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Site-saturation studies of β-lactamase: Production and characterization of mutant β-lactamases with all possible amino acid substitutions at residue 71

(mutagenesis/enzymatic catalysis/protein structure–function relationships/protein stability)

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ABSTRACT **A mutagenic technique that "saturates" a particular site in a protein with all possible amino acid substitutions was used to study the role of residue 71 in β-lactamase (EC 3.5.2.6). Threonine is conserved at residue 71 in all class A β-lactamases and is adjacent to the active site Ser-70. All 19 mutants of the enzyme were characterized by the penam and cephem antibiotic resistance they provided to *Escherichia coli* LS1 cells. Surprisingly, cells producing any of 14 of the mutant β-lactamases displayed appreciable resistance to ampicillin; only cells with mutants having Tyr, Trp, Asp, Lys, or Arg at residue 71 had no observable resistance to ampicillin. However, the mutants are less stable to cellular proteases than wild-type enzyme is. These results suggest that Thr-71 is not essential for binding or catalysis but is important for stability of the β-lactamase protein. An apparent change in specificity indicates that residue 71 influences the region of the protein that accommodates the side chain attached to the β-lactam ring of the substrate.**

The creation of mutant proteins with specific changes in amino acid sequence by oligonucleotide-directed mutagenesis (1, 2) affords a general method for studies of the relationship between structure and function. Recently, this approach has been applied to several enzymes, including trypsin (3), dihydrofolate reductase (4), aspartate carbamoyltransferase (5), tyrosyl-tRNA synthetase (6, 7), triosephosphate isomerase (8, 9), lysozyme (10), and β-lactamase (11, 12). Insights obtained from x-ray crystallography, computer modeling, sequence homologies, and catalytic mechanism greatly assist in predicting the consequences of a particular mutation on protein function. However, even then a particular amino acid substitution often causes a surprising change in activity; a thorough study of the role of an important residue requires substitution with all 19 other amino acids (site saturation). Such a study was recently reported (13) for residue 12 in the c-Ha-*ras* 1 gene; in this case the mutants were generated by oligonucleotide-directed mutagenesis. All 19 amino acid substitutions were also produced for residue 222 in subtilisin by a technique similar to the one used here (14).

As an approach to structure–function studies, site saturation becomes particularly attractive under two conditions: (*i*) a procedure exists for efficiently generating the appropriate mutants and (*ii*) the protein in question affects the phenotype of cells such that one can easily screen for mutants that perform a particular function. One can extend this approach to the simultaneous saturation of two or more sites in a protein to assess the effect of combinations of residues on protein function or, perhaps, to create a novel activity.

Site saturation can be accomplished by introducing into the gene at the codon for the residue of interest a mixture of nucleotides: A, T, G, and C at the first two positions, G and C at the third position. This mixture of oligonucleotides, which codes for all 20 amino acids and the amber codon, can be readily introduced into the gene as a "cassette" when unique restriction sites are conveniently nearby. (If desired, appropriate mixtures of oligonucleotides can be used to encode a particular subset of amino acids.)

We have applied site saturation to the study of pBR322-encoded β-lactamase (EC 3.5.2.6). This is an RTEM-1 enzyme (12, 15) originally taken from R factor R1 (16, 17) that catalyzes the hydrolysis of the β-lactam ring of penam and cephem antibiotics (18). This activity confers resistance to these antibiotics on cells that produce the enzyme and thereby provides a convenient screening procedure to assess the activities of mutants. The class A β-lactamases contain a conserved triad (Ser-Thr-Xaa-Lys) (19) at the catalytic site; this site includes Ser-70, whose hydroxyl group opens the β-lactam ring (20, 21). This study focuses on the conserved Thr-71, whose role in the activity of β-lactamase is unknown.

Restriction sites that flank the codon for Thr-71 were introduced into pBR322 by oligonucleotide-directed mutagenesis; an *Ava* I site at nucleotide 3972 and a *Sca* I site at nucleotide 3937 were introduced. The DNA fragment between nucleotides 3972 and 3937 (Pro-62 to Val-74 in β-lactamase) was removed and replaced with a mixture of synthetic double-stranded oligonucleotides that included 32 codons for residue 71 (codons for 20 amino acids and the amber codon). The resulting mixture of plasmids was used to transform *Escherichia coli* and the colonies were screened for their sensitivity to penam and cephem antibiotics. Surprisingly, cells containing any of 14 of the mutant β-lactamases displayed appreciable resistance to ampicillin; only cells containing mutants with Tyr, Trp, Asp, Lys, or Arg at residue 71 had no observable resistance to ampicillin. The mutant proteins show significantly increased sensitivity to proteolysis.

MATERIALS AND METHODS

Restriction enzymes and the large (Klenow) fragment of DNA polymerase I were purchased from Boehringer Mannheim. The T4 DNA ligase and T4 DNA kinase were obtained from Bethesda Research Laboratories. All antibiotics were from Sigma. The [α-^{32}P]dTTP, 3000 μCi/mmol (1 Ci = 37 GBq), was purchased from Amersham and [γ-^{32}P]ATP was from ICN.

Oligonucleotides were synthesized by using the phosphoramidite chemistry (22) on the Applied Biosystems (Foster City, CA) DNA synthesizer, model 380A. Mixed oligonucleotides were produced by using an equimolar mixture of the four nucleotide phosphoramidites or an equimolar mixture of G and C nucleotide phosphoramidites in a normal

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coupling reaction. The oligonucleotides were purified by preparative polyacrylamide gel electrophoresis.

Bacteriophage M13 mp 8 was grown in *E. coli* JM 103 (23) in 2× YT medium and prepared according to standard procedures (24). Plasmid pBR322 was grown in *E. coli* LS1 (25) in LB medium (28). Plasmid purification (26) and cell transformations (27) were done by using standard procedures. Selection involved growth in the presence of tetracycline at 15 mg/liter or ampicillin at 100 mg/liter unless otherwise stated. Numbering of pBR322 nucleotides is the one commonly used (28).

Oligonucleotide-directed mutagenesis was carried out on M13 mp 8 containing the 752-base-pair *Pst* I–*Eco*RI fragment of pBR322. An *Ava* I site was introduced by changing T to C at residue 3972 and a *Sca* I site was introduced by changing A to T at residue 3939. Procedures used for the mutagenesis are described elsewhere (29, 30). To remove the preexisting *Sca* I site at 3846 on pBR322, the 61-base-pair segment between *Hin*cII at 3907 and *Sca* I at 3846 was removed and replaced by a 61-base-pair fragment that changed G to A at 3846.

To introduce the 20 amino acids at site 71, the following mixture of oligonucleotides was used.

$$5'\text{-CC-GAG-GAA-CGT-TTT-CCA-ATG-ATG-AGC}\begin{bmatrix}\text{AA}\\ \text{CCC}\\ \text{GGG}\\ \text{TT}\end{bmatrix}\overset{*}{\text{TTC}}\text{-AAA-GT}$$
$$\text{C-CTT-GCA-AAA-GGT-TAC-TAC-TCG}\begin{bmatrix}\text{AA}\\ \text{CCC}\\ \text{GGG}\\ \text{TT}\end{bmatrix}\text{AAG-TTT-CA-5}'$$

The asterisk indicates a silent mutation that removes a *Dra* I restriction site at 3943 and also decreases the self-complementarity of 6 (and possibly as many as 10) bases near this end of the individual strands. After purification, as described above, the mixtures of oligonucleotides were individually phosphorylated. They were then annealed by mixing 0.4 pmol/μl of each strand and heating to 95°C in 10 mM $MgCl_2$/50 mM Tris·HCl, pH 8, followed by gradual cooling to 20°C over a 45-min period. The resulting mixture of double-stranded oligonucleotides (0.4 pmol) was mixed with the *Sal* I to *Sca* I fragment (0.04 pmol) and the *Ava* I to *Sal* I fragment (0.04 pmol) from pBR322-CR7 and pBR322-XN1, respectively (see Fig. 1). This mixture was ligated in 10 mM $MgCl_2$/50 mM Tris·HCl, pH 8/0.5 mM ATP/5 mM dithiothreitol containing 10 units of T4 DNA ligase in a total volume of 50 μl at 15°C for approximately 18 hr. The reaction mixture was extracted with phenol and the DNA was precipitated by addition of ethanol. The DNA was redissolved in 20 μl of 1 mM Tris·HCl, pH 8/0.1 mM EDTA; an aliquot (5 μl) was used for transformation of *E. coli* LS1 cells. After the transformation, 1/10th of the cells were plated on tetracycline, producing 423 colonies. From these, 108 individual colonies were picked and inoculated onto tetracycline-containing plates and were also tested for activity on the various substrates of β-lactamase. Plasmids derived from these 108 colonies were sequenced.

For sequencing, the plasmids were digested with *Ava* I and the resulting 2962-base-pair fragment was isolated from a 1.2% agarose gel by using DEAE-paper or the International Biotechnologies (New Haven, CT) UEA electroelutor. The fragment was labeled at T-3973 by using [α-^{32}P]dTTP and the Klenow fragment of DNA polymerase I. The labeled fragments were sequenced by using standard techniques (31).

In L agar plates (28), an antibiotic concentration gradient was generated by raising one end of a Petri dish (8.5-cm diameter) 5 mm and pouring 15 ml of L agar containing an appropriate concentration of antibiotic into the tilted dish. These were allowed to dry overnight. The plates were then placed on a flat horizontal surface and 15 ml of L agar was poured on top; they were used immediately upon hardening. An aliquot (50 μl) of a 1:10^5 dilution of a saturated culture (approximately 2 × 10^9 cells per ml) was spread over half of the plate. A standard mutant was plated on the other half; standards used were as follows: for ampicillin and benzylpenicillin at 37°C and for 6-aminopenicillanic acid at 30°C, Thr-71 → Leu; for 6-aminopenicillanic acid at 37°C, Thr-71 → Cys; for ampicillin and benzylpenicillin at 30°C, Thr-71 → Glu.

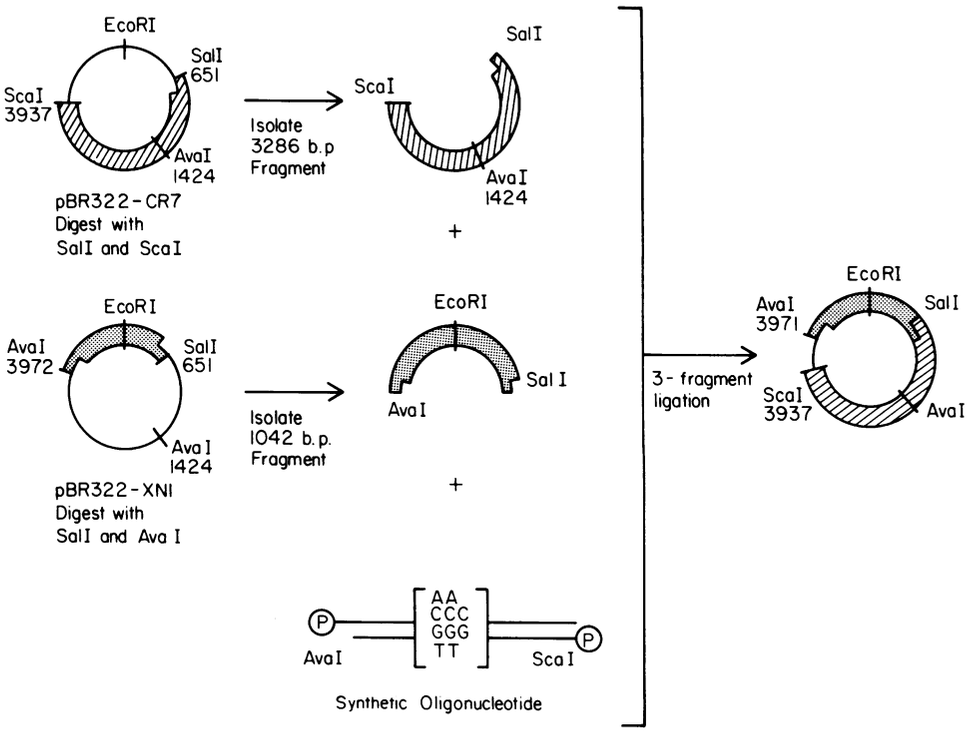

FIG. 1. Design of three-fragment ligation for inserting the mixture of oligonucleotides. bp, Base pair.

Protein for electrophoretic transfer blots was prepared by pelleting 1×10^9 *E. coli* LS1 cells from a late-logarithmic-phase culture containing tetracycline at 15 mg/liter in LB medium. These cells were resuspended in 100 μl of 10% (vol/vol) glycerol/5% (vol/vol) 2-mercaptoethanol/3% sodium dodecyl sulfate/62.5 mM Tris·HCl, pH 6.8, and incubated 10 min at 95°C. An aliquot (20 μl) was loaded onto a 15-cm 12% polyacrylamide gel with a 2-cm 4% stacking gel. After electrophoresis, protein blots were prepared according to the Bio-Rad procedure (32); the transfer was at 50 V for 5 hr. Visualization was according to the Vectastain procedure (33) using rabbit antibody to β-lactamase.

RESULTS

Introduction of the *Ava* I 3972 and *Sca* I 3937 sites and removal of the *Sca* I 3845 site were verified by restriction mapping of the plasmids. The absence of other changes in the β-lactamase gene was verified by sequencing one of the mutants from the *Pst* I site through the residues of the structural gene encoding the protein N terminus.

After ligation of the mixture of oligonucleotides into the plasmid, transformation of competent *E. coli* LS-1 cells gave 423 colonies that were resistant to tetracycline. From these 423 colonies, 108 were picked and inoculated onto tetracycline plates and were also tested for resistance to ampicillin (10, 50, 100, and 500 mg/liter), benzylpenicillin (100 mg/liter), 6-aminopenicillanic acid (10, 25, 50, and 100 mg/liter), cephalothin (25, 50, and 100 mg/liter), and cephalexin (25 and 50 mg/liter). Ampicillin and cephalothin plates were incubated at 30°C and 37°C; all others were incubated only at 37°C. This phenotype screening indicated that a change in antibiotic specificity and reduced stability, particularly at 37°C, were characteristics of some of the mutants.

Plasmids derived from 105 of the colonies were sequenced from *Ava* I (3972) through *Sca* I (3937); Table 1 lists the codon frequencies in this collection of colonies. The sequences were matched to the phenotypes determined above. Mutants with Tyr, Trp, Asp, Lys, or Arg at position 71 gave no resistance to any of the five antibiotics. A mutant with Phe was resistant to ampicillin at 100 mg/liter at 30°C but sensitive even to low levels of ampicillin at 37°C. Mutants with Gly, Gln, or Glu produced resistance up to 100 mg of ampicillin or benzylpenicillin per liter at 37°C. Mutants with Ala, Val, Leu, Ile, Pro, His, Cys, Ser, Thr, or Asn gave resistance to ampicillin at >500 mg/liter and benzylpenicillin at >100 mg/liter. Only mutants with His, Cys, Ser, or Thr were resistant to 6-aminopenicillanic acid at >100 mg/liter, those with Gly, Ala, Val, Leu, Ile, Pro, Glu, or Asn were resistant to 25 mg/liter, and those with Met or Gln (although resistant to ampicillin) showed no resistance to 6-amino-penicillanic acid. Only the wild-type enzyme (Thr-71) conferred resistance to cephalothin and cephalexin at 37°C. A mutant, Thr-71 → Ser, was resistant to cephalothin at 50 mg/liter at 30°C, but none of the other mutants grew in the presence of this antibiotic even at 30°C.

Table 1. Distribution of codons for residue 71

Codon	No.	Codon	No.	Codon	No.	Codon	No.
AAC	2	CAC	3	GAC	2	TAC	8
AAG	6	CAG	3	GAG	3	TAG	2
ACC	4	CCC	1	GCC	5	TCC	5
ACG	2	CCG	2	GCG	4	TCG	3
AGC	3	CGC	0	GGC	2	TGC	4
AGG	4	CGG	2	GGG	1	TGG	5
ATC	6	CTC	1	GTC	0	TTC	1
ATG	7	CTG	2	GTG	2	TTG	6

In addition to these 101 point mutations, there were three deletions at the *Sca* I site, six insertions at the *Ava* I site, one deletion at the *Ava* I site, four insertions in the codon for residue 71, and one insertion in the synthetic segment.

More accurate values for the level of antibiotic resistance provided to cells by the mutant proteins were determined by spreading cells onto an agar plate with a continuous concentration gradient of antibiotic. The maximal concentration at which colonies were established could then be observed. These phenotypic characteristics are summarized in Table 2. The values were independent of dilutions of cells higher by a factor of 2 or lower by a factor of 10 than those used in the experiments summarized in Table 2. Particularly noteworthy are the resistances at 37°C of cells producing the mutants Thr-71 → Ile and Thr-71 → His. In addition, the differences in levels of resistance at 30°C versus those at 37°C are especially dramatic.

Protein boiled in sodium dodecyl sulfate after extraction from cultures of the various mutants grown at 30°C and 37°C was electrophoresed in denaturing sodium dodecyl sulfate/polyacrylamide gels and then transferred to nitrocellulose. Fig. 2 shows antibody stains of these blots. Protein of the same size as β-lactamase is present for all mutants, but the quantity varies substantially for different mutants; this variation is greatly reduced at 30°C. We believe that the protein responsible for the band slightly above β-lactamase is pre-β-lactamase because it represents a protein of appropriate size and because this band is absent for the Thr-71 → amber mutant. The blots suggest that the mutant proteins are processed normally but that mature mutant proteins are degraded, probably by *E. coli* proteases.

DISCUSSION

Importantly, for site-saturation studies, one should use mixtures of synthetic oligonucleotides having all specified codons at nearly equal frequencies; if codons for certain amino acids are present at low frequencies, the corresponding mutants will be underrepresented, difficult to find, and

Table 2. Maximal level of resistance of strains with mutations at β-lactamase residue 71

Amino acid	Maximal antibiotic concentration (mg/liter) at which colonies grew					
	Ampicillin		Benzyl-penicillin		6-Amino-penicillin	
	30°C	37°C	30°C	37°C	30°C	37°C
Gly	>500	Trace	>500	50	35	20
Ala	>500	75	>500	100	Trace	Trace
Val	>500	150	>500	175	75	Trace
Leu	>500	100	>500	125	80	Trace
Ile	>500	>500	>500	>500	90	40
Met	>500	125	>500	150	30	0
Pro	>500	200	>500	350	50	40
Phe	60	0	0	0	0	0
Trp	0	0	0	0	0	0
Tyr	0	0	0	0	0	0
His	>500	>500	>500	>500	>250	>250
Cys	>500	>500	>500	>500	>250	100
Ser	>500	>500	>500	>500	>250	>250
Thr	>500	>500	>500	>500	>250	>250
Asn	270	Trace	210	Trace	55	Trace
Gln	130	20	70	Trace	55	0
Asp	0	0	0	0	0	0
Glu	200	Trace	70	Trace	Trace	Trace
Lys	0	0	0	0	0	0
Arg	0	0	0	0	0	0
Stop	0	0	0	0	0	0

A > indicates that this value was the highest level of antibiotic tested.

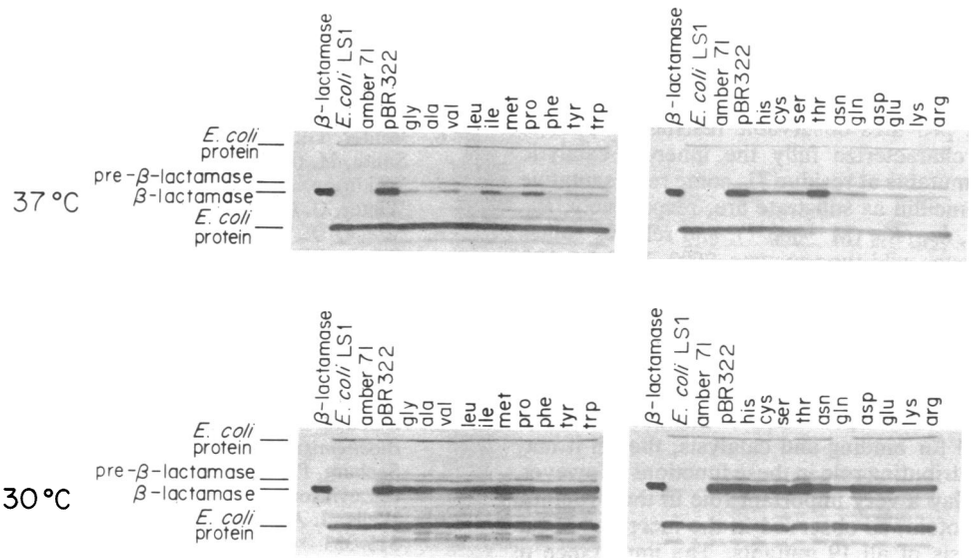

FIG. 2. Blots of β-lactamases with the 19 amino acid substitutions at residue 71. The blots were treated with rabbit anti-β-lactamase antibody and visualized by using the horseradish peroxidase assay (see ref. 33).

may go untested in the phenotype screening, particularly when multiple sites are involved. Nearly equimolar mixtures of oligonucleotides can be obtained by careful synthesis (S. Horvath, personal communication) and then used in cassette mutagenesis to generate the various mutations with known frequencies. In a Poisson distribution the probability of finding a particular codon at least once among 101 colonies is 96%. As shown in Table 1, 94% (30/32) of the codons were observed one or more times. The absence of two codons, therefore, was probably the consequence of a small sample size.

In sequencing plasmids from 105 individual colonies we found none in which the 35-base-pair segment contained unexpected base substitutions. However, 14 frameshift mutants resulting from single-base deletions or additions were observed (see Table 2). The 9 additions and deletions at restriction sites may occur during processing of the synthetic fragment or during ligation; similar deletions have been observed, for example, in ligation of a synthetic fragment into the *Pst* I site of the gene for subtilisin (14). The other 5 mutations that occur within the synthetic fragment probably result from imperfections in the DNA synthesis. None of the colonies containing plasmids with these frameshift mutations show any resistance to penam or cephem antibiotics.

The antibiotic resistances of colonies containing the 19 mutant β-lactamases were initially determined by picking colonies and patching them onto agar plates with various levels of antibiotics. More accurate values were measured by spreading mutant colonies onto agar plates having a linear concentration gradient of antibiotics (see Table 2). This latter method gives consistently lower values for antibiotic resistance, as in this case individual cells must establish colonies, whereas when colonies are picked and applied in patches many cells are initially present at a single site, allowing them to cooperate in inactivating sufficient antibiotic to allow growth. Both sets of data are, however, useful, as colonies with mutants having low lactamase activity would be classified as inactive if only plating were used. In Table 2, colonies that grew when picked, but not when plated, are designated as having trace activity.

The antibiotic resistance of colonies containing mutant plasmids reflects many factors: plasmid replication, transcription, stability and ease of translation of the mutant mRNAs, stability of the pre-β-lactamases in the cytoplasm, possible differences in the rate of processing and secretion, stability of the proteins in the periplasm, and, finally, intrinsic enzymatic activity. With regard to the steps from transcription to translation, the codon used for a given amino acid at residue 71 did not alter observed antibiotic resistance; in all cases, a given amino acid mutant confers the same level of antibiotic resistance regardless of which codon was used (see Table 1).

Mutations may affect processing and secretion of proteins (34) and thereby alter the relative concentrations of mutant β-lactamases in the periplasm. Although mutants such as Thr-71 → Ser and Ser-70 → Thr and the double mutant Ser-70 → Thr, Thr-71 → Ser seem to be processed and secreted normally in *Salmonella typhimurium* (12), these mutations are unlikely to affect these processes as drastically as mutations such as Thr-71 → Trp, Tyr, Lys; even in these cases, however, abundant processed protein is apparent in cells growing at 30°C (see Fig. 2).

Resistance to thermal denaturation and proteolysis can greatly influence the *in vivo* concentration of mutant proteins (12, 35). Analysis by protein blots (Fig. 2) for cell lysates of colonies growing at 30°C and 37°C show significant differences in the concentrations of mutants. A band representing the precursor protein shows similar intensities for the mutants and wild type; the presence of normal amounts of precursor proteins suggests that processing and secretion have not been drastically altered for the mutants. However, the amounts of protein in the periplasm vary greatly for the mutants as contrasted to wild type, particularly for cells growing at 37°C, indicating that the mutants are more susceptible to proteolysis than the wild-type enzyme.

A surprising number of mutants with amino acid substitutions at Thr-71 display lactamase activity; indeed, of the 19 substitutions at residue 71 only 5 (Trp, Tyr, Lys, Arg, or Asp) give proteins that confer no observable resistance to ampicillin or benzylpenicillin. Enzymes with Gln, Glu, Asn, or Phe at residue 71 provide low-level resistance, but enzymes with all other amino acids at this site have high activity toward benzylpenicillin and ampicillin. Some preliminary conclusions about structure–function relationships can be drawn from these data. Mutants having amino acids with charged or very large side chains at residue 71 have at best very low activity, otherwise both polar and nonpolar side chains seem compatible with appreciable activity. When 6-aminopenicillanic acid is used to select for activity, mutants with amino acids having nonpolar side chains show decreased activity, whereas mutants with amino acids having polar side chains retain activity toward this antibiotic. All mutants show a dramatically decreased ability to confer resistance toward the

cephem antibiotics cephalothin and cephalexin. At 30°C, wild-type β-lactamase (Thr-71) confers resistance to at least 100 mg/liter while the mutant Thr-71 → Ser confers resistance only to low levels of the cephem antibiotics; at 37°C only wild-type enzyme provides observable resistance. Though we have yet to characterize fully the inherent catalytic activity of all the mutants at residue 71, some representative data for benzylpenicillin as substrate are, respectively, k_{cat} (sec^{-1}), K_m (μM), k_{cat}/K_m ($M^{-1} \cdot sec^{-1}$), and relative values for k_{cat}/K_m as follows: wild-type enzyme, 2000, 26, 7.7×10^7, and 1; Thr-71 → Ser, 300, 21, 1.6×10^7, and 0.21 (12); Thr-71 → Ile, 1530, 350, 4.4×10^7, and 0.57 (Y. H. Chang and J.H.R., unpublished results).

Because many mutants at residue 71, including those having amino acids with polar and nonpolar side chains, show appreciable activity toward penams, we conclude that Thr at 71 is not essential for binding and catalysis, though it may perform some contributing role in these functions. However, residue 71 does play a very important role in the structural stability of the protein, as observed in the increased sensitivity to proteolysis of all 19 mutants. The importance of branching at the β-carbon of Thr with a polar hydroxyl group and nonpolar methyl group is demonstrated, for example, by the increasing antibiotic resistance of cells growing at 37°C provided by the series of enzymes with Gly < Ala < Leu < Val. The increased thermal and proteolytic sensitivity relative to wild type of the mutant Thr-71 → Ser (12), lacking only the methyl group of Thr, further supports this view. The relatively high catalytic activities of Thr-71 → Cys and Thr-71 → Ser demonstrate the importance of the polar group. The reduced activity toward 6-aminopenicillanic acid of enzymes having amino acids at residue 71 with nonpolar side chains suggests that the side chains of residue 71 influence the hydrophobic or hydrophilic characteristics of the pocket that accommodates the substituent attached to the β-lactam ring of the substrates. This possibility is further emphasized by the lower activity of enzymes with Glu or Gln at residue 71 toward benzylpenicillin (neutral, nonpolar substituent) than toward ampicillin (positively charged, polar substituent).

All mutant enzymes confer on cells at best very low resistance toward cephalothin and cephalexin; this likely results from the inherently lower activity of β-lactamase against cephem antibiotics (36) and accords with results observed for mutations in other regions of the enzyme (37). This behavior also demonstrates the importance of the conserved residues at the catalytic site in providing an enzyme with high levels of catalytic activity and stability.

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