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Active-Site Mutants of β-Lactamase: Use of an Inactive Double Mutant To Study Requirements for Catalysis†

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ABSTRACT: We have studied the catalytic activity and some other properties of mutants of *Escherichia coli* plasmid-encoded RTEM β-lactamase (EC 3.5.2.6) with all combinations of serine and threonine residues at the active-site positions 70 and 71. (All natural β-lactamases have conserved serine-70 and threonine-71.) From the inactive double mutant Ser-70 → Thr, Thr-71 → Ser [Dalbadie-McFarland, G., Cohen, L. W., Riggs, A. D., Morin, C., Itakura, K., & Richards, J. H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6409–6413], an active revertant, Thr-71 → Ser (i.e., residue 70 in the double mutant had changed from threonine to the serine conserved at position 70 in the wild-type enzyme), was isolated by an approach that allows identification of active revertants in the absence of a background of wild-type enzyme. This mutant (Thr-71 → Ser) has about 15% of the catalytic activity of wild-type β-lactamase. The other possible mutant involving serine and threonine residues at positions 70 and 71 (Ser-70 → Thr) shows no catalytic activity. The primary nucleophiles of a serine or a cysteine residue [Sigal, I. S., Harwood, B. G., & Arentzen, R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7157–7160] at position 70 thus seem essential for enzymatic activity. Compared to wild-type enzyme, all three mutants show significantly reduced resistance to proteolysis; for the active revertant (Thr-71 → Ser), we have also observed reduced thermal stability and reduced resistance to denaturation by urea.

The application of in vitro mutagenesis techniques to problems in enzymology has recently acquired momentum as it allows study of diverse problems in protein biochemistry through the experimental tools of molecular biology. Such problems include studies of the role of specific residues in catalysis as in the case of trypsin and chymotrypsin (Craik et al., 1984), dihydrofolate reductase (Villafranca et al., 1983), tyrosyl-tRNA synthetase (Winter et al., 1982; Wilkinson et al., 1984), and β-lactamase (Dalbadie-McFarland et al., 1982; Sigal et al., 1982, 1984), the role of disulfide bridges in protein stability (Villafranca et al., 1983; Perry et al., 1984; Dalbadie-McFarland, 1985), and the structural requirements for functional leader sequences in the secretion of proteins such as β-lactamase (Kadonaga et al., 1984) and the outer membrane lipoprotein of *Escherichia coli* (Inouye et al., 1982). In this approach, the strategy of specific in vitro mutagenesis allows a systematic study of structural variants resulting from precise amino acid substitution(s) introduced within a protein in a rational manner. In a complementary strategy, requiring a selectable phenotype, one demands a particular function of a protein, generates structural variants by various techniques of random mutagenesis, and then screens the resultant variants for those that can perform the required function.

In a combination of these two strategies, specific in vitro mutagenesis can be used first to produce an inactive protein; then numerous methods can generate random mutants that can be screened for recovery of activity which can reflect either changes at the site of the original mutation(s) or changes at other sites. Implementation of this approach can be particularly effective if reversion from the inactive mutant to the wild-type enzyme is prevented as this avoids screening against a background of many active revertants which simply have regained the sequence, structure, and function of the parental protein. In achieving this objective, one can use, as the inactive progenitor, a mutant that requires at least two base changes to re-form the wild-type and allow random mutagenesis to occur under conditions in which only single base changes are likely in the mutants being screened.

An enzyme of considerable medical significance and

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mechanistic interest, β-lactamase also satisfies many of the requirements for study by the approach outlined. The enzyme confers a selectable phenotype and is an appropriate subject for in vitro mutagenesis; its gene is cloned and sequenced (Sutcliffe, 1978), and the enzyme is abundantly expressed, particularly when the expression is controlled by a highly efficient promoter such as tac (DeBoer et al., 1983) or trp (Sigal et al., 1984). An appropriate, catalytically inactive double mutant has been prepared that has Ser-70 → Thr, Thr-71 → Ser (5′-AGC ACT-3′ → 5′-AC*C T*CT-3′) (Dalbadie-McFarland et al., 1982). Mechanistic studies have strongly implicated Ser-70 as the residue directly involved in opening the β-lactam ring of penicillins and cephalosporins (Fisher et al., 1980; Knott-Hunziker et al., 1979). This residue is also part of a triad conserved in all lactamases of this class, *-Ser-Thr-*(Phe)-*Lys-* (Ambler, 1980). These two observations initially directed our attention to this region of the molecule, the site of several reported mutations. The β-lactamase double mutant Ser-70 → Thr, Thr-71 → Ser is catalytically inactive (Dalbadie-McFarland et al., 1982); thiolactamase (Ser-70 → Cys; Sigal et al., 1982) retains a relatively low level of activity (Table II); a mutant β-lactamase, from *Staphylococcus aureus*, only provisionally characterized as Thr-71 → Ile, is reported to be inactive (Ambler, 1980).

In this work, we have obtained the active mutant Thr-71 → Ser by the approach outlined. Spontaneous random mutation of the double mutant (Ser-70 → Thr, Thr-71 → Ser) led to a conversion of residue 70 back to serine, as in the wild type (i.e., 5′-ACC TCT-3′→5′-AGC TCT-3′). Necessarily, given the approach used, the serine of the double mutant is retained at position 71; this pseudorevertant therefore has the sequence -Ser-Ser-Phe-Lys- in the region of the active site and, as described below, shows considerable catalytic activity (~ 15% that of the wild type).

To complete our study of the combination of serine and threonine residues in this region, we also prepared the mutant Ser-70 → Thr by site-directed mutagenesis; Ser-70 → Thr shows no activity as a catalyst of penicillin hydrolysis.

Mutations may change properties of an enzyme other than catalysis such as the rate of processing and secretion, thermal stability, or resistance to proteolysis; consequently, these behavioral aspects of the mutants were studied as well as catalysis. Though these mutants seem to be processed and secreted in an essentially normal manner, they show considerably reduced resistance to thermal denaturation and proteolysis relative to wild-type β-lactamase.

Materials and Methods

Bacterial Strains. Mutagenesis and/or reversion experiments were done in *E. coli* strain LS1. *E. coli* JM103 served as a host for phage M13mp8 (Messing, 1983) used in DNA sequencing. Culture media was L broth or L agar unless otherwise indicated. *Salmonella* strains DB 4673, DB 4381, DB 6142, DB 7000, and DB 7609 and P22 phage derivatives P22bla 1752 and 4393 (a deletion mutant of 1752; Koshland & Botstein, 1980) were used in the transfer of β-lactamase mutants to P22bla and in pulse–chase experiments.

For growth of plasmids containing the tac promoter, *E. coli* strain D1210 was used as a host. This is a lac I^q derivative of HB 101.

DNA. Plasmid DNA was prepared by the alkaline lysis method (Maniatis et al., 1982). The plasmids used were pBR322 and its derivative carrying the double mutation Ser-70 → Thr, Thr-71 → Ser (Dalbadie-McFarland et al., 1982). The plasmid pKK carrying the wild-type β-lactamase gene under the control of the tac promoter (DeBoer et al., 1983) was obtained from John Rossi. Plasmid pKR2 has the *Eco*RI–*Pst*I fragment of the active-site revertant (Thr-71 → Ser) cloned into the corresponding site in pKK. Sequence determination was done by the dideoxy method (Sanger et al., 1977) on a 700 base pair (bp)[1] *Eco*RI–*Pst*I fragment subcloned into M13mp8; a synthetic oligonucleotide (5′-GCG GCG ACC GAG TTG-3′) complementary to the insert at a site 50 bp away from the sequence of interest was used as a primer.

Enzymes. Restriction enzymes and exonuclease III were obtained from BRL, T4 DNA ligase was obtained from New England Biolabs, and DNA polymerase (Klenow fragment) was from Boehringer-Mannheim.

Site-Specific Mutagenesis. Oligonucleotide-directed mutagenesis was carried out as previously described (Dalbadie-McFarland et al., 1982).

β-Lactamase Purification. The isolation procedure used was a modification of the method of Fisher et al. (1980), with two significant differences that were due to a significantly higher affinity of the pBR322 lactamase for DE-52 cellulose compared to that of RP1-encoded enzyme (Fisher et al., 1980) which probably reflects the difference in isoelectric points. The pBR322 enzyme (TEM-1) has a p*I* of 5.4, and the TEM-2, RP1 enzyme has a p*I* of 5.6 as a result of a single amino acid substitution at residue 39; TEM-1 has Gln (Sutcliffe, 1978) (we have confirmed the prediction from the DNA sequence of Gln at this position by determination of the amino acid sequence of the enzyme encoded by pBR322), and TEM-2 has Lys at this position (Ambler & Scott, 1978). The two changes are the use of ammonium sulfate precipitation in place of the first DE-52 column and an increase in the final buffer concentration of the DE-52 gradient column to 200 mM to elute the TEM-1 enzyme.

E. coli strain D1210 containing plasmid pKK or pKR2 was grown to late log phase in 11 l FB medium (25 g of tryptone, 7.5 g of yeast extract, 6 g of NaCl, 50 mL of 1 M Tris-HCl, pH 7.5, and 10 mg of ampicillin/L) in a New England Biosystems fermenter with vigorous aeration. The growth temperature was 37 °C for pKK and 30 °C for pKR2.

After the mixture was cooled to 4 °C, IPTG was added to 0.1 mM. After 30 min at 4 °C, the cells were collected by centrifugation and washed with 50 mM Tris-HCl, pH 7.0, and 0.1 mM IPTG.

Osmotic extrusion was done as described by Fisher et al. (1980). Sufficient solid ammonium sulfate was added to the active enzyme supernatant to reach 20% (w/v). After being stirred for 12 h at room temperature, the precipitate was collected by centrifugation and discarded. Additional ammonium sulfate was added to the supernatant to reach 66% (w/v). After being stirred at room temperature for 12 h, the precipitate was collected by centrifugation at 16000*g* for 45 min. This fraction was redissolved in distilled water and the insoluble portion removed by centrifugation at 10000*g* for 20 min. Activity recovered from the ammonium sulfate precipitation step was 70–100% with a 7-fold increase in specific activity. After removal of the ammonium sulfate residue by dialysis against 25 mM triethanolamine hydrochloride, pH 7.25, this solution was concentrated to 25 mL by ultrafiltration

[1] Abbreviations: Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; IPTG, isopropyl thiogalactoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; bp, base pair(s); EDTA, ethylenediaminetetraacetic acid. "RTEM β-lactamase" refers to enzymes whose isoelectric points and substrate profiles are similar to those properties for the plasmid-encoded β-lactamase isolated from the TEM strain of *Escherichia coli* (Datta & Kontomichalou, 1965). The numbering system used for RTEM-1 β-lactamase reflects a consensus for the various class A β-lactamases (Ambler, 1980).

(Amicon PM-10 membrane) and applied to a 2.5 × 30 cm DEAE-cellulose (DE-52) column equilibrated with the same buffer. The column was developed by using a linear triethanolamine gradient (25–200 mM, pH 7.25). The active fractions were pooled and concentrated to 2 mL by ultrafiltration, applied in two equal portions to a 1 × 50 cm column of Ultra Gel 54, and eluted with 20 mM Tris-HCl, pH 7.0. About 20 mg of pure protein can be isolated from an 11-L culture, representing an overall yield of approximately 40%. Inactive mutants were isolated by a similar procedure without using overproducing plasmid constructs and with a reduction in overall yield to 2–5 mg from an 11-L culture.

The stability of the revertant enzyme improves significantly after the ammonium sulfate precipitation, suggesting that the loss of activity may be due to degradation by proteases normally found in the periplasmic space of *E. coli*.

Enzyme Assays. During purification, the inactive mutant β-lactamases were assayed either by direct SDS–PAGE on 12% acrylamide gels and comparison with wild-type controls or by precipitating with rabbit anti-β-lactamase antibodies (see Immunoprecipitation).

Activities were determined by measuring the change in absorbance with time at 240 nm of a solution containing 1 mM benzylpenicillin (Sigma) and 100 mM sodium phosphate, pH 7.0. Standard assay temperature was 30 °C, maintained with a jacketed water bath. Protein concentrations were determined by using the abosrbance at 281 nm and published conversion factors (Fisher et al., 1980).

Immunoprecipitation. Antigen (~20 μg) and 15 μL of rabbit anti-β-lactamase antiserum were incubated on ice for 60 min in a 100-μL volume; 200 μL of reconstituted goat anti-rabbit immunoglobin immunobeads (Bio-Rad) was added to each sample and incubated on ice for an additional 30 min. The precipitate was collected and washed 3 times by resuspension in buffer (0.01 M Tris-HCl, pH 7.3, 0.15 M NaCl, and 0.5% Triton X-100). After removal of the final wash, 100 μL of elution buffer (0.063 M Tris-HCl, pH 6.8, 2% SDS, and 2% fresh β-mercaptoethanol) was added, and the pellet was resuspended by vortexing. The samples were boiled in a water bath for 2 min, and the precipitate was removed by centrifugation.

After addition of glycerol and Pyronin-Y, the supernatants were analyzed by electrophoresis through 12% SDS–polyacrylamide gels and visualized by silver staining (Merril et al., 1981).

Immune Blots. After electrophoresis on 12% SDS–polyacrylamide gels, protein was electrophoretically transferred to nitrocellulose (Schleicher & Schuell BA85) by using a Bio-Rad Trans-blot cell following manufacturer's instructions. The nitrocellulose was washed twice with 0.05% Tween-20 (Sigma), 10 mM sodium phosphate, pH 7.5, and 0.9% sodium chloride and then incubated overnight at 22 °C with 1% bovine serum albumin and 1% normal goat serum in the same buffer. After being washed 3 times in buffer, 50 mL of a 1:1000 dilution of rabbit anti-β-lactamase was added and the membrane incubated at 37 °C for 2.5 h. Bound antibody was visualized by using an anti-rabbit immunoperoxidase system (Vectastain ABC kit, Vector Laboratories).

Pulse–Chase Experiments. β-Lactamase mutants were transferred to phage P22bla, a variant of the temperate *Salmonella* phage P22 (Koshland & Botstein, 1980; Dalbadie-McFarland, 1985). Labeling of phage-encoded proteins after infection and pulse–chase experiments were done as described (Koshland & Botstein, 1982). A 30-s pulse of [^{35}S]methionine was used, and samples were taken every 20 s after addition of the chase mixture unless otherwise indicated. Each sample was transferred immediately into an Eppendorf tube containing gel-loading sample buffer preheated to 90 °C. The samples were analyzed by SDS–polyacrylamide gel electrophoresis using 12% gels (Laemmli, 1970); the gels were fixed in 10% acetic acid and 50% ethanol before being dried and exposed to Kodak XAR5 film at room temperature. Autoradiograms were traced by using a Cary 219 spectrophotometer with the appropriate attachments.

Determination of k_{cat} and K_m. All measurements were done at 30 °C in 0.1 M potassium phosphate, pH 7.0, by using a Beckman Acta CIII with 1-cm path-length cells. Hydrolytic cleavage of benzylpenicillin was observed at 240 nm, and cleavage of cephalothin was observed at 260 nm. The $\Delta\epsilon$ values used were 500 M^{-1} cm^{-1} for benzylpenicillin (Samuni, 1975) and 7700 M^{-1} cm^{-1} for cephalothin. Comparison of initial and final absorbances at these wavelengths confirmed the $\Delta\epsilon$ values.

Thermal Stability. Purified β-lactamase (0.1 mg/mL in 0.1 M potassium phosphate, pH 7.0) was incubated at the indicated temperatures. After the various times indicated, samples were removed and immediately assayed at 30 °C for remaining activity with benzylpenicillin as substrate.

Thermolysin Digestion. Thermolysin (0.2 mg) was added to 2 mL of β-lactamase solution (0.6 mg of β-lactamase, 2 mM calcium acetate, and 0.1 M Tris-HCl, pH 7.0), and the mixtures were incubated at the indicated temperature. After appropriate intervals, aliquots were removed and rapidly quenched by adding EDTA (pH 7.5) to 0.01 M and cooling on ice. Aliquots were assayed for remaining β-lactamase activity at 30 °C.

Stability in Urea. The enzymatic activities toward benzylpenicillin of the wild-type enzyme and the Thr-71 → Ser mutant were determined in the presence of various concentrations of urea in 0.1 M potassium phosphate, pH 7.0, at 30 °C and at protein concentrations of 0.1 mg/mL or below.

Resistance to Cellular Proteases. E. coli LS1 cells harboring pBR322 (wild-type or active-site mutants) were grown in L broth with 25 μg/mL tetracycline at 37 °C with vigorous shaking. Samples (1.5 mL) were taken at late log phase (A_{550} = 1.1–1.2) and 12 h later. Cells were pelleted, resuspended in 50 μL of gel-loading buffer (0.063 M Tris-HCl, pH 6.8, 2% SDS, and 5% β-mercaptoethanol), and boiled 10 min. Aliquots of supernatant (20 μL) were analyzed by immune blotting.

Results and Discussion

Active Revertant (Thr-71 → Ser). To explore ways in which the inactive double mutant (Ser-70 → Thr, Thr-71 → Ser) might revert to an active enzyme, *E. coli* strain LS1 harboring the double mutant plasmid was cultured in L broth to a cell density of 5 × 10^8 cells/mL and then plated on L agar containing 10 mg/L ampicillin. After incubation of the plates overnight at 37 °C, seven colonies were found that were resistant to this level of antibiotic. From each of these revertants, plasmid DNA was isolated and used to transform competent *E. coli* which were then plated on L agar containing 20 mg/L tetracycline. Transformants were obtained from six of the plasmids, and, of these six, five grew on both ampicillin and tetracycline. Therefore, five of the revertants had ampicillin resistance encoded on the plasmid.

Each of these transformants was colony-purified, and plasmid DNA was prepared. The resulting plasmids were digested with *Eco*RI and analyzed by gel electrophoresis on 1.2% agarose; one of these five plasmids appeared to have different size and restriction sites than pBR322 and has not

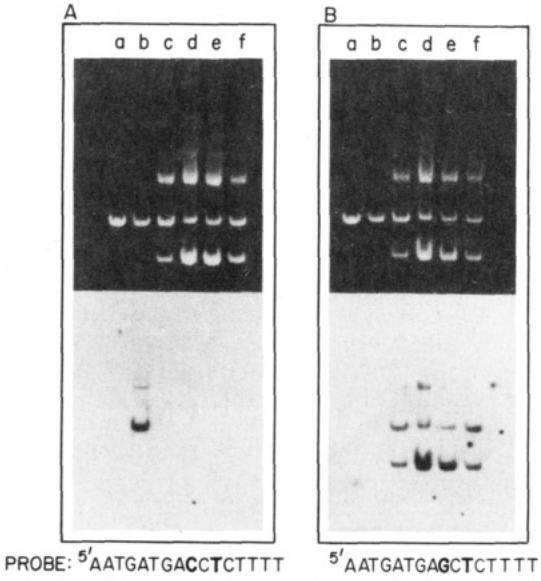

FIGURE 1: Hybridization of oligonucleotides coding for the sequences Thr-70-Ser-71 (panel A) and Ser-70-Ser-71 (panel B) to DNA from four revertants to ampicillin resistance. Plasmid DNA was linearized with *Eco*RI and analyzed by agarose gel electrophoresis. The gels were stained with ethidium bromide and photographed. They were then treated for 20 min at room temperature with 0.15 M NaCl and 0.5 M NaOH and neutralized in 0.15 M NaCl and 0.5 M Tris-HCl, pH 7.4. The gels were dried in a gel dryer, and the resulting membrane was hybridized to each of the oligonucleotides indicated. Hybridization conditions were as previously described (Dalbadie-McFarland et al., 1982). Each panel shows the ethidium bromide stained gel (top) and an autoradiogram of the dried gel after hybridization (bottom). Plasmid DNAs are (a) pBR322 (···AGCACT···), (b) DNA from the double mutant Ser-70 → Thr, Thr-71 → Ser [22G9 (···ACCTCT···)], and (c–f) DNA from the Amp^R revertants R2, R3, R4, and R6. Additional bands can be seen, one above and one below the linearized plasmid band; these correspond to supercoiled plasmid dimer and/or relaxed plasmid monomer (above) and to supercoiled plasmid monomer (below), due to incomplete digestion by *Eco*RI.

been further characterized. The four remaining plasmids were characterized by hybridization experiments, and one was further analyzed by DNA sequencing.

Figure 1 shows the results of a hybridization experiment in which the plasmid DNA from the four revertants and two controls, wild-type pBR322 and the parent double mutant, were probed with two synthetic oligonucleotides: 5′-^{32}P-A ATG ATG AC*C TC*T TTT-3′ previously used to obtain the double mutant (Ser-70 → Thr, Thr-70 → Ser) and 5′-^{32}P-A ATG ATG AGC T*CT TTT-3′ (Thr-71 → Ser). (The corresponding wild-type sequence found in pBR322 is 5′-A ATG ATG AGC ACT TTT-3′.)

The probe that matches the sequence of the parental DNA binds at discriminating temperatures only to this double mutant and not to wild-type pBR322 or to any of the four revertants (Figure 1, panel A). The probe that binds (equally well) to all four revertants (Figure 1, panel B) does not bind to either of the controls and corresponds to a sequence differing from the double mutant by a single base, coding for serine at both positions 70 and 71. DNA sequencing (Sanger et al., 1977) of one of the four revertants confirmed the sequence 5′-AGC T*CT-3′, which codes for Ser-70-Ser-71 at the active site (i.e., Thr-71 → Ser), in agreement with the hybridization results. These experiments emphasize that sequences differing by only one base can be reliably distinguished by hybridization to oligonucleotide probes (Wallace et al., 1979, 1981).

Only certain amino acid substitutions could have arisen by the method of random mutagenesis used in this experiment; these are summarized in Table I which lists those codons that

Table I: Codons Arising by a Single Base Substitution in Codons 70 and 71 of the β-Lactamase Double Mutant Ser-70 → Thr, Thr-71 → Ser

ACC (Thr-70) →	ACA (Thr)	ACG (Thr)	ACT (Thr)
	AAC (Asn)	AGC (Ser)	ATC (Ile)
	GCC (Ala)	CCC (Pro)	TCC (Ser)
TCT (Ser-71) →	TCA (Ser)	TCG (Ser)	TCC (Ser)
	TAT (Tyr)	TGT (Cys)	TTT (Phe)
	ACT (Thr)	GCT (Ala)	CCT (Pro)

can arise by a single base change for residue 70 or 71 in the double mutant. Given these restrictions, two serine codons are possible (AGC or TCC); however, only one (AGC) was obtained. This could reflect a common origin for all four of these revertants, or it could result from a higher incidence of CG to GC transversions, involving a transient CC or GG mismatch, than of AT to TA transversions, involving a transient AA or TT mismatch. In this regard, CC and AG mismatches are not corrected by the *Streptococcus pneumoniae* Hex mismatch repair system, whereas AA, and other, mismatches are corrected (Claverys et al., 1983). Similarly in *E. coli*, corrections of AA, GG, and TT mismatches occur at a significantly higher frequency than does correction of CC mismatches (Kramer et al., 1984; Dohet et al., 1985).

This approach allows only a few amino acid substitutions (five at position 70 and six at 71) as Table I illustrates. Accordingly, the possibly active reversion involving the change Thr-70 → Cys, to produce the double mutant Ser-70 → Cys, Thr-71 → Ser, would *not* be a likely mutant to have been generated by this procedure.

Inactive Mutant. An additional mutant, Ser-70 → Thr, which completes the possible sequence permutations of serine and threonine at positions 70 and 71 of β-lactamase and which can arise by a single base change (Table I), was not obtained in the phenotypic screen for revertants; therefore, we presumed it to be inactive. This mutant was later directly obtained by oligonucleotide-directed mutagenesis; it was shown to be catalytically inactive, but expressed and secreted normally (see below).

Requirement for Catalytic Activity at Residue 70. The only catalytically active lactamases now known have a residue at position 70 with a primary nucleophile (–CH_2OH or –CH_2SH) separated from the polypeptide backbone by a single methylene group. The requirement of such a group accords with the postulated mechanism that involves attack of this nucleophile on the carbonyl carbon of the β-lactam ring to generate an intermediate acylenzyme (Fisher et al., 1980); the steric hindrance to such attack within the constraints of the active site, caused by an additional methyl group, as in threonine, might therefore be expected to abolish activity.

Isolation and Purification of Mutants. The catalytic activity of the mutant Thr-71 → Ser rapidly decays in *E. coli* grown at the normal 37 °C; yields can be markedly improved by growing at 30 °C. In this sense, the Thr-71 → Ser mutant has a temperature-sensitive phenotype in its ampicillin resistance. Synthesis of enzyme can also be greatly improved when the structural gene is transferred to a plasmid where it is under control of the tac promoter and inducing synthesis of the active revertant at 4 °C; enzyme used in subsequent characterizations was produced from such a plasmid.

Processing and Secretion. The apparently low production of mutant enzyme under normal conditions might indicate difficulties in processing and secretion. Some dependence of this aspect of protein synthesis on the structure of the processed protein, as well as of the leader sequence, has ample precedent, for example, in maltose binding protein (Bassford et al., 1979) and in β-lactamase (Koshland & Botstein, 1982). For this

Table II: Comparison of Catalytic Parameters of Wild-Type and Active Revertant β-Lactamase

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Benzylpenicillin			
Ser-Thr (wild type)	26 ± 10	2000 ● 200	7.7×10^7
Ser-Ser	21 ± 8	330 ± 100	1.6×10^7
Cys-Thr (Sigal et al., 1984)	60 ± 10	20	3.3×10^5
Cephalosporins			
Ser-Thr (wild type) (cephalothin)	250 ± 100	230 ± 50	9.2×10^5
Ser-Ser (cephalothin)	300 ± 100	30 ± 10	1×10^5
Cys-Thr (Sigal et al., 1974) (cephaloridine)	1800 (1000)[a]	2 (1700)[a]	1.1×10^3 (1.7×10^6)[a]
Benzylpenicillin/Cephalosporin			
Ser-Thr (wild type) (benzylpenicillin/cephalothin)	0.10	8.7	84
Ser-Ser (benzylpenicillin/cephalothin)	0.07	11.0	160
Cys-Thr (Sigal et al., 1984) (benzylpenicillin/cephaloridine)	0.03 (0.05)[a]	0.1 (1)[a]	300

[a] Wild-type value.

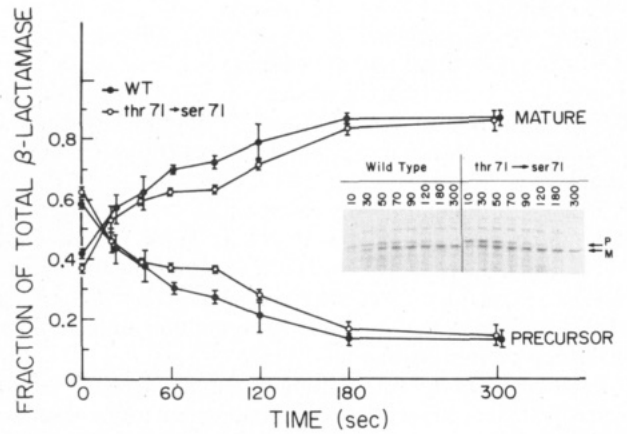

FIGURE 2: Pulse–chase analysis of wild-type and mutant β-lactamase gene products. Irradiated cells were infected with the appropriate P22bla phage and labeled with [^{35}S]methionine for 3 s as described in the text and in the literature (Koshland & Botstein, 1980). The length of the chase (in seconds) is indicated on the abscissa and above the corresponding lane in the autoradiogram (inset).

reason, we studied this aspect of the mutants in *Salmonella typhimurium* after transferring the β-lactamase gene to bacteriophage P22 as described. Processing was monitored by pulse–chase experiments using [^{35}S]methionine. At 37 °C, the incorporation of label into both the precursor and mature forms of the enzyme was so rapid that even sampling at 15-s intervals after a 30-s pulse of [^{35}S]methionine gave relatively poor resolution but showed no differences between wild-type and mutant enzyme. At 30 °C, resolution was much improved; Figure 2 shows such an autoradiogram and summarizes the quantitative results in which the value for each point was normalized to the sum of all label present in each lane of the gel. Three independent experiments confirm this result; there is no significant difference in the processing and secretion of the wild-type enzyme and the Thr-71 → Ser mutant. Similarly, the inactive mutant Ser-70 → Thr is processed and secreted normally.

Recognition by Antibody against β-Lactamase. All three active-site mutants cross-react with comparable affinity to antibody raised in rabbits against wild-type β-lactamase (data not shown).

Kinetic Parameters of Active Revertant (Thr-71 → Ser). Table II collects the appropriate kinetic parameters for the catalytically active mutant Thr-71 → Ser for benzylpenicillin and cephalothin. Also included for comparison are the values for the other known active-site mutant thiolactamase Ser-70 → Cys (Sigal et al., 1984). The relative activities toward penicillin and cephalosporin are also shown. The inhibition constant for boric acid was 1 mM, unchanged from that of the wild type (Kiener & Waley, 1978).

Table III: Thermal Stability of Active β-Lactamases

wild-type Ser-Thr		
temp (°C)	$t_{1/2}$ (min)	mutant Ser-Ser $t_{1/2}$ (min)
40		9
45		5
50	>30	<1
55	13	
60	1–2	

Table IV: Resistance of Wild-Type and Revertant β-Lactamase to Thermolysin Digestion

	$t_{1/2}$ (min)	
temp (°C)	wild type	revertant (Ser-Ser)
37	50	3.5
44	18	<1

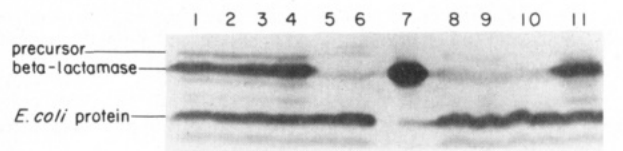

FIGURE 3: Resistance of wild-type and mutant β-lactamases to cellular proteases in vivo. *E. coli* LS1 harboring plasmids encoding either wild-type or mutant β-lactamase genes were cultured and treated as described under Materials and Methods. After cell lysis, proteins were analyzed by SDS–PAGE followed by immune blotting with antibody raised against β-lactamase. The figure is a photograph of a peroxidase-stained nitrocellulose membrane from the immune blotting procedure showing β-lactamase, its precursor, and an *E. coli* protein which also reacts with the antibody. Samples in lanes 1–6 were taken at late log phase; lane 7, pure β-lactamase control (0.14 μg); samples in lanes 7–12 were taken 12 h later. The following amino acid dyads correspond to the active-site sequence (residues 70 and 71) of each of the β-lactamases tested: samples 1 and 8, Thr-Ser; 2 and 9, Thr-Thr; 3 and 10, Ser-Ser; 4 and 11, Ser-Thr (wild type); 5, Ser-stop (control) (S. Schultz, unpublished results); 6, *E. coli* LS1 control (no plasmid).

Thermal Behavior. Table III summarizes the relative thermal stability of wild-type and mutant enzymes; whereas the parent enzyme is stable for periods longer than 30 min at 50 °C, the active mutant permanently loses activity at this temperature in less than 1 min. This thermal denaturation appears to be concentration dependent; more dilute solutions are more resistant to denaturation (data not shown). This concentration dependence is similar to behavior seen for the λ repressor (Hecht et al., 1984). The thermally denatured enzyme shows no change in mobility on 12% SDS–PAGE. Even at 4 °C, purified revertant enzyme in 20 mM Tris-HCl, pH 7.0, and at protein concentrations of 0.1 mg/mL or above

was not stable and became completely inactivated over several weeks, again with no change in mobility on SDS–PAGE.

Proteolytic Stability. All of the mutants discussed in this paper show a marked increase in susceptibility to proteolytic degradation. For example, the Thr-71 → Ser mutant enzyme is dramatically more sensitive to thermolysin digestion at both 37 and 45 °C (Table IV) than the wild-type β-lactamase.

Figure 3 shows the increased susceptibility of the three active-site mutants to proteolytic digestion in *E. coli*. In late log phase, the mutant proteins are present, but in observably lower quantities than wild-type enzyme. After growth to stationary phase, the mutant proteins have almost completely vanished, while wild-type enzyme is still present in normal amounts. Fragments of lower molecular weight that react with antibody, and are absent from control cells, are also present; these presumably result from proteolysis.

A similar but less dramatic loss of enzyme activity is seen if extruded enzyme is incubated in *E. coli* periplasmic fluid (data not shown).

Urea Stability. The Thr-71 → Ser revertant enzyme is also more sensitive to urea-induced denaturation than the wild-type enzyme. At 30 °C and pH 7.0, the Thr-71 → Ser enzyme is inactive at urea concentrations above 2 M, while wild-type enzyme is inactivated only at urea concentrations above 4 M. Both enzymes readily regain normal activity upon 50-fold dilution of urea, even after 48-h exposure at 37 °C. (Note that these studies involve protein concentrations lower by a factor of at least 10 than those used in the studies of thermal stability.)

Conclusions. Of four mutants at the catalytic site of β-lactamase, only the two with a primary nucleophile at residue 70 (Ser-70 → Cys and Thr-71 → Ser) are active; the other two (Ser-70 → Thr and the double mutant Ser-70 → Thr, Thr-71 → Ser) show no ability to catalyze the hydrolysis of the β-lactam ring of penicillin. A primary nucleophile at residue 71 cannot substitute for the primary nucleophile required at residue 70 to confer enzymatic activity.

The major effect on catalysis by conversion of the threonine residue at position 71 to serine (a loss of a single methyl group in an enzyme of 28 500 daltons) is on the value of k_{cat} (reduced to about 15% that of wild type). The preference for penicillin compared to cephalosporin for this mutant remains that characteristic of wild-type RTEM lactamases.

Though the mutation Thr-71 → Ser does not drastically reduce catalytic activity, this change seriously destabilizes the three-dimensional structure of the protein. [In interesting contrast, the mutant Ser-70 → Cys, which retains Thr-71, has enhanced thermal stability and resistance to proteolysis (Sigal et al., 1984).] In the presence of proteases, as in the environment of the periplasmic space, the instability of the Thr-71 → Ser mutant is significantly amplified by proteolytic attack on the mutant protein which may be partially unfolded at 37 °C due to a stretch of polypeptide chain in the region of the active site that has an abnormally high mobility relative to this region of the wild-type protein. Possibly, the mutant proteins have a conformation resembling pre-β-lactamase, which has no catalytic activity and is highly susceptible to proteolytic attack (Koshland & Botstein, 1982; Roggenkamp et al., 1985). In the wild-type enzyme, the additional methyl group of the threonine residue (71) probably acts almost as a keystone to interlock with other segments of the β-lactamase polypeptide chain and stabilize the three-dimensional structure; in the Thr-71 → Ser mutant, the absence of this interaction would therefore destabilize the three-dimensional structure in the region of the active site. We would emphasize that any such local instabilities may well be propagated throughout the entire molecule and may thereby become manifest at sites surprisingly remote from the site of the amino acid change itself.

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Registry No. Ser, 56-45-1; Thr, 72-19-5; boric acid, 10043-35-3; benzylpenicillin, 61-33-6; cephalothin, 153-61-7.

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Roles of the Two Copper Ions in Bovine Serum Amine Oxidase†

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ABSTRACT: With a view to obtaining information on the roles of the two copper ions in bovine serum amine oxidase (BSAO), spectroscopic and magnetic studies on several BSAO derivatives have been carried out. Cu-depleted BSAO (Cu-depBSAO) exhibits no enzyme activity and only a low absorption intensity at ca. 475 nm, which is the characteristic absorption maximum of the chromophore in BSAO. The binding of 1 mol of Cu to 1 mol of Cu-depBSAO slightly but definitely increases the enzyme activity and the absorptivity, although they are much lower than those of native BSAO. The incorporation of 2 mol of Cu into Cu-depBSAO gives rise to a similar high activity and absorptivity as those of the native enzyme. Electron paramagnetic resonance (EPR) spectra of the BSAO derivatives reveal that two copper ions in the enzyme molecule are environmentally identical. Titrations of BSAO, Cu-depBSAO, and Cu-half-depleted BSAO (Cu-half-depBSAO), containing 1 mol of copper per mole of protein, with phenylhydrazine (an inhibitor of BSAO) indicate that only 1 mol of phenylhydrazine reacts with 1 mol of the enzyme. In other words the enzyme possesses only one chromophore or one active site, though the molecule is composed of two electrophoretically identical subunits. The binding constants between phenylhydrazine and BSAO, Cu-depBSAO, or Cu-half-depBSAO were estimated to be 5×10^6, 5×10^4, and 1×10^5 M^{-1}, respectively. The binding of phenylhydrazine to the chromophore is assisted by the presence of two copper ions by a factor of 100. One of the most important roles of the copper ions in BSAO is likely to retain the chromophore in a favorable geometric and electronic structure for binding the substrate.

Copper-containing amine oxidases [amine:oxygen oxidoreductase (deaminating) (copper-containing), EC 1.4.3.6] catalyze the oxidative deamination of amines by accepting two electrons from amines and transferring them to molecular oxygen, as expressed by the equation (Malmström et al., 1975)

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$$

They are known to contain nonblue and electron paramagnetic resonance (EPR)[1] detectable copper (Yamada et al., 1963, 1969; Mondovi et al., 1967; Lindström et al., 1974; Suzuki et al., 1980, 1983) and an organic chromophore responsible for their yellowish pink color (Yamada & Yasunobu, 1963; Adachi & Yamada, 1969; Lindström & Pettersson, 1973; Ishizaki & Yasunobu, 1976; Finazzi-Agrò et al., 1977; Suzuki et al., 1981, 1982, 1983). Reactions of the copper with complexing agents (Yamada & Yasunobu, 1962; Lindström & Pettersson, 1974; Lindström et al., 1974) or of the chromophore with carbonyl reagents (Yamada & Yasunobu, 1963; Falk, 1983) lead to an inactivation of the enzyme. Therefore, the presence of both the copper and the chromophore seems to be essential for the activity of amine oxidases. Our recent investigation on metal substitution and depletion of bovine serum amine oxidase (BSAO) established that there are two forms of the chromophore, namely, yellowish pink (oxidized) and pale yellow (reduced) forms. The chromophore and

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[1] Abbreviations: BSAO, bovine serum amine oxidase; Cu-depBSAO, Cu-depleted BSAO; Cu-excessBSAO, BSAO containing excess Cu; Cu-half-depBSAO, Cu-half-depleted BSAO; Cu-recBSAO, Cu-reconstituted BSAO; EPR, electron paramagnetic resonance; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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