity) and were apparently homogeneous in Coomassie Blue-stained sodium dodecyl sulfate–polyacrylamide gels. The peak of activity was eluted from the S-Sepharose column at around

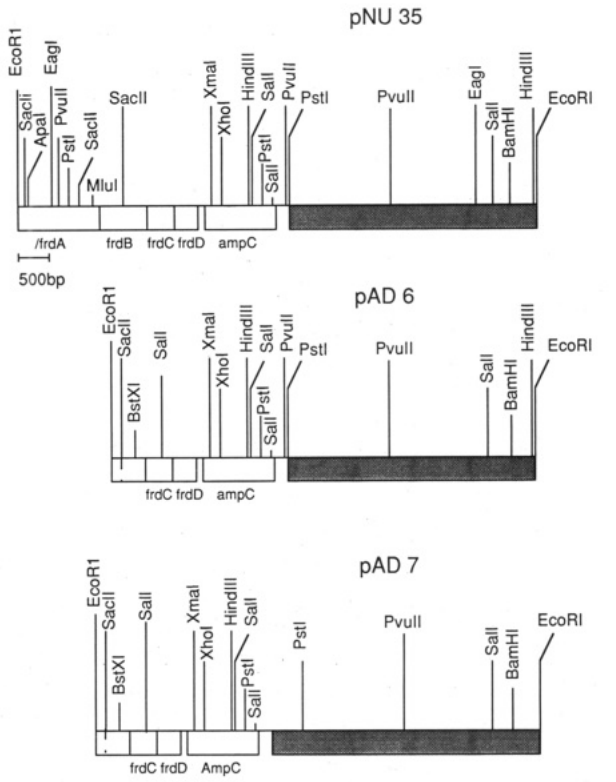


FIGURE 1: Construction of plasmid pAD7. Linear restriction maps of the parent plasmid pNU35 and the truncated, modified derivatives pAD6 and pAD7 are shown.

75 mM Tris and from the phenylboronate column at around 0.3 M borate for the wild-type enzyme. The mutant proteins had a much decreased affinity for the phenylboronate column, and part of the activity could be eluted with 0.5 M NaCl.

(*B*) *CD Spectroscopy.* (*i*) *Secondary Structure Content of the Proteins.* The wild-type and mutant proteins had identical CD spectra in the far-UV region (Figure 2). In 20 mM potassium phosphate, the molar ellipticity reached a maximum of 8000 at about 198 nm and a minimum of –4000 at 215 nm. The spectral data were used to obtain predicted fractional secondary structure content by the method of Provencher and Glöckner (1988). The estimates for the wild-type protein were as follows: α-helix, 0.07–0.16; β-sheet, 0.47–0.51; remainder, 0.37–0.41. The mutants gave estimates in similar ranges. These differ quite considerably from the fractional secondary structure content obtained from the X-ray crystal structure, which is as follows: α-helix, 0.32; β-sheet, 0.14; remainder, 0.54. The correspondence between the two sets of estimates was rather disappointing, but interpretation of CD spectra from proteins with a relatively low, mixed content of α-helix and β-sheet is difficult without a closely matched data base.

Transferring the proteins to 0.5 M potassium phosphate buffer, in an attempt to mimic the crystallization conditions, did not significantly alter the shape of the spectrum. The small differences between the spectra obtained under different conditions suggested that (i) there was no significant difference between the structure of the wild-type and mutant proteins and (ii) buffer conditions did not greatly perturb the structure of any of the proteins.

(*ii*) *Environments of Aromatic Side Chains.* The spectra of the Tyr150Ser and Tyr150Glu mutants were different from that of the wild-type protein in the near-UV region (Figure 2c). The difference spectra exhibited a minimum at 278 nm for both Tyr150Ser and Tyr150Glu mutants [Δ(molar

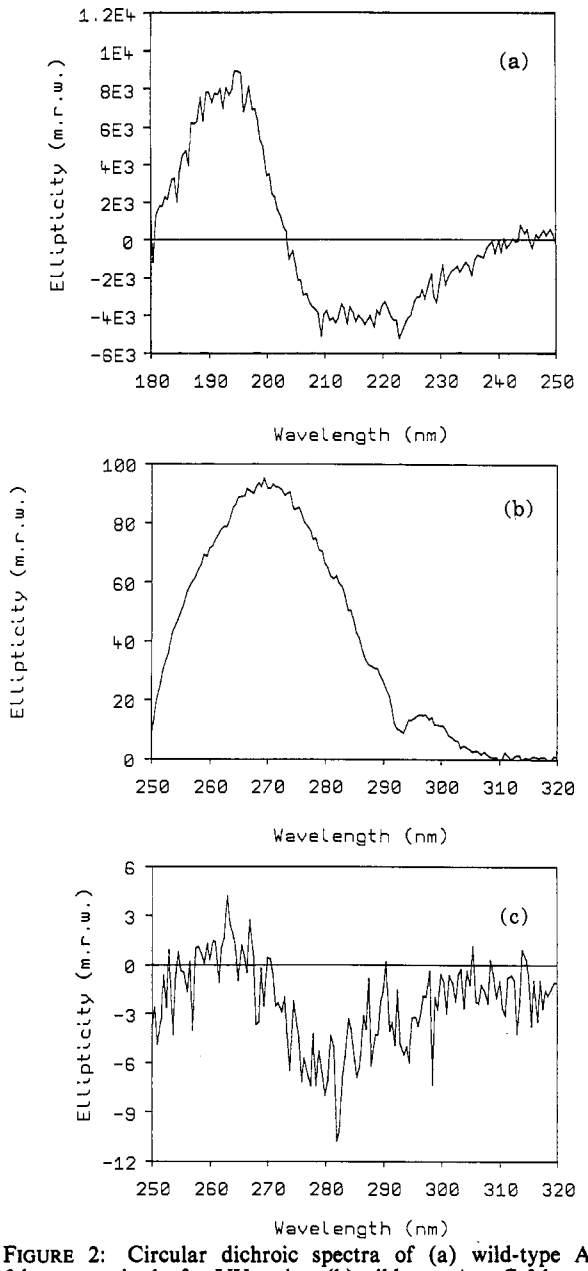


FIGURE 2: Circular dichroic spectra of (a) wild-type AmpC β-lactamase in the far-UV region, (b) wild-type AmpC β-lactamase in the near-UV region, and (c) difference spectrum for the Tyr150Glu – wild-type in the near-UV region.

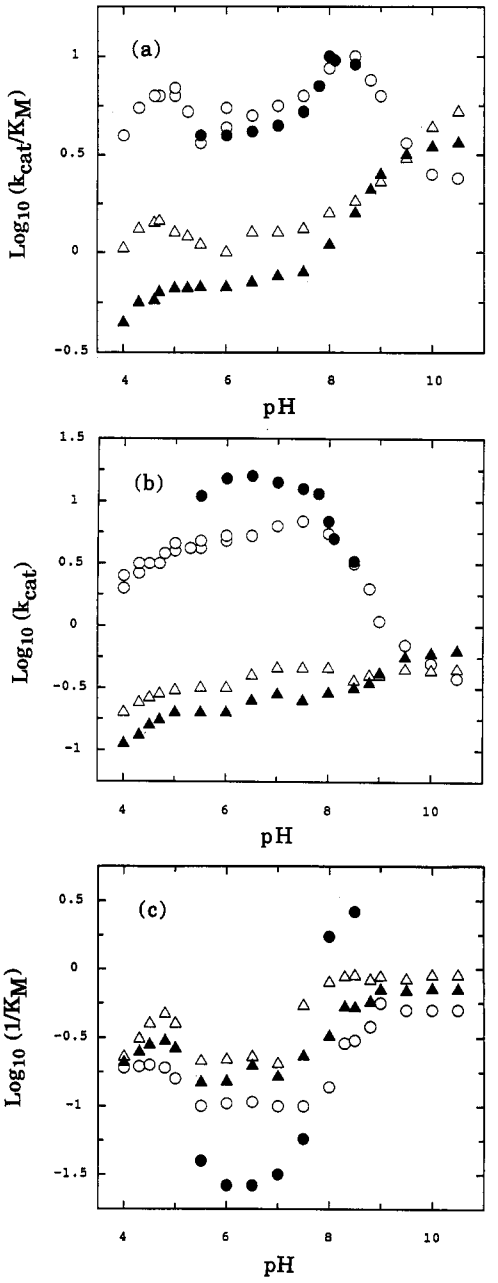


FIGURE 3: The pH dependence of the rate of hydrolysis of cephalothin by wild-type (○ in 25 mM Tris–acetate buffers, ● in 0.1 M sodium phosphate buffers), Tyr150Ser (△), and Tyr150Phe (▲) enzymes in Tris–acetate buffers. (a) The variation of k_{cat}/K_M with pH, (b) the variation of k_{cat} with pH, and (c) the variation of K_M with pH.

ellipticity) = –8.8 and –7.5, respectively]. This is within the range of wavelengths where tyrosine residues contribute to the spectrum and where a change was expected for the Tyr150Ser and Tyr150Glu mutants. Smaller differences were seen with the Tyr150Phe mutant [Δ(molar ellipticity) = –2 at 275 nm], but this was expected since replacement of one aromatic residue by another should have a smaller effect on the spectrum.

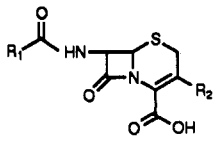
(3) Effects of the Mutations on Kinetic Properties. (A) Steady-State Kinetics Observed with Rapidly Hydrolyzed Substrates. The catalytic efficiency (k_{cat}/K_M) was decreased up to 1000-fold in the Tyr150 mutants. The major change was a decrease in k_{cat}, although the mutations always produced some changes in K_M. The magnitude of the changes in both parameters depended on the nature of the substrate as well as the substitution in the mutant enzyme. With rapidly hydrolyzed cephalosporins there was no correlation between the changes in either of the kinetic parameters and the structures of the cephalosporin side chains (Table 1).[1]

Overall, the magnitudes of the changes in k_{cat} (for the most part between 200- and 2000-fold) were less than might have been expected for substitution of a catalytically important residue. For example, alteration of the histidine residue that acts as general base in the catalytic triad of subtilisin resulted in a 10^6-fold decrease in catalytic activity (Carter & Wells, 1988).

The changes in K_M, which lay between a 5-fold increase and 200-fold decrease, were unexpected, as Tyr150 does not make a significant contact with the substrate in the complex

[1] Abbreviations: CENTA, 7-(thien-2-ylacetamido)-3-[[(3-carboxy-4-nitrophenyl)thio]methyl]-Δ^3-cephem-4-carboxylic acid; FAP, 6-β-[[3-(2-furyl)acryloyl]amino]penicillanic acid; PADAC, 7-(thien-2-ylacetamido)-3-[[2-[[4-(*N*,*N*-dimethylamino)phenyl]azo]pyridinium-1-yl]methyl]-Δ^3-cephem-4-carboxylic acid.

Table 1: Kinetic Parameters for the Reactions of Rapidly Hydrolyzed Substrates



R_1	R_2	enzyme	buffer	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (μM^{-1} s^{-1})
cephalothin		wild type	P_i[a]	757 ± 32	380 ± 63	1.99 ± 0.2
				28 ± 1.2	8.8 ± 0.3	3.1 ± 0.2
			Tris	430 ± 12	48 ± 2.3	8.95 ± 0.61
		Tyr150Phe	Tris	0.288 ± 0.012	187 ± 12	0.00153 ± 0.0006
		Tyr150Ser	Tris	4.8 ± 0.03	217 ± 16	0.022 ± 0.008
		Tyr150Glu	Tris	3.53 ± 0.09	98 ± 7	0.036 ± 0.05
cephaloridine		wild type	Tris	180 ± 8	210 ± 9	0.86 ± 0.04
		Tyr150Phe	Tris	0.72 ± 0.11	350 ± 16	0.0021 ± 0.0004
		Tyr150Ser	Tris	0.36 ± 0.05	170 ± 8	0.0021 ± 0.0006
		Tyr150Glu	Tris	0.1 ± 0.02	98 ± 6	0.001 ± 0.0003
nitrocefin		wild type	P_i[a]	639 ± 16	360 ± 58	1.78 ± 0.083
				39 ± 3	2.4 ± 0.7	15.7 ± 3.5
			Tris	420 ± 60	380 ± 42	1.11 ± 0.03
				25 ± 0.9	6.5 ± 0.6	3.85 ± 0.11
		Tyr150Phe	Tris	3.9 ± 0.3	4.2 ± 0.5	0.93 ± 0.02
		Tyr150Ser	Tris	2.11 ± 0.12	108 ± 21	0.019 ± 0.003
		Tyr150Glu	Tris	0.51 ± 0.03	356 ± 34	0.0014 ± 0.0004
PADAC		wild type	Tris	835 ± 64	310 ± 24	2.69 ± 0.43
		Tyr150Phe	Tris	0.50 ± 0.03	380 ± 28	0.0013 ± 0.0005
		Tyr150Ser	Tris	1.14 ± 0.08	203 ± 19	0.0056 ± 0.0007
		Tyr150Glu	Tris	0.45 ± 0.03	310 ± 43	0.0014 ± 0.0003
CENTA		wild type	Tris[a]	1312 ± 25	833 ± 53	1.55 ± 0.027
				293 ± 15	49 ± 5	5.98 ± 0.1
		Tyr150Phe	Tris	15 ± 2	35 ± 4	0.43 ± 0.03
		Tyr150Ser	Tris	12 ± 4	33 ± 2	0.36 ± 0.02
		Tyr150Glu	Tris	0.047 ± 0.005	4.6 ± 0.1	0.010 ± 0.003
cefadroxil	CH_3	wild type	Tris	167 ± 21	565 ± 36	0.296 ± 0.051
		Tyr150Phe	Tris	0.08 ± 0.01	324 ± 12	0.00025 ± 0.00009
		Tyr150Ser	Tris	1.15 ± 0.04	3.28 ± 0.13	0.35 ± 0.02
		Tyr150Glu	Tris	3.2 ± 0.1	230 ± 12	0.014 ± 0.005
cephalexin	CH_3	wild type	Tris	53 ± 6	8 ± 1	6.6 ± 0.5
		Tyr150Phe	Tris	6.7 ± 0.3	43 ± 7	0.15 ± 0.04
		Tyr150Ser	Tris	8.3 ± 0.2	12.1 ± 1.8	0.68 ± 0.05
		Tyr150Glu	Tris	0.24 ± 0.07	6.4 ± 0.5	0.038 ± 0.007

[a] Biphasic Eadie–Hofstee plots. The two sets of kinetic parameters correspond to intercept and slope of asymptotes to the curve at high and low substrate concentrations, respectively.

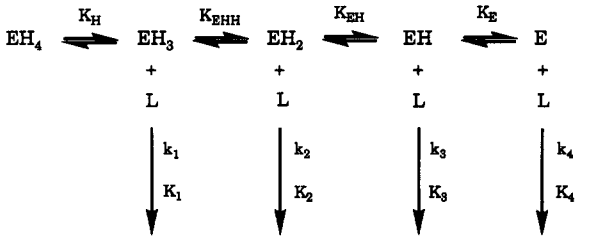
Table 2: Kinetic Parameters Describing the pH–Activity Profile[a]

	substrate																	
	cephalothin									benzylpenicillin								
	wild type			Tyr150Ser			Tyr150Phe			wild type			Tyr150Ser			Tyr150Phe		
enzyme species	k_{cat}	K_M	pK	k_{cat}	K_M	pK	k_{cat}	K_M	pK	k_{cat}	K_M	pK	k_{cat}	K_M	pK	k_{cat}	K_M	pK
EH_4			3.8			4.3			3.9			3.90			4.1			4.2
EH_3	38	3.4	5.69	3.2	6.7	4.9	2.5	3.6	nd[b]	9.1	4	5.23	1	4.0	5.1	0.3	1.2	nd
EH_2	45	8.9	7.84	5.8	2.2	8.7	3.3	17	8.3	15	10	8.00	0.8	13	8.6	1.2	8	8.2
EH	55	1.7	9.33	5.4	4.5	nd	6.8	6.2	nd	17	5	9.50	0.45	3.5	nd	1.1	3.3	nd
E	4.6	1.7		nd	nd		nd	nd		2	4		nd	nd		nd	nd	

[a] The kinetic parameters were obtained by weighted fit of the data to simple models for the pH dependences of k_{cat}, K_M, and k_{cat}/K_M over a limited range of pH and then over the full range of pH by iteration. Standard errors are therefore not given. The values quoted for pK were obtained from the k_{cat}/K_M data. The value against EH_4 is for K_H in Scheme 1, and similarly, that against EH_3 is for K_{EHH}, that against EH_2 is for K_{EH}, and that against EH is for K_E. [b] nd indicates that no evidence for these ionizations or enzyme species was detected.

of aztreonam with the *Citrobacter freundii* enzyme (Oefner *et al.*, 1990). Interpretation of the effects of the mutations on K_M was complicated by the biphasic dependence of the rate of hydrolysis on substrate concentration observed with the wild-type enzyme (Page, 1993). This behavior, which indicates two kinetically distinct pools of enzyme (Ainsley *et al.*, 1972; Meunier *et al.*, 1974), was less evident or was absent from the kinetics of the mutants (Table 1). This suggests that the kinetic properties of one form of the enzyme had been so altered that either it was indistinguishable from the other or it no longer made any contribution in the concentration range tested.

Scheme 1: Reaction Scheme Showing the Suggested Ionization States of the Wild-Type Enzyme



(B) pH Dependence of the Rate of Hydrolysis. The pH dependence of hydrolysis by the wild-type protein was quite complex (Figure 3). A strong influence of buffer composition on the kinetic parameters was observed with the wild-type protein (Tables 1 and 2). In particular, phosphate-containing buffers gave higher values of k_{cat} and K_M for the wild-type enzyme, while the mutant proteins were less sensitive to the buffers used and approximately the same results were obtained in all the buffers used. The pH profile of k_{cat}/K_M (Figure 3a) shows two prominent maxima, around pH 5 and 8, together with a shoulder around pH 6.7. In addition, the activity tends to a limiting value at high pH. Thus, a minimum of four protonation reactions, with five protonated states, is necessary to explain this profile (Scheme 1). The five states are: EH_4 at extreme acid pH (apparently inactive with low k_{cat} and high K_M), EH_3 giving the maximum around pH 5 (high k_{cat}, low K_M), EH_2 giving the shoulder around pH 6.7 (high k_{cat}, high K_M, especially in phosphate), EH giving the maximum around pH 8 (high k_{cat} and low K_M) and E, giving the limiting activity at alkaline pH (low k_{cat} and high K_M). The kinetic parameters associated with each of these forms were determined by weighted fit of data to simple models over a limited pH range and then, by iteration, to the complete models given in eqs 1–3. The values determined in this way are given in Table 2.

$$k_{cat} = \frac{K_H}{K_H + [H^+]} \times \frac{k_1[H^+]^3 + k_2K_{EHH}[H^+]^2 + k_3K_{EHH}K_{EH}[H^+] + k_4K_{EHH}K_{EH}K_E}{K_{EHH}K_{EH}K_E + K_{EHH}K_{EH}[H^+] + K_{EHH}[H^+]^2 + [H^+]^3} \quad (1)$$

$$K_M = \frac{K_1[H^+]^3 + K_2K_{EHH}[H^+]^2 + K_3K_{EHH}K_{EH}[H^+] + K_4K_{EHH}K_{EH}K_E}{K_{EHH}K_{EH}K_E + K_{EHH}K_{EH}[H^+] + KE_{HH}[H^+]^2 + [H^+]^3} \quad (2)$$

$$\frac{k_{cat}}{K_M} = \frac{K_H}{K_H + [H^+]} \times \frac{k_1[H^+]^3 + k_2K_{EHH}[H^+]^2 + k_3K_{EHH}K_{EH}[H^+] + k_4K_{EHH}K_{EH}K_E}{K_1[H^+]^3 + K_2K_{EHH}[H^+]^2 + K_3K_{EHH}K_{EH}[H^+] + K_4K_{EHH}K_{EH}K_E} \quad (3)$$

The pH profile was altered in the Tyr150 mutants. The most important difference was the absence of the decline in activity at alkaline pH, suggesting that the contribution from EH ⇌ E ionization was abolished and the pK_a for the EH_2 ⇌ EH transition is shifted to a somewhat higher value. The absence of the EH ⇌ E ionization from the pH profile of the mutants suggests that it is controlled by Tyr150. Indeed, the observed pK_a value (9.3) is in the right range to represent ionization of the phenolic hydroxyl group itself, given that it

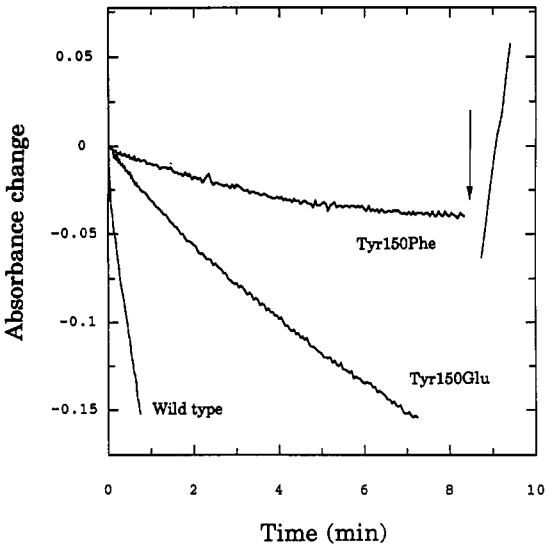


FIGURE 4: Hydrolysis of ceftriaxone by wild-type and mutant β-lactamases. The absorption change at 265 nm was monitored during the reaction between 100 μM ceftriaxone and 2.5 μM enzyme (as indicated). At the point in the reaction with Tyr150Phe enzyme marked by an arrow, nitrocefin was added to a final concentration of 100 μM and the absorbance change at 492 nm was monitored.

is juxtaposed to two lysine residues. For example, a very reactive tyrosine in bovine serum albumin, juxtaposed to two lysine residues and a more basic arginine, has a pK_a of 8.7 (Means & Bender, 1975). Whatever its identity, the ionizable group must be protonated for high activity of the wild-type protein, which is inconsistent with the proposed role of Tyr150.

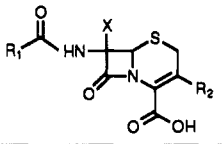
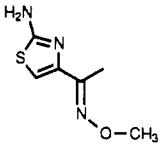
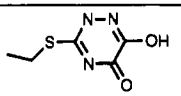
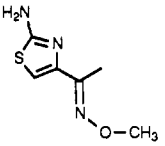
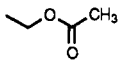
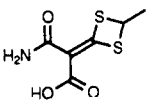
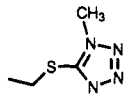
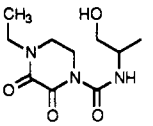
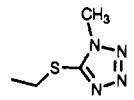
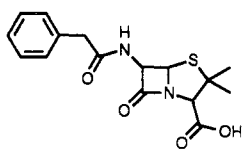
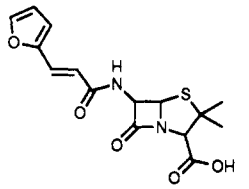
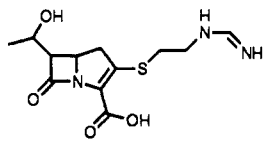
(C) Steady-State Kinetics of Reaction with Slowly Hydrolyzed Substrates. It was expected that mutation of Tyr150 would have less effect on k_{cat}/K_M for slowly hydrolyzed substrates where the breakdown of the acyl intermediate is rate-limiting, as it was not clear how effective Tyr150 would be in activating the water molecule for hydrolysis. As with the rapidly hydrolyzed substrates, the catalytic efficiency (k_{cat}/K_M) was decreased as much as 1000-fold in the Tyr150 mutants. The changes in k_{cat} were much less than observed with the rapidly hydrolyzed substrates and lay between a 5-fold decrease and a 5-fold increase (Table 3). The major change in k_{cat}/K_M was due to an up to 200-fold increase in K_M, which, as remarked above, was unexpected.

(D) Partitioning Behavior. The reaction with many of the slowly hydrolyzed substrates proceeded with a rapid initial burst in hydrolysis having an amplitude corresponding to between 1 and 1000 turnovers of the enzyme, according to the substrate and mutant. The rate of hydrolysis slowly decayed until a new, slower steady-state rate was established (Figure 4).

The possibility that this burst is due to product inhibition is eliminated by a number of standard controls (Fersht, 1985; Charnas & Then, 1988; Page, 1993), such as linearity of the reaction in the steady state until a significant amount of the substrate was consumed, the addition of preformed hydrolysis product before addition of the substrate (which had no effect, data not shown), and introduction a second portion of enzyme once the steady state had been achieved, which produced an identical burst of hydrolysis and a doubling of the steady-state rate of hydrolysis (data not shown).

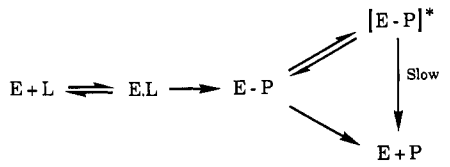
The burst of hydrolysis has been attributed to the presence of two or more conformations of the enzyme with different kinetic properties (Faraci & Pratt, 1985; Charnas & Then, 1988; Matagne *et al.*, 1990; Page, 1993). In the reaction with some cephalosporins, it has been suggested that the two

Table 3: Kinetic Parameters for the Reactions of Slowly Hydrolyzed Substrates

substrate				kinetic parameters			
R_1		R_2	enzyme	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($\mu M^{-1}\ s^{-1}$)	Hill coeff
	ceftriaxone (X = H)		wild type	0.069 ± 0.002	0.81 ± 0.04	0.085 ± 0.003	NA[a]
			Tyr150Phe	0.15 ± 0.03	109 ± 12	0.0014 ± 0.0002	
			Tyr150Ser	0.10 ± 0.01	2.5 ± 0.6	0.040 ± 0.007	
			Tyr150Glu	0.05 ± 0.01	1.8 ± 0.2	0.028 ± 0.05	
	cefotaxime (X = H)		wild type	0.23 ± 0.02	1.9 ± 0.4	0.12 ± 0.03	NA
			Tyr150Phe	0.37 ± 0.04	56 ± 8	0.0066 ± 0.0014	
			Tyr150Ser	0.25 ± 0.07	2.7 ± 0.3	0.093 ± 0.020	
			Tyr150Glu	0.09 ± 0.02	8.9 ± 1.9	0.010 ± 0.003	
	cefotetan (X = OCH_3)		wild type	0.06 ± 0.01	10 ± 1	(6.3 ± 0.9) × 10^{-3}	NA
			Tyr150Phe	0.013 ± 0.005	124 ± 23	(0.10 ± 0.01) × 10^{-3}	
			Tyr150Ser	0.037 ± 0.008	18 ± 2	(2.1 ● 0.3) × 10^{-3}	
			Tyr150Glu	0.011 ± 0.004	54 ± 9	(2.0 ± 0.3) × 10^{-3}	
	cefbuperazone (X = OCH_3)		wild type	0.31 ± 0.04	200 ± 10	(1.6 ± 0.2) × 10^{-3}	NA
			Tyr150Phe	1.4 ± 0.2	690 ± 80	(2.0 ± 0.5) × 10^{-3}	
			Tyr150Ser	0.53 ± 0.08	340 ± 68	(1.6 ± 0.3) × 10^{-3}	
benzylpenicillin:			wild type	15 ± 0.9	5.1 ± 0.2	2.9 ± 0.4	NA
			Tyr150Ser	0.43 ± 0.05	3.5 ± 0.1	0.12 ± 0.03	
			Tyr150Glu	0.24 ± 0.03	12.3 ± 2.1	0.02 ± 0.002	
			Tyr150Phe	1.05 ± 0.08	3.3 ± 0.5	0.32 ± 0.04	
FAP:			wild type	15 ± 3	7 ± 1	2.14 ± 0.3	NA
			Tyr150Ser	5 ± 1	10 ± 2	0.5 ± 0.1	
			Tyr150Glu	5 ± 2	23 ± 5	0.22 ± 0.06	
			Tyr150Phe	0.012 ± 0.004	10 ± 2	0.0012 ± 0.0005	
imipenem:			wild type	3.0 ± 0.4	235 ± 65	(12.8 ± 2.5) × 10^{-3}	1.08
			Tyr150Ser	4.7 ± 0.9	1247 ± 130	(3.8 ± 0.6) × 10^{-3}	1.30
			Tyr150Phe	5.5 ± 0.6	47020 ± 1400	(0.12 ± 0.04) × 10^{-3}	1.79

[a] NA indicates that normal kinetics were observed and therefore the Hill coefficient is not applicable.

Scheme 2: Acyl-Enzyme Branched Pathway



conformations are due to chemical rearrangement in the acyl-enzyme complex (Scheme 2), which creates a more stable acyl-enzyme intermediate (Faraci & Pratt, 1985, 1986).

However, the burst kinetics are a fairly general property of the mechanism of class C β-lactamases, including reactions with substrates and inhibitors that do not undergo this chemical rearrangement, and it is likely that the free form enzyme also exists in at least two conformations (Scheme 3).

In the model presented in Scheme 2, the slow steady-state rate of hydrolysis is due to the rate-limiting breakdown of the acyl-intermediate [E–P]*. Thus, most of the enzyme will be in this form during the slow phase of the reaction. In the model described by Scheme 3, the slow steady-state rate of hydrolysis is due to redistribution of the enzyme between the fast and slow catalytic cycles. It is possible for there to be a significant amount of free enzyme present during the steady state if the substrate cannot combine with one form of the enzyme and the isomerization reactions are slow.

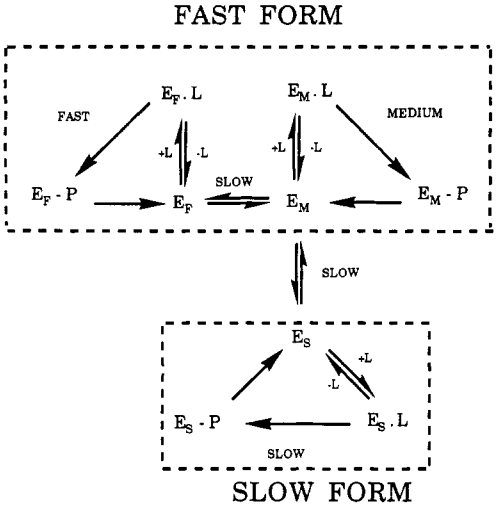
Ceftriaxone appeared to convert the Tyr150Phe enzyme into a virtually unreactive state after several turnovers (Figure 4). Addition of a readily hydrolyzed substrate (for example, nitrocefin) to the reaction mixture demonstrated that at least half of the original activity of enzyme was still present. The presence of a significant fraction of free enzyme is inconsistent with the possibility that the apparent inactivation is caused by the reaction branching at the acyl-enzyme, to form a slowly deacylating species. The time course can be explained by the presence of two interconverting kinetic forms of the enzyme in the initial reaction mixture. Both forms react readily with nitrocefin, but one form has less activity toward substrates

Table 4: Parameters Describing the Partitioning Behavior of Wild-Type and Mutant Proteins

	enzyme							
	wild type		Tyr150Ser		Tyr150Phe		Tyr150Glu	
substrate	k_b (min^{-1})	Amp[a]	k_b (min^{-1})	Amp	k_b (min^{-1})	Amp	k_b (min^{-1})	Amp
ceftriaxone	no burst		0.8 ± 0.1	1.3 ± 0.3	1.6 ± 0.4	2.4 ± 0.6	0.42 ± 0.05	3.5 ± 0.4
cefbuperazone	0.67 ± 0.08	21 ± 1.9	0.34 ± 0.07	33 ± 8	0.14 ± 0.02	1.8 ± 0.3	0.33 ± 0.04	15 ± 3
cefmenoxime	no burst		0.5 ± 0.1	2.1 ± 0.3	0.22 ± 0.05	1.2 ± 0.2	0.45 ± 0.06	3.6 ± 0.3
FAP	1.73 ± 0.24	5250 ± 360	2.6 ± 0.4	6700 ± 700	0.069 ± 0.008	8400 ± 780	0.35 ± 0.04	7300 ± 690

[a] Amplitude of the burst of hydrolysis in moles hydrolyzed per mole of protein.

Scheme 3: Suggested Reaction Scheme Accounting for the Nonstoichiometric Bursts in Cephalosporin Hydrolysis [Redrawn from Page (1993)][a]



[a] It is postulated that the enzyme has three reactive states denoted by E_F, E_M, and E_S; E_F and E_M are only readily distinguished with certain substrates and rapid reaction techniques. Thus, in this work they are grouped as the fast form of the enzyme.

like ceftriaxone. Other substrates showed similar reaction kinetics (Table 4).

The Tyr150 mutations decreased the time constant describing the decay of the initial rate of hydrolysis and affected the amplitude of the burst. In several instances, a burst phase was observed with the mutant proteins where none was evident in the reaction of the parent enzyme (Table 4). These observations suggest that mutation of Tyr150 directly influences the isomerization reaction, making it slower. However, an indirect effect exerted through changes in the kinetic parameters of the two pools of enzyme cannot be excluded.

Ceftriaxone is a simple competitive inhibitor of the hydrolysis of nitrocefin by the wild-type enzyme (Figure 5a and Table 5). The reactions with the Tyr150Ser mutant showed two phases (Figure 5b). Initially, ceftriaxone acted as a slow binding inhibitor with progressive irreversible inhibition. This phase of reaction accounted for about 50% of the nitrocefin hydrolysis activity. In the second phase ceftriaxone acted as a simple competitive inhibitor. The Tyr150Phe mutant also showed biphasic time courses in these experiments but was only very weakly inhibited in the second phase of the reaction (Figure 5c). Similar reactions were observed with other third-generation cephalosporins and the monobactams aztreonam and carumonam (Table 5).

The conspicuously biphasic time courses observed with the mutants in these experiments suggested that these substrates combine with two distinct forms of the enzyme. The incomplete inhibition of the Tyr150Phe mutant is not easily explained by models in which only the acyl intermediate undergoes an isomerization reaction but is readily explained

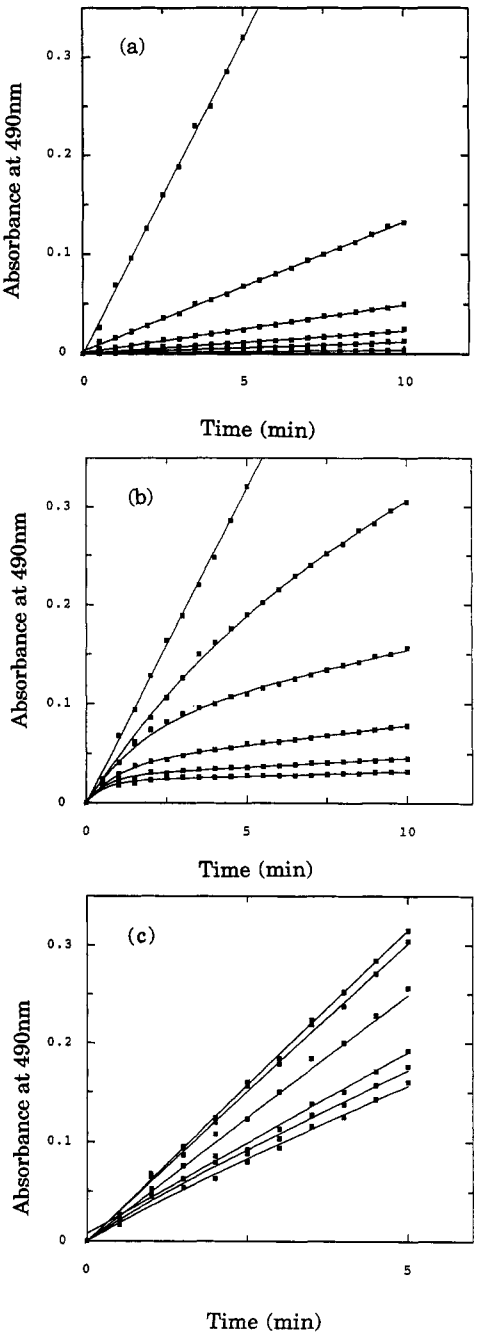


FIGURE 5: Alternative substrate inhibition of nitrocefin hydrolysis by wild-type and mutant β-lactamases. The time course of hydrolysis of 100 μM nitrocefin (as increasing absorbance at 492 nm) is shown for (a) wild-type, (b) Tyr150Ser, and (c) Tyr150Phe enzymes in the presence of the following concentrations of ceftriaxone: 0, 2, 5, 10, 20, and 50 μM for the curves in order of decreasing activity. In (a), the data are fitted to a linear increase in absorbance, while in (b) and (c), the data obtained in the presence of ceftriaxone are fitted to an exponential burst plus a steady-state rate of increase in absorbance [$A_t = A_1(1 - e^{-k_b t}) + A_2 t$].

Table 5: Competitive Inhibition Constants for Slowly Hydrolyzed Substrates

substrate	enzyme	K_I for initial phase (μM)	K_I in steady state (μM)	time constant for conversion (min^{-1})
ceftriaxone	wild type	0.296 ± 0.081	monophasic	
	Tyr150Ser	0.71 ± 0.13	2.2 ± 0.3	1.1 ± 0.2
	Tyr150Phe	0.395 ± 0.042	169 ± 42	0.15 ± 0.03
cefmenoxime	wild type	1.7 ± 0.4	monophasic	
	Tyr150Ser	30 ± 7	15 ± 4	0.29 ± 0.05
	Tyr150Phe	1230 ± 275	>10000	0.08 ± 0.02
cefbuperazone	wild type	0.098 ± 0.03	>500	0.23 ± 0.04
	Tyr150Ser	0.12 ± 0.02	250 ± 69	0.35 ± 0.09
	Tyr150Phe	0.15 ± 0.04	>1000	0.54 ± 0.06
aztreonam	wild type	0.011 ± 0.005	monophasic	
	Tyr150Ser	0.44 ± 0.06	5.6 ± 0.9	0.83 ± 0.07
	Tyr150Phe	379 ± 65	>10000	0.34 ± 0.03
	Tyr150Glu	289 ± 42	1500	0.69 ± 0.07

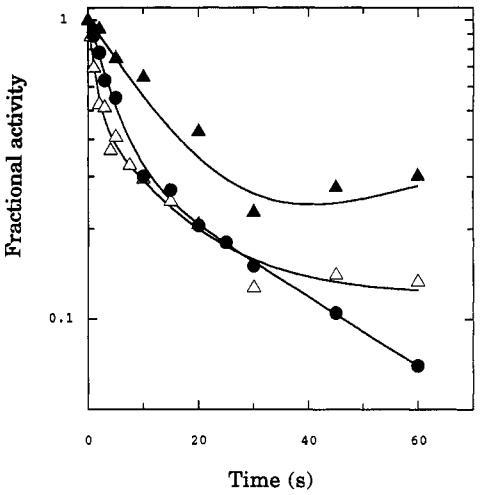


FIGURE 6: Acylation of wild-type and mutant β-lactamases by ceftriaxone. The extents of acylation of (●) wild-type, (△) Tyr150Ser, and (▲) Tyr150Phe enzymes were monitored as loss of activity toward nitrocefin, as described in the text. The ceftriaxone concentration was 2.5, 50, and 200 μM for the wild-type, Tyr150Ser, and Tyr150Phe enzymes, respectively. The data for the wild type were fitted to a double exponential decay of activity: $A_t = A_1(e^{-k_1 t}) + A_2(e^{-k_2 t})$. The observed values of the parameters were as follows: $A_1 = 0.71 \pm 0.06$, $A_2 = 0.34 \pm 0.06$, $k_1 = 0.24 \pm 0.04\ s^{-1}$, $k_2 = 0.026 \pm 0.007\ s^{-1}$. The data for the Tyr150Ser mutant were fitted to a double exponential decay to a limiting level of activity (equivalent to the steady-state level of free enzyme): $A_t = A_{ss} + A_1(e^{-k_1 t}) + A_2(e^{-k_2 t})$. The observed values of the parameters were as follows: $A_{ss} = 0.12 \pm 0.03$, $A_1 = 0.53 \pm 0.08$, $A_2 = 0.36 \pm 0.07$, $k_1 = 0.74 \pm 0.17\ s^{-1}$, $k_2 = 0.077 \pm 0.03\ s^{-1}$. The data for the Tyr150Phe mutant were fitted to a single exponential decay plus a slow recovery of activity: $A_t = A_1(e^{-k_1 t}) + A_2 t$. The observed values of the parameters were as follows: $A_1 = 0.53 \pm 0.04$, $k_1 = 0.24 \pm 0.07\ s^{-1}$, $A_2 = 0.004\ s^{-1}$.

by a model with two forms of the enzyme, one of which does not react with the substrates.

(E) Stability of the Acyl-Enzyme Complex Formed with Slowly Hydrolyzed Substrates. Ceftriaxone rapidly acylated the wild-type enzyme following a biphasic time course (Figure 6). The biphasic nature of the time course was more pronounced in the reactions of the Tyr150 mutants. The Tyr150 mutants reacted more rapidly than the wild type in the fast phase of acylation and exhibited decreased affinity for ceftriaxone in the second phase (Table 6). Similar observations where made with other slow substrates. While normally half of the enzyme reacted rapidly and half reacted more slowly, sometimes different amplitudes of reaction were observed (for example, with cefbuperazone). Such behavior is expected in a model with two interconverting forms of enzyme when the rate of acylation is comparable to the rate of interconversion (Ratzikis, 1984; Ainsley *et al.*, 1974). It occurs because the more rapid acylation of one form displaces the conformational equilibrium toward the rapidly reacting form.

The wild-type and mutant enzymes sometimes had different end points of the acylation reaction (Table 7), which reflect differing steady-state levels of acylation during steady-state turnover. These suggest that the balance between the rates of formation and breakdown of the acyl intermediate had been altered. While this was partly due to the effect of the substitution on the rate of acylation, the deacylation rate was increased in some cases (Table 7).

DISCUSSION

We set out to test the hypothesis put forward by Oefner *et al.* (1990) that Tyr150 could act as a general base in catalysis of β-lactam hydrolysis in a similar way to that in which the essential histidine of serine proteases acts to accelerate peptide bond hydrolysis. Tyr150 was replaced by Phe, which retains the general shape of the tyrosine residue and would, for example, preserve hydrophobic contacts with the substrate. It was also replaced by serine and glutamate, both of which would retain hydrogen bonding potential although they are somewhat smaller. The effects of the mutations on activity were rather complicated, and it was important to establish that the mutations had not altered the protein structure to a significant extent. Circular dichroic measurements indicated that there was no great perturbation of secondary structure, and confirmation of this has been obtained recently from X-ray crystallographic studies of the mutant proteins (J. J. Daly, A. D'Arcy, C. Oefner, and F. Winkler, unpublished observations). These indicate that there are no changes in the position of the principal active site residues and, for the most part, only minor rearrangements on the periphery of the binding site. The loop formed by residues 280–290, which forms one edge of the active site, is rather disordered and may adopt at least two conformations, one of which is identical to that of the wild type. Such local conformation changes could readily explain the two kinetic forms of the protein discussed here.

Overall, the mutations produced smaller changes in k_{cat} than were expected for a residue acting in a similar way to the histidine of the catalytic triad of a serine protease. The magnitude of the changes was far less than observed for replacement of histidine in subtilisin (Carter & Wells, 1988). There was some implication of this residue in substrate recognition. Since the biggest effect of substrate recognition was seen in the Tyr150Phe, which has the least steric change,

Table 6: Kinetic Parameters Describing the Acylation Reaction

		fast phase			slow phase		
substrate	enzyme	k_{on} (s^{-1})	K_S (μM)	amplitude[a]	k_{on} (s^{-1})	K_S (μM)	amplitude
cefbuperazone	wild type	20 ± 3	430 ± 37	0.86 ± 0.05	0.18 ± 0.03	500 ± 64	0.14 ± 0.04
	Tyr150Ser	22 ± 4	321 ± 25	0.81 ± 0.08	0.19 ± 0.04	100 ± 18	0.19 ± 0.05
	Tyr150Phe	0.06 ± 0.01	177 ± 15	0.48 ± 0.04	<0.1	>1000	nd[b]
ceftriaxone	wild type	3.3 ± 0.2	14.7 ± 1.5	0.51 ± 0.05	1.8 ± 0.1	28 ± 6	0.49 ± 0.06
	Tyr150Ser	4.67 ± 0.71	90 ± 17	0.89 ± 0.11	0.008 ± 0.001	120 ± 34	0.11 ± 0.07
	Tyr150Phe	>5	>10000	0.5	<0.001	>1000	nd
	Tyr150Glu	>5	>1000	0.5	<0.01	>1000	nd
aztreonam	wild type	1.4 ± 0.3	21 ± 4	0.45 ± 0.08	0.29 ± 0.05	105 ± 23	0.54 ± 0.10
	Tyr150Ser	3.3 ± 0.4	22 ± 3	0.5 ± 0.1	0.52 ± 0.07	169 ± 19	0.28 ± 0.05
	Tyr150Phe	0.35 ± 0.05	210 ± 43	0.5 ± 0.1	<0.01	<1000	nd

[a] The fraction of total activity lost during this phase of reaction. [b] Complete acylation could not be demonstrated at the highest concentration tested.

Table 7: Stability of the Acyl-Enzyme Complex Formed with Wild-Type and Mutant β-Lactamases

substrate	enzyme	fractional steady-state level of acylation at saturating substrate[a]	estimated deacylation rate[b]
ceftriaxone	wild type	0.99	<0.01
	Tyr150Ser	0.87	0.021
	Tyr150Phe	0.35	0.032
	Tyr150Glu	0.75	0.027
cefmenoxime	wild type	nd[c]	>1
	Tyr150Ser	0.83	0.3
cefbuperazone	wild type	0.97	0.68
	Tyr150Ser	0.93	0.5
	Tyr150Phe	0.41	0.93
cefotetan	wild type	0.69	0.24
	Tyr150Ser	0.99	0.13
	Tyr150Phe	0.98	0.05
aztreonam	wild type	>0.99	<0.0001
	Tyr150Ser	0.93	0.03
	Tyr150Phe	0.67	0.66
	Tyr150Glu	0.78	0.1
moxalactam	wild type	>0.99	<0.001
	Tyr150Ser	0.98	0.27
	Tyr150Phe	0.93	0.003
	Tyr150Glu	0.95	0.05

[a] Determined in acylation experiments such as shown in Figure 6. [b] Determined from the rate of acylation and the ratio of active to inactive protein (Christensen *et al.*, 1990). [c] Acyl intermediate not sufficiently stable to be trapped by this method.

it seems that this is unlikely to be due a direct interaction between residue 150 and the substrate.

Nonstoichiometric bursts of hydrolysis were observed, and mutation of Tyr150 has some influence on the characteristics of these reactions. The burst phenomenon has been attributed to the enzyme existing in alternative conformations in slow equilibrium with one another (Page, 1993). Addition of substrate to this mixture will start parallel catalytic cycles, running at different rates (Scheme 3). After repeated turnovers, the enzyme will partition among these according to the relative rates of turnover of the cycles, and therefore, most of the enzyme will accumulate in the slowest cycle. Mutation of tyrosine 150 appeared to slow down the rate of interconversion between the forms and markedly affected the properties of the slow form, rendering it unable to bind certain types of substrate (particularly those with the side chain of third-generation cephalosporins). It is probable that the changes in substrate recognition mentioned above are due to the altered recognition properties of this form of the enzyme.

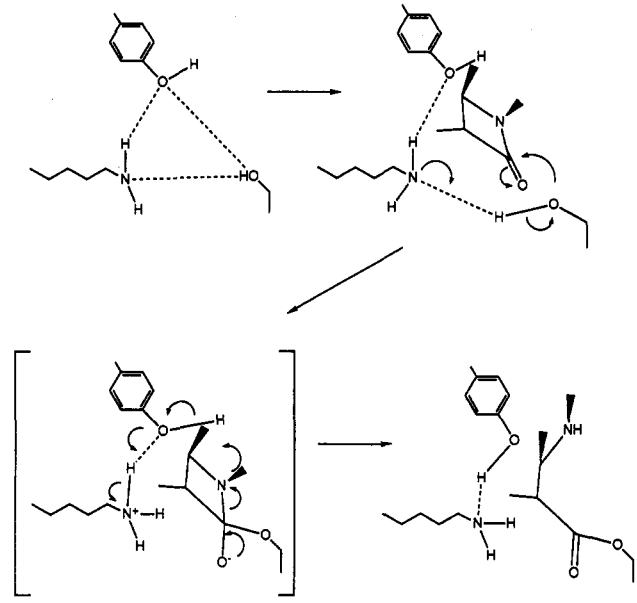


FIGURE 7: Schematic drawing of the postulated sequence of reactions during formation of the acyl-enzyme intermediate with wild-type β-lactamase [adapted from Oefner *et al.* (1990)].

It appears that tyrosine does not play such an essential role in catalysis as might be expected if it were acting as a general base, transiently accepting a proton during attack on the β-lactam ring (Oefner *et al.*, 1990). Instead, the residue must be perceived as an important, but not indispensable, member of the network of hydrogen bonds that runs across the catalytic center from Asn152 to the carbonyl of Thr316. Both the amino group of Lys67 and the hydroxyl group of Tyr150 in the native protein are members of this network and are close enough to form hydrogen bonds to Ser64. The presence of a basic group in position 67 has been shown to be important for activity in the *C. freundii* enzyme (Tsukamoto *et al.*, 1990), and it might be that this is the primary acceptor for the proton from serine 64, while Tyr150 might be more important for delivery of a proton to the nitrogen atom of the β-lactam as it leaves (Figure 7). Tyr150 is well placed to carry out this reaction in the wild-type enzyme, and such a role would be consistent with the changed pH–activity profiles of the mutants, which suggest that Tyr150 should be protonated for high activity. This role could be fulfilled, albeit with reduced efficiency, by a water molecule in the mutants (which crystallography and modeling suggest there would be space for, even in the Tyr150Phe mutant). The somewhat enhanced deacylation rates observed with the mutants could also be due to increased accessibility of the acyl intermediate to water.

Tyrosine 150 of class C β-lactamases is homologous to Tyr159 of *Streptomyces* R61 DD-peptidase (Joris *et al.*, 1988), and the two residues probably occupy similar positions in the active site (Kelly *et al.*, 1986). Some of the effects we report here are similar to those observed after mutation of Tyr159 of the *Streptomyces* peptidase (Wilkin *et al.*, 1993). In particular, the alteration of the pH profile, the sometimes dramatic effect of the Tyr → Phe substitution (compared to the effects of the Tyr → Ser substitution), and the modest increases in deacylation rates are all rather similar in the two sets of mutants. It is likely that some aspects of the function of the tyrosine residues in the two proteins are similar, especially perhaps a role in protonation of the nitrogen during formation of the acyl intermediate (Figure 7; Wilkin *et al.*, 1993).

In the class A enzymes for which the crystal structures are known, serine hydroxyl groups occupy the position of the hydroxyl group of Tyr150 (Moews *et al.*, 1990; Herzberg, 1991), and it has been suggested that the two hydroxyl groups might fulfil similar roles by functioning either as a general base in catalysis (Oefner *et al.*, 1990) or, at least, to protonate the nitrogen as it leaves during formation of the acyl intermediate (Jacob *et al.*, 1990; Lamotte-Brasseur *et al.*, 1991). The effects of mutagenesis of this serine residue on activity of the class A enzyme from *Streptomyces albus* G are different to those observed here (Jacob *et al.*, 1990). For example, the mutant proteins were markedly less stable than the wild-type protein, suggesting that some perturbation of the structure had occurred, which was not the case with the *Escherichia coli* AmpC enzyme. There were no large changes in k_{cat} for reaction of the class A enzyme with rapidly hydrolyzed substrates, and K_M was slightly affected according to the substitution made and the substrate being assayed. Thus, there is no compelling evidence that these residues fulfill a similar function in the two classes of β-lactamases.

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REFERENCES

Ainsley, G. R., Shill, J. P., & Neet, K. E. (1972) *J. Biol. Chem. 347*, 7088–7096.
Ambler, R. P. (1980) *Philos. Trans. R. Soc. London, Ser. B, Biol. Sci. 289*, 321–331.
Bush, K. (1989a) *Antimicrob. Agents Chemother. 33*, 259–263.
Bush, K. (1989b) *Antimicrob. Agents Chemother. 33*, 264–270.
Bush, K. (1989c) *Antimicrob. Agents Chemother. 33*, 271–276.
Carter, P., & Wells, J. A. (1988) *Nature 332*, 564–568.
Charnas, R. L., & Then, R. L. (1988) *Rev. Infect. Dis. 10*, 752–760.
Christensen, H., Martin, M. T., & Waley, S. G. (1990) *Biochem. J. 266*, 853–861.
Dideberg, O., Charlier, P., Wéry, J.-P., Dehottay, P., Dusart, J., Erpicum, T., Frère, J.-M., & Ghuysen, J.-M. (1987) *Biochem. J. 245*, 911–913.
Faraci, W. S., & Pratt, R. F. (1985) *Biochemistry 24*, 903–910.
Faraci, W. S., & Pratt, R. F. (1986) *Biochemistry 25*, 2934–2941.
Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd ed., W. H. Freeman, New York.
Grundström, T., Jaurin, B., Edlund, T., & Normark, S. (1980) *J. Bacteriol. 143*, 1127–1134.
Herzberg, O. (1991) *J. Mol. Biol. 217*, 701–719.
Jacob, F., Joris, B., Lepage, S., Dusart, J., & Frère, J. M. (1990) *Biochem. J. 271*, 399–406.
Jaurin, B., Grundström, T., Edlund, T., & Normark, S. (1981) *Nature 290*, 221–225.
Joris, B., Ghuysen, J.-M., Dive, G., Renard, A., Dideberg, O., Charlier, P., Frère, J.-M., Kelly, J. A., Boyington, J., Moews, P., & Knox, J. (1988) *Biochem. J. 250*, 313–324.
Kelly, J. A., Dideberg, O., Charlier, P., Wéry, J., Libert, M., Moews, P., Knox, J., Duez, C., Fraipont, C., Joris, B., Dusart, J., Frère, J.-M., & Ghuysen, J.-M. (1986) *Science 231*, 1429–1431.
Lamotte-Brasseur, J., Dive, G., Dideberg, O., Charlier, P., Frère, J.-M., & Ghuysen, J.-M. (1991) *Biochem. J. 279*, 213–221.
Leatherbarrow, R. L. (1989) Grafit, Erithacus Software, Staines, U.K.
Lindberg, F., & Normark, S. (1987) *J. Bacteriol. 169*, 758–763.
Matagne, A., Misselyn-Baudin, A.-M., Joris, B., Erpicum, T., Granier, B., & Frère, J.-M. (1990) *Biochem. J. 265*, 131–146.
Means, G. E., & Bender, M. L. (1975) *Biochemistry 14*, 4989–4994.
Messing, J. (1983) *Methods Enzymol. 101* (Part C), 20–78.
Meunier, J.-C., Bue, J., Navarro, A., & Ricard, J. (1974) *Eur. J. Biochem. 49*, 209–223.
Moews, P. C., Knox, J. R., Dideberg, O., Charlier, P., & Frère, J.-M. (1990) *Proteins: Struct., Funct., Genet. 7*, 156–171.
Normark, S., & Burman, L. G. (1977) *J. Bacteriol. 132*, 1–7.
Oefner, C., D'Arcy, A., Daly, J. J., Gubernator, K., Charnas, R. L., Heinze, I., Hubschwerlen, C., & Winkler, F. K. (1990) *Nature 343*, 284–288.
Page, M. G. P. (1993) *Biochem. J. 295*, 295–304.
Provencher, S. W., & Glöckner, J. (1981) *Biochemistry 20*, 33–37.
Rakitzis, E. T. (1984) *Biochem. J. 217*, 341–351.
Richmond, M. H., & Sykes, R. B. (1973) *Adv. Microbiol. Physiol. 9*, 31–88.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Sanger, F., Nicklen, & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Taylor, J. W., Ott, J., & Eckstein, F. (1985) *Nucleic Acids Res. 13*, 8764–8785.
Tsukamoto, K., Tachibana, K., Yamazaki, N., Ishii, Y., Ujiie, K., Nishida, N., & Sawai, T. (1990) *Eur. J. Biochem. 188*, 15–22.
Wilkin, J.-M., Jamin, M., Damblon, C., Zhao, G. H., Joris, B., Duez, C., & Frère, J.-M. (1993) *Biochem. J. 291*, 537–544.
Yanisch-Perron, C., Vieira, J., & Messing, J. (1985) *Gene 33*, 103–119.