Effects of site-specific mutagenesis of tyrosine 105 in a Class A β-lactamase

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Tyr-105 is a conserved residue in the Class A β-lactamases and is in close proximity to the active-site. Tyr-105 in β-lactamase from *Bacillus licheniformis* was converted into Phe by site-directed mutagenesis. This mutation caused no significant effect on the structure of the enzyme and had only small effects on the catalytic properties. In particular, in comparison to the wild-type, $k_{cat.}$ for benzylpenicillin was increased slightly, whereas it was decreased slightly for several other substrates. For each substrate examined, K_m increased 3–4-fold in the mutant compared with the wild-type enzyme. Examination of the effect of pH on the catalytic reaction revealed only small perturbations in the pK values for the acidic and basic limbs of the $k_{cat.}/K_m$ pH profiles due to the mutation. Overall effects of the Y105F substitution on the catalytic efficiency for different pencillin and cephalosporin substrates ranged from 14% to 56% compared with the wild-type activity. We conclude that Tyr-105 is not an essential residue for β-lactamase catalysis, but does contribute to substrate binding.

INTRODUCTION

There are several residues both within and near the catalytic site of Class A β-lactamases which are highly conserved (Joris et al., 1988). The degree of conservation observed for these residues often extends past the boundaries of Class A enzymes, frequently including Class C and D β-lactamases and the penicillin-binding proteins. Such a high degree of conservation is generally taken to indicate that a residue is playing a pivotal role in either the catalytic reaction or the structure of the enzyme. One such conserved residue is Tyr-105 [in the numbering system of Ambler (1980)].

In addition to the ubiquitous nature of Tyr-105, there are other lines of evidence which indicate that this residue may play an important role in the action of these enzymes. Its proximity to the catalytic site as well as its proximity to Val-103 (Herzberg and Moult, 1987) suggests that perturbations at this site might disrupt activity. Val-103 lines a gully in the catalytic site where model-building studies suggest that it may be a key residue in determining binding properties as well as substrate specificity (Herzberg and Moult, 1987; Moews et al., 1990; Knox and Moews, 1991). Tyr residues are commonly observed to participate in hydrogen-bond formation through their hydroxyl group. Thus, in addition to acting as acid/base catalyses, they can particulate in maintaining the structural integrity of proteins (intramolecular hydrogen-bonding) or in the binding of substrate (intermolecular bonding). If the role of a given Tyr residue is important in maintaining enzyme structure then replacement by Phe could disrupt the local, if not the overall, organization of the enzyme, resulting in decreased catalytic efficiency of the enzyme.

The hydrolysis of β-lactams catalysed by Class A β-lactamases has been shown to involve a covalent acyl-enzyme intermediate with Ser-70. Although it has been postulated that the alkaline limb of the pH-activity profiles for the Class A β-lactamases arises from the deprotonation of Lys-234 or Lys-73, this may not be the case (Ellerby et al., 1990). An alternative candidate residue responsible for the decrease in catalysis at high pH (pH ≈ 9; range 8.5–10.5) is Tyr-105. Chemical-modification studies initially implicated Tyr as an essential catalytic residue in β-lactamase catalysis (Ambler, 1975), but subsequent more careful investigation indicated that Tyr was not essential for catalysis, the loss of activity on nitration of Tyr being due to intermolecular crosslinking (Bristow and Virden, 1978). Acetylation of Tyr resulted in loss of catalytic activity in the RTEM enzyme but not that from *Bacillus cereus* (Wolozin et al., 1982). The chemical-modification studies were assumed to result in modification predominantly at Tyr-105. The proximity of Tyr-105 to the other identified catalytic residues, Glu-166, Ser-130 and Lys-73, and its conserved nature, suggest that it may play a key structural or catalytic role in the catalytic mechanism. The present study was undertaken to clarify the role of Tyr-105 in catalysis and structural stability by its conversion into Phe through site-directed mutagenesis.

EXPERIMENTAL

Materials

Benzylpenicillin and phenoxymethylpenicillin were purchased from Sigma, nitrocefin was gift from Glaxo, PADAC was obtained from Calbiochem and sequenase was purchased from U.S. Biochemicals. The restriction enzymes, Klenow fragment and DNA ligase used for mutagenesis were obtained from New England Biolabs.

Mutagenesis

The Y105F mutant β-lactamase was made by the methods described previously (Escobar et al., 1991). Mutations were introduced via the Kunkel method (Kunkel, 1985) and sequenced with Sequenase. The primer used for mutagenesis was 5′-GAT-CTT-GTT-AAC-(TTC)-AAC-CCG-ATT-ACG-3′. This sequence replaces Tyr-105 with Phe (in parentheses) and introduces a *Hpa* I site (underlined). Thus, digestion of the mutant sequence with *Hpa* I allows for restriction purification (Wells et al., 1986). The Y105F sequence was confirmed by dideoxy-sequencing (Sanger et al., 1977) with the aid of Sequenase. The primer

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sequence used was 5′-ACA-GAA-ATC-AAT-AGA-AGA-TC-3, and was designed to sit 40 bp upstream of the mutagenesis site.

Purification of β-lactamase

The wild-type and mutant enzymes were purified as follows. *Bacillus licheniformis* β-lactamase was expressed in *Bacillus subtilis*. Fresh streaks were inoculated into 6 l of rich media (Ellerby et al., 1990) and grown on a 37 °C shaker for 12–14 h. Cells were removed by centrifugation (8000 rev./min for 30 min) and the cell-free supernatant dialysed against 0.02 M sodium acetate, pH 4.8. CM-Sepharose matrix (CL-6B from Sigma) was pre-equilibrated with the same buffer. The protein was equilibrated with CM-Sepharose matrix (15 ml of gel) for 1 h by gently swirling on a shaker at room temperature. This enzyme–matrix mixture was batch-loaded on to a CM-Sepharose column (50 ml of gel in 2.5–19.5 cm column) and eluted with a linear salt gradient of 0–0.25 M NaCl. Fractions containing β-lactamase activity were pooled and exchanged via ultrafiltration with an Amicon YM10 filter into 0.02 M Tris buffer, pH 7.2. This was equilibrated with DEAE-Sepharose (0.02 M Tris, pH 7.2, for 1 h with 10 ml of gel), batch-loaded on to a DEAE-Sepharose column (same size as above) and eluted with a linear salt gradient of 0–0.25 M NaCl. Fractions containing β-lactamase activity were pooled and exchanged into 0.05 M potassium phosphate buffer, pH 7.0, by ultrafiltration with a YM10 filter. Both wild-type and mutant enzymes were purified to homogeneity as determined by SDS/PAGE using the Pharmacia PhastSystem.

Kinetics

The pH rate profile for Y105F was obtained from complete progress curves and analysed by the method of Koerber and Fink (1987). All points were determined in triplicate. The hydrolysis of phenoxymethylpenicillin was monitored at 240 nm and 30 °C with a Hewlett–Packard 8452A diodearray spectrophotometer using a 1 cm path-length cell. All buffers contained 0.5 M KCl and were brought to a final concentration of 50 mM buffer. The buffers used for the pH profile were sodium acetate for pH 4.0–5.5, potassium phosphate for pH 6.0–7.5, Tris/HCl for pH 8.0–8.5 and Ches for pH 9.0–9.5. The kinetic properties of the mutant were also analysed with benzylpenicillin, nitrocefin and PADAC. These studies were done as described above in 50 mM potassium phosphate, 0.5 M KCl, pH 7.0. All four of these β-lactams are good substrates of the wild-type enzyme. The extinction coefficients and wavelengths used have been reported previously (Ellerby et al., 1990).

Circular dichroism measurements

The far-u.v. c.d. spectra were measured at pH 4.0, 7.0 and 9.0 using an AVIV model 60 DS instrument at 30 °C. Protein concentrations (final) were 11.5 mM for the Y105F mutant at all three pHs. The cell path length was 0.1 cm. The buffers used were: 50 mM potassium phosphate for pH 7.0, 50 mM sodium acetate for pH 4.0 and 50 mM Ches for pH 9.0. All these buffers contained 0.5 M KCl.

RESULTS AND DISCUSSION

Structural effects

As demonstrated in Figure 1 the secondary structure of β-lactamase is not appreciably affected by the Y105F mutation. The c.d. spectra of Y105F at pH 4, 7 and 9 are superimposable on that of the wild type under native conditions (pH 7.0). This finding argues against a key role for the hydroxyl group of Tyr-105 in maintaining the structure of the Class A β-lactamases, at least in a global sense. These results, however, do not preclude the possibility that there has been a small structural perturbation of the region neighbouring site 105. If this perturbation does not affect the overall secondary structure of the protein it might not by visualized by c.d.

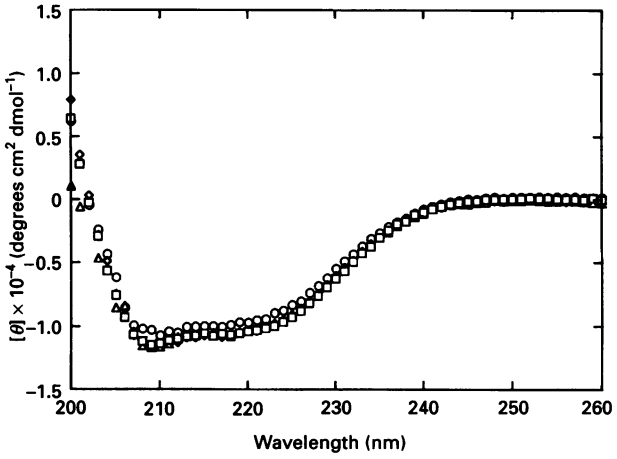

Figure 1 Circular dichroism spectra of Y105F at pH 4, 7 and 9

Spectra are superimposable on that of the native wild-type enzyme within experimental error. Studies were done at 30 °C. ◇, Y105F at pH 7; △, Y105F at pH 4; ○, Y105F at pH 9.0; □, wild-type enzyme at pH 7.0.

Table 1 Substrate specificity profile for wild-type and Y105F *B. licheniformis* β-lactamase

Units for $k_{cat.}$, K_m and $k_{cat.}/K_m$ are s^{-1}, mM and $M^{-1} \cdot s^{-1}$ respectively.

	Wild-type β-lactamase*			Y105F β-lactamase			
Substrate	$k_{cat.}$	K_m	$k_{cat.}/K_m$	$k_{cat.}$	K_m	$k_{cat.}/K_m$	Rel†
Benzylpenicillin	2650	0.12	2.15×10^7	3760	0.33	1.12×10^7	0.52
Phenoxymethylpenicillin	2552	0.1	2.48×10^7	2305	0.2	1.15×10^7	0.46
Nitrocefin	1088	0.041	2.64×10^7	720	0.19	3.8×10^6	0.14
PADAC	667	0.14	4.63×10^6	457	0.26	1.76×10^6	0.40

* Wild-type values are from Ellerby et al. (1990).

† Rel values are ratios of $k_{cat.}/K_m$ (Y105F)/$k_{cat.}/K_m$ (wild type). S.D. $< \pm 6.0\%$ for wild type and $< \pm 10.0\%$ for Y105F.

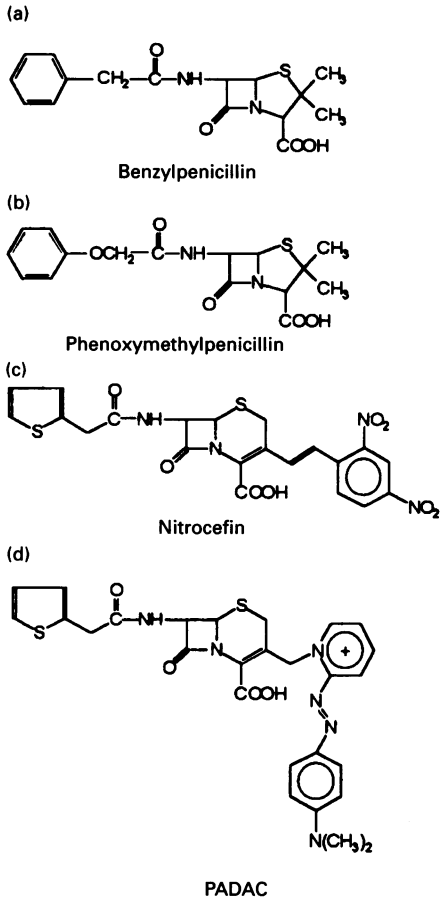

Figure 2 Substrates used to characterize Y105F β-lactamase

Two representative penicillins (a,b) and two cephalosporins (c,d) were used to check for differential effects of the mutation on catalysis for these two types of β-lactam antibiotics.

Kinetics

As shown in Table 1 the $k_{cat.}$ values for the reaction of Y105F with the four substrates used in this study (Figure 2) approximate those of the wild-type enzyme. Interestingly, although the $k_{cat.}$ is slightly decreased for three of the four substrates used in this study the value for benzylpenicillin has increased by ~25%. Moreover, as indicated by the values for K_m in Table 1, it appears that all the substrates bind less tightly to Y105F. The values of K_m are increased between 2- and 3-fold in the Y105F mutant when compared with the wild-type values. The observed differences in $k_{cat.}$ and K_m might indicate that the structure of the catalytic site has been altered. However, since the detected differences in the kinetic constants are small (within the same order of magnitude) any modifications to the structure of the catalytic site are probably small.

Activity (k_{cat} and $k_{cat.}/K_m$)–pH profiles were fit to the expression in eqn. (1).

$$k_{obs.} = k_{lim.}/(1+[H^+]/K_1+K_2/[H^+]) \quad (1)$$

The $k_{cat.}$–pH profile of the Y105F enzyme is very similar to that of the wild-type enzyme with phenoxymethylpenicillin (Figure 3). The pK_1 with Y105F is 4.5±0.1, which is the same as the wild-type value (Ellerby et al., 1990). The ionization constant, pK_2, for the alkaline limb is increased slightly however, with a value of 8.6±0.1 for Y105F compared with 8.1±0.1 for the wild-type enzyme (Table 2). The limiting values for $k_{cat.}$ are similar in the wild type and Y105F enzymes.

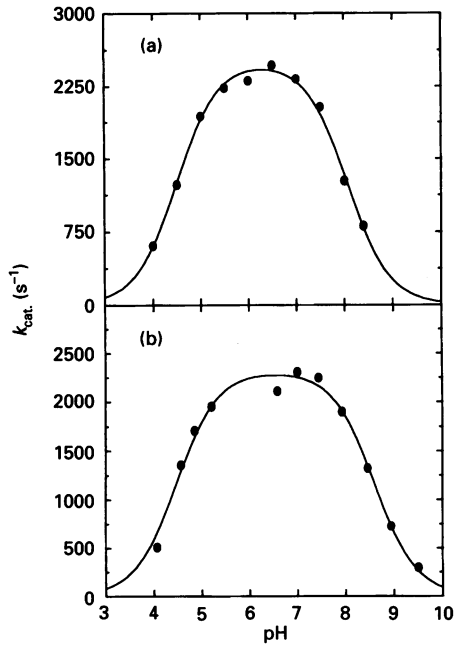

Figure 3 $k_{cat.}$–pH profiles for (a) wild-type and (b) Y105F β-lactamase

Studies done with phenoxymethylpenicillin at 30 °C. pK_a values for the two profiles are listed in Table 2.

Table 2 pKa values for wild type and Y105F β-lactamase with phenoxymethylpenicillin

	Wild type*		Y105F	
	pK_1	pK_2	pK_1	pK_2
$k_{cat.}$	4.5±0.1	8.1±0.1	4.5±0.1	8.6±0.1
$k_{cat.}/K_m$	5.0±0.1	8.6±0.1	5.3±0.1	8.8±0.1

* Wild-type values are from Ellerby et al. (1990)

By comparing the K_m–pH profiles for the mutant Y105F and wild-type enzymes we can see that there seems to be a slight decrease in the affinity of Y105F for phenoxymethylpenicillin (Figure 4), the K_m is approximately 2-fold greater for Y105F than wild type across the pH range studied. However, the pH-dependence of K_m of the mutant and wild-type enzymes are very similar. The effect on K_m could represent either an effect on K_s, the intrinsic affinity of the enzyme for the substrate, or may just be reflection of a change in the deacylation rate, k_3, if acylation is not rate-limiting. For the acyl-enzyme kinetic scheme:

$$E+S \overset{K_s}{\rightleftharpoons} ES \overset{k_2}{\rightarrow} EA \overset{k_3}{\rightarrow} E+P$$

$K_m = k_3K_s/(k_2+k_3)$. If k_3 were rate-limiting, then $k_{cat.}$ would equal k_3; since $k_{cat.}$ for phenoxymethylpenicillin is decreased only by 10% in the mutant, one would expect K_m to be decreased comparably. Thus the effect of the Y105F mutation on K_m, for this substrate at least, appears to reflect an effect on K_s.

The $k_{cat.}/K_m$–pH profile of the mutant Y105F is also similar to

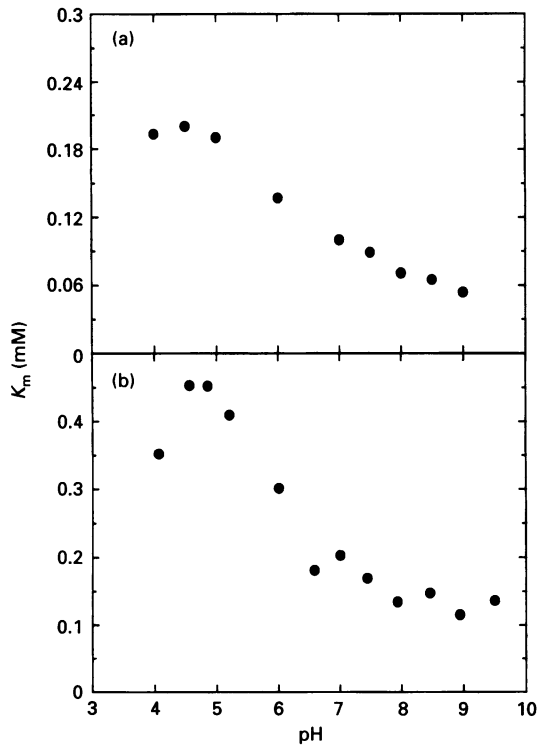

Figure 4 K_m–pH profile for (a) wild-type and (b) Y105F β-lactamase with phenoxymethylpenicillin at 30 °C

Wild-type values from EHerby et al. (1990).

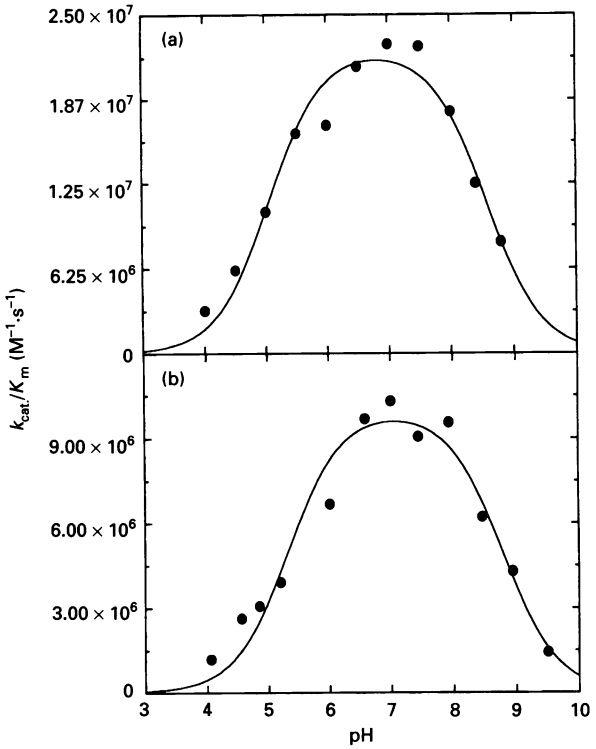

Figure 5 $k_{cat.}/K_m$–pH profile for (a) wild-type and (b) Y105F β-lactamase with phenoxymethylpenicillin at 30 °C

Wild-type values are from Ellerby et al. (1990).

that of the wild-type enzyme (Figure 5). The values for pK_1 and pK_2 for $k_{cat.}/K_m$ (Table 2) are similar, indicating that the pK values of the key ionizing groups in the catalytic mechanism are unaffected by the substitution of Phe for Tyr-105. The limiting values of the Y105F profile are approximately one-half those of the wild-type profile (Table 1). This difference arises from the increased K_m values seen in Figure 3. Most importantly, we note that the alkaline limb has not been abolished by the mutation. Thus it is clear that the ionization of the Tyr-105 hydroxyl group is not responsible for the alkaline limb of the pH-rate profiles.

Observations in other studies on β-lactamase catalysis suggest that electrostatic effects are very important in its catalysis. Although Tyr-105 is not an essential catalytic residue, as confirmed by these results, it is apparent, nevertheless, that this residue does affect the catalytic process. In the case of the activated cephalosporin substrate, nitrocefin, the overall catalytic efficiency of the mutant is only 14% of that of the wild-type. For the other substances examined, the catalytic efficiency was 40–50% of that of the wild-type enzyme. In each case, the major effect of the mutation was on K_m, suggesting that the main effect of the substitution of Phe for Tyr-105 is perturbation of substrate binding.

Why is Tyr-105 so highly conserved?

Clearly the mutation of Tyr-105 to Phe does not severely disrupt the function of β-lactamase. If the degree to which this residue is conserved throughout the β-lactamase family indicates its importance to the action of these enzymes, then we can conclude that it is not the hydroxyl moiety of Tyr-105 which is important. Consequently, it is likely that it is the hydrophobic phenyl group that is contributing to the stability and integrity of the active site. It is interesting to note that a Tyr residue has been implicated in the catalytic mechanism of the Class C β-lactamases (Oefner et al., 1990).

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REFERENCES

Ambler, R. P. (1975) Biochem. J. **151**, 197–218
Ambler, R. P. (1980) Phil. Trans. R. Soc. London B **289**, 321–331
Bristow, A. F. and Virden, R. (1978) Biochem. J. **169**, 381–388
Ellerby, L. Escobar, W., Fink, A., Mitchinson, C. and Wells. J. (1990) Biochemistry **29**, 5797–5806
Escobar, W., Tan, A. and Fink, A. (1991) Biochemistry **30**, 10783–10787
Herzberg, O. and Moult, J. (1987) Science **236**, 694–701
Joris, B., Ghuysen, J.-M., Dive, G., Renard, A., Dideberg, O., Charlier, P., Frere, J.-M., Kelly, J. A., Boyington, J. C., Moews, P. C. and Knox, J. R. (1988) Biochem. J. **250**, 313–324
Knox, J. R. and Moews, P. C. (1991) J. Mol. Biol. **220**, 435–455
Koerber, S. C. and Fink, A. L. (1987) Anal. Biochem. **165**, 75–87
Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 488–492
Moews, P. C., Knox, J. R., Dideberg, O., Charlier, P. and Frere, J.-M. (1990) Proteins Struct. Funct. Genet. **7**, 156–171
Oefner, C., D'Arcy, A., Daly, J. J., Gubernator, K., Charnas, R. L., Heinze, I., Hubschwerten, C. and Winkle, F. K. (1990) Nature (London) **343**, 284–288
Sanger, F., Nicklen, S. and Coulsen, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
Wells, J. A., Cunningham B. C., Graycar, T. P. and Estell, D. (1986) Philos. Trans. R. Soc. London, A **317**, 415
Wolozin, B., Myerowitz, R. and Pratt, R. (1982) Biochim. Biophys. Acta **701**, 153–163

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