THE EXPRESSION, STABILITY, AND ENZYMATIC ACTIVITY OF SPECIFIC BETA-LACTAMASE MUTANTS.

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Charlotte, thank you for still putting up with me. Without your help, I wouldn't have made it.

The Expression,Stability, and Enzymatic Activity of Specific Beta-Lactamase Mutants

ABSTRACT

Three catalytic site mutants (ser 70 to thr, thr 71 to ser, and the double mutant ser 70 to thr-thr 71 to ser) of the pBR 322 beta-lactamase were purified to homogeneity. These mutant proteins were unstable and could only be obtained from E.coli at 30o C, rather than the normal growth temperature of 37o. The use of the strong tac promoter increased the yield of the thr 71 to ser mutant protein. This promoter was coupled with a kanamycin resistance gene to create a new vector for the expression of inactive beta-lactamase mutants. A new purification protocol for the pBR 322 lactamase was devised. By replacing an early ion-exchange chromatography column with an ammonium sulfate precipitation, it was possible to isolate unstable beta-lactamases in high yield.

The ser 70 to thr mutant and the double mutant show no catalytic activity and do not form an acylenzyme intermediate. However, they do still retain the ability to bind benzylpenicillin. The thr 71 to ser mutant is active. The K_m values for benzylpenicillin and cephalothin hydrolysis are unchanged from the wild-type enzyme, while the k_{cat} values are approximately 15 % of the wild-type value. This mutant also cleaves the poor substrate cefoxitin, again with no significant change in K_m, but with

kcat reduced to 8 % of the wild-type value. Examination of the pre-steady state burst during cefoxitin hydrolysis showed that the thr 71 to ser enzyme acylated at the same rate and to at least 80 % of the extent of the wild-type enzyme. Direct measurement of the deacylation rate confirmed that a reduction in the deacylation rate is responsible for the lowered reaction rate in this mutant protein. Additionally, this protein lost catalytic activity at elevated temperature more rapidly than the wild-type enzyme.

The denaturation of the active thr 71 to ser mutant was observed in more detail. This enzyme thermally denatures at 45°, a temperature 10 to 15° lower than that required to denature the wild-type enzyme. This mutant is also more susceptible to digestion by thermolysin, trypsin, and those proteases present _in vivo_ in the periplasmic space of _E.coli_. The enzyme also loses activity at a urea concentration of 2 M, whereas the wild-type enzyme is still active at urea concentrations greater than 4 M. The inactive mutants ser 70 to thr and ser 70 to thr-thr 71 to ser are even more susceptible to proteolytic attack by _E.coli_ proteases _in vivo_.

A mutant pBR 322 beta-lactamase lacking the disulfide bond found in the wild-type enzyme was also purified to homogeneity. This protein showed no alterations in catalytic activity relative to the wild-type enzyme at

temperatures below 40°. Above this temperature, this enzyme rapidly lost activity. This enzyme was also more susceptible to proteolytic attack at elevated temperature. However, this enzyme is more resistant to thermal and proteolytic denaturation than the thr 71 to ser mutant beta-lactamase.

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Nomenclature

The beta-lactamase amino acid residues are numbered according to the consensus sequence (R.P.Ambler, Phil.Trans. Roy.Soc.London Ser B 289:321-331, 1980). Mutations in this protein are presented in the form S70T, where the first letter represents the amino acid in the wild-type protein, the number is the position of the amino acid, and the second letter represents the amino acid present in the mutant enzyme.

Abbreviations

Tris-HCl,tris(hydroxymethyl)aminomethane hydrochloride; IPTG, isopropylthiogalactoside; SDS-PAGE,sodium dodecylsulfate polyacryamide gel electrophoresis; EDTA,ethylenediaminetetraacetic acid.

CHAPTER I

INTRODUCTION

The linear sequence of amino acids in a protein uniquely determines its precise three-dimensional structure. A major goal of biochemistry is to learn the principles by which the primary amino acid sequence of a protein dictates this three-dimensional structure and how this structure in turn dictates the wide variety of biological functions proteins perform.

The three-dimensional structures of a few of the myriad of naturally occurring proteins have been directly determined using diffraction techniques. Other physical methods, such as ultraviolet,visible, and nuclear magnetic resonance spectroscopy, have provided clues to the relationships between structure and function in solution for a larger group of proteins. However, historically the most widely used technique for examining structure-function relationships in proteins has been to chemically perturb the structure (with varying degrees of specificity) and then examine the altered protein for resulting changes in function.

An especially interesting subset of altered proteins are those in which a specific amino acid is replaced by one or more of the 19 other amino acids naturally occuring in proteins. These changes, along with deletions and insertions, are the only possible alterations in protein primary structure that could be produced by an organism. The production of a serine to cysteine "chemical mutation" using the tech-

niques of organic chemistry has been achieved in subtilisin (1,2). Similar mutations have been reported in trypsin (ser to cys)(3) and in papain (cys to ser)(4). An "enzymatic muta-tion" was described for the soybean trypsin inhibitor, in which specific enzymatic digestions were followed by resynthesis to produce an arg to lys mutation(5). However, these two approaches are far from general. Until recently, most amino acid substitutions were provided by non-specific mutations. Additionally, homologous proteins from different species have been compared, allowing inferences to be made about the roles of conserved (and non-conserved) amino acids. This comparative approach has provided valuable insights into the biochemistry of cytochrome c(6) and serine proteases(7), to name two examples.

Oligonucleotide-directed site specific mutagenesis has proven to be a powerful technique for examining the roles specific amino acid residues play in a wide variety of protein functions. This power derives from the ability of this technique to uniquely alter specific residues. This technique has been the subject of several recent reviews(8-10). If the protein studied confers a selectable phenotype on the host cell, active revertants from an inactive mutant previously generated using site-specific mutagenesis can be isolated(11). Using this approach, one can couple phenotypic information from this classic genetic technique with the detailed investigation of the functional properties of a

purified mutant protein. This synthesis of molecular genetics with protein biochemistry can reveal alterations in protein function due to subtle structural changes whose synthesis would not otherwise be attempted.

In addition to its value in studying the functional roles played by amino acids, site-directed mutagenesis has also provided information about the role specific amino acids play in stabilizing protein molecules against denaturation. Attempts have been made to increase the thermal stability of proteins by introduction of cysteine residues at positions which would allow disulfide bonds to form(12-14); however, this procedure requires accurate structural information on the protein in question. Frequently, the additional disulfide provides no additional stability to the altered protein.

One of the first proteins to be extensively examined for alterations in catalysis and stability caused by structural changes produced using site directed mutagenesis was the beta-lactamase encoded by the E.coli plasmid pBR322(11,15). The remainder of the introduction will review the properties of this protein, and other bacterial proteins that interact with penicillin.

One of the major factors which gives bacteria resistance to penicillin and related ß-lactam antibiotics is the presence of ß-lactamases. Due to this clinical importance, ß-lactamases have been the subject of a vast amount of research. Through this research, a significant knowledge

about the mechanism of this enzyme has been obtained. However, the roles specific amino acid residues, other than serine 70, play in the reactions catalyzed by this enzyme are not well understood. In addition to providing a basis for the rational design of specific enzyme inhibitors and resistant antibiotics, exploring the mechanism of ß-lactamases may also contribute to an understanding of the mechanisms used by other hydrolytic enzymes, especially those acting on other amides, such as serine proteases.

ß-Lactamases(EC 3.5.2.6) catalyze the hydrolytic cleavage of the cyclic amide bond in the four-membered ring of penicillins and cephalosporins, as shown in Figure I-1. The study of this enzyme dates from 1940, when Abraham and Chain discovered that extracts from various bacteria inactivated penicillin(16). It soon became apparent that these penicillin-inactivating enzymes varied in physical properties, depending on which organism was used as a source. ß-Lactamases are found in blue-green algae, virtually all bacteria, and most actinomycetes(17). The presence of ß-lactamase in these diverse organisms, many of which would not be expected to encounter ß-lactam antibiotics in their normal environment, suggests that some of these ß-lactamases may have other, as yet unknown substrates(18).

Penicillin and related ß-lactam antibiotics block bacterial growth by inhibiting the enzymes involved in cell wall biosynthesis. Tipper and Strominger proposed that this

inhibition is based on the structural resemblance between the ß-lactam region of penicillin and the D-ala-D-ala peptide bond cleaved in formation of crosslinks in bacterial cell wall peptidoglycan(19).

If a bacterium is exposed to radioactive penicillin a number of proteins, specific for each species, are found which covalently bind the antibiotic(Reviewed in 17). These penicillin-binding proteins(PBPs) fall into two functional classes. The larger proteins (molecular weight 90-100,000) are peptidoglycan transpeptidases. These proteins have only recently been isolated and their properties are largely unknown. The smaller proteins (molecular weight 25-50,000) are D-ala-D-ala carboxypeptidases. A number of these proteins have been isolated and extensively characterised. Although these functional definitions overlap (for example, most carboxypeptidases are capable of transpeptidation under certain conditions), the physiological role of each enzyme is specific. Though not all PBPs have identifiable functions, those known to be required for bacterial growth cannot be replaced by other proteins. It is this inhibion of the irreplaceable enzymes needed for the synthesis and maintainance of cell walls that allows penicillin to kill bacteria.

Many different classification schemes for organizing the various ß-lactamases have been proposed. Classification schemes for ß-lactamase are based on molecular weight, isoelectric point, presence of metal ions, and substrate pro-

file(20). It should be noted that one species of bacteria can produce more than one type of ß-lactamase. The scheme I will use here splits ß-lactamases into three categories, A, B, and C, based on size, substrate hydrolysis profile, and sequence homology(21,22). However, it is likely that there are additional, as yet uncharacterized, classes.

The type A ß-lactamases have an active site serine and a molecular weight of about 30,000. These enzymes are found in both gram positive and gram negative bacteria. Membrane associated forms are also known. These are the best characterized ß-lactamases. The pBR322 ß-lactamase examined in this work is a class A enzyme.

The type B ß-lactamases are metalloproteins with a molecular weight of about 22,000 and only two examples are known. They show little substrate specificity, cleaving all beta-lactams equally well, and are unaffected by the usual lactamase inhibitors. These enzymes require zinc(II) or cobalt(II) and appear to function as general acid-base catalysts with a branched pathway mechanism(23). No evidence of an acylenzyme intermediate in this class of ß-lactamase has been found.

The type C ß-lactamases are similar to the type A enzymes in that both types produce an acylenzyme intermediate using a serine residue. The type C lactamases differ in that their molecular weight is about 39,000 and they are sensitive to exogenous nucleophiles. For example, the E.coli chromoso-

mal lactamase (ampC) can transfer the penicilloyl group of its acylenzyme intermediate to alcohols, resulting in ester formation(24). The ampC enzyme can also hydrolyze penicilloyl esters. No evidence for these two reactions has been found in type A ß-lactamases. The rate of cephem cleavage is increased for class C lactamases when hydroxylamine is added to the solvent, while the class A lactamases show no rate enhancement under these conditions. The class C lactamases generally hydrolyze cephems rapidly relative to their hydrolysis rate for penams; in contrast, the class A lactamases hydrolyze penams much more rapidly than they hydrolyze cephems.

One of the factors contributing to the clinical importance of ß-lactamases is the ability of the genes encoding these proteins to migrate from one bacterial species to another. Many plasmid encoded ß-lactamases are expressed in multiple host bacterial species. Changing of the host species doesn't affect the specific properties of a plasmid encoded ß-lactamase, but it can affect the amount of enzyme produced(25,26). In addition, ß-lactamases were among the first selectable markers found on transposable sequences. These TnA transposable sequences can mediate the movement of ß-lactamase genes between plasmids and bacterial chromosomes(27).

The pBR322 ß-lactamase was originally isolated from the Tn1(formerly TnA) transposon carried by plasmid R1 drd 19.

This enzyme and many similar plasmid encoded ß-lactamases are often referred to as RTEM ß-lactamases, named after the type strain of E.coli from which the first enzyme of this type was isolated(28). These RTEM ß-lactamases are similar in size (Molecular weight 25-30,000), substrate profile, and isoelectric point(pH 5.4-5.6). These enzymes hydrolyze penicillins much faster than they hydrolyze cephalosporins. Isoelectric focusing separates these enzymes into two subclasses, the first with an isoelectric point of 5.4(RTEM1), the second with an isoelectric point of 5.6(RTEM-2). The pBR322 enzyme used in this project is the RTEM-1 enzyme. This protein is synthesized as a 286 amino acid preprotein containing a 23 amino acid leader sequence which is removed when the protein is exported to the periplasmic space of E.coli(29,30) The RTEM proteins are the only lactamases that possess a disulfide bond(31,32).

Five RTEM enzymes have been sequenced and they are homologous in primary structure(21). Two of these enzymes-(the pBR322 enzyme and the RP1 lactamase) are almost identical and differ only at one amino acid residue. At residue 37, the pBR 322 enzyme possesses a gln, while the RP 1 enzyme has a lys at this position. Comparison of these enzymes with the other RTEM enzymes (Staphylococcus aureus PC1, Bacillus cereus I, and B.licheniformis) yields amino acid sequence homologies of 20 to 46 %.

When the sequences of ß-lactamases and pencillin binding

proteins are compared, one region of homology, centered on the active site serine, is found(33). Covalent labeling with presumed active site directed reagents has confirmed the identity of this serine as the site of acylenzyme formation. In both types of protein, a conserved lysine residue is found at the third position to the carboxyl side of the acylated serine residue. ß-Lactamases and D-ala carboxypeptidases also show a striking resemblance when their secondary structures are compared(34).

The class A and C ß-lactamases and penicillin binding proteins can be viewed as two extremes of a common reaction pathway. In both proteins, penicillins form acylenzyme intermediates. In the ß-lactamases, these intermediates rapidly hydrolyze, resulting in catalytic hydrolysis of penicillins. In the case of the penicillin binding proteins, these acylenzyme intermediates have a long life. Addition of a good nucleophile such as hydroxylamine speeds the breakdown of this acylenzyme intermediate. Thus penicillin can be viewed as a poor substrate acting as a competitive inhibitor of normal cell-wall synthesis reactions. Support for this model comes from the observation that penicillin binding proteins act as poor ß-lactamases, and that ß-lactamases can, however poorly, hydrolyze depsipeptides that are analogs of the substrates cleaved by D-ala-D-ala carboxypeptidases(35). This supports the hypothesis that ß-lactamases originated as a result of mutations to penicillin binding proteins. These

mutations have resulted in the increase of the deacylation rate of penicillin bound as an acyl enzyme.

The ß-lactamase gene encoded on plasmid pBR322(bla) has proven to be a convenient system for the study of many biochemical processes. It possesses an easily transferred coding sequence that can be readily expressed in many organisms, both procayotic and eucaryotic. The enzyme confers an easily selectable phenotype on the host organism and a number of chromogenic substrates are available for accurate determination of the amount of active enzyme. This gene has been used to examine protein secretion(36), expression of fusion proteins(37), and as a marker for experiments examining control of gene expression(38).

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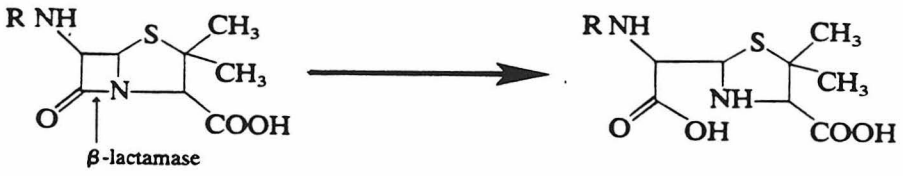
Figure I-1: The inactivation of (a) penicillin and (b) cephalosporin by beta-lactamase. In both cases, the beta-lactam bond is broken by a hydrolytic mechanism. In (a), the product penicilloic acid is relatively stable, while the corresponding cephalosporin product in (b) is unstable and decomposes spontaneously. R and R′ represent variable side chains.

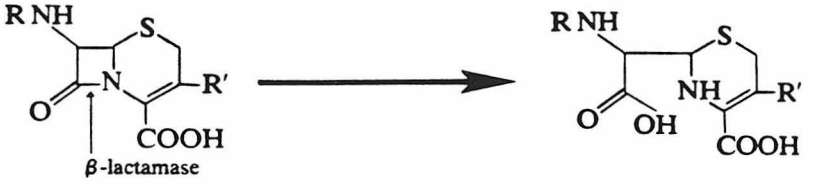
A



Penicillin

Penicilloic acid

B



Cephalosporin

Cephalosporoic acid

CHAPTER II

THE EXPRESSION AND PURIFICATION OF UNSTABLE BETA-LACTAMASE MUTANTS

Introduction

One of the major advantages of using plasmid-encoded ß-lactamase as a model system for site-directed mutagenesis and site saturation is the ability to easily detect changes in the phenotype of the host bacterial cell. This allows one to rapidly screen for activity in mutant enzymes and to search for active revertants of known inactive mutants. However, the use of phenotype alone is inadequate to describe the alterations in function of mutant proteins. The efficacy of a ß-lactam antibiotic depends on its ability to pass through the cell wall or outer membrane of bacteria, to resist the effect of ß-lactamase activity, and to interact with the target proteins in the inner membrane. Changes in the permeability of the outer cell membrane are particularly important as resistance mechanisms in gram negative bacteria such as E.coli. Alterations in all of these three processes have produced ß-lactam resistant phenotypes(Reviewed in 1).

Altered ß-lactamases that lack activity or confer reduced resistance to selecting antibiotics may have any of several types of defects. The altered protein may be synthesized or secreted at a reduced rate, or the protein may be improperly localized. The mutant protein may be catalytically competent, yet due to reduced ability to withstand thermal or proteolytic degradation, confer an antibiotic sensitive phenotype on the host cell. Lastly, of course, the

mutant protein may be inherently catalytically inactive.

The active-site mutants of ß-lactamase discussed here show no alterations in rate of secretion or ability to serve as substrate for signal peptidase. Though pulse-chase experiments show that these mutants are synthesized and exported normally and are stable for short time periods(2), these experiments do not reveal the catalytic competence of these mutant enzymes or the ability of these proteins to withstand thermal and proteolytic degradation over longer time periods.

To resolve these ambiguities, it is necessary to produce interesting mutant enzymes in sufficient quantity and purify them to homogeneity without causing denaturation, if possible. In this chapter, I will discuss the techniques used to produce and purify unstable mutant ß-lactamases. In addition, immunological assays that do not depend on enzyme activity were developed.

Materials and Methods

Bacterial cell lines. E.coli strain LS1(a derivative of HB101)(3) was used for all plasmid preparations and as a host cell for enzyme production using the intrinsic pBR322 promoters. E.coli strain D1210 was used as a host cell for all plasmids containing the tac promoter. Protease deficient E.coli strains SG 4121 and SG 3325 were generous gifts from J. Rossi.

Plasmids. All DNA fragments were purified on agarose

gels using standard procedures(4). Fragments were isolated from the agarose using an electroeluter(International Biotechnologies, Inc. model UEA). Table II-1 shows the plasmids used and their origins.

Growth media. For all enzyme production fermentations, rich media were used. If tetracycline(20 mg/l) was used as the selecting antibiotic, FB [1 l of water containing 25 g tryptone, 7.5 g yeast extract, 6 g sodium chloride, and 50 ml 1 M Tris-HCl(ph 7.5)] was used. For selection using ampicillin (10-20 mg/l) or kanamycin(30-50 mg/l), XB [1 l of water containing 25 g tryptone, 7.5 g yeast extract, 20 ml 1M magnesium sulfate, and 50 ml 1 M Tris-HCl(ph 7.5)] was used. A New Brunswick Magnaferm fermenter(12 l) was used with vigorous aeration (10 l/min) and stirring (200 rpm). The use of double distilled ultrapure water in the growth media increased the yield of beta-lactamase.

For production of enzyme, frozen starter cultures of E. coli containing appropriate plasmid were used. These were produced by adding 5 ml MLT [10 g tryptone, 5 g yeast extract, 5 g sodium chloride, 100 ml 1 M Tris-HCl(pH 7.4) per liter] to a fresh agar plate and gently suspending the cells. One ml of this suspension was added to 0.1 ml sterile glycerol and quickly frozen. Fifty µl of this suspension was added to 10 ml broth to initiate growth.

Tryptone and yeast extract were from Difco Laboratories, and antibiotics were obtained from Sigma. All buffer pH

values were measured at 22°C.

Osmotic shock procedure. E. coli were grown to the desired stage and harvested by gentle centrifugation(GSA rotor, 5 krpm for 5-10 min or J-6 rotor, 4.2 krpm for 15-20 min). The collected cells were washed with 50 mM Tris-HCl, ph 7.0 and concentrated by centrifugation as above. The collected cells were gently suspended in hyperosmotic sucrose[450 g sucrose, 0.5 g Na_2EDTA, 25 mM Tris-HCl(pH 7.0) per 1] and gently stirred at room temperature for 30 minutes. The cells were gathered by centrifugation(GSA rotor, 10 krpm, 30 min) and resuspended in ice-cold water. This cell slurry was vigorously stirred in the cold room for 30 minutes. The cells were removed by centrifugation (GSA, 10 krpm, 15-20 min) and the supernatant used for further purification. Sodium azide (0.2 % w/v) was added at this point as a preservative.

Sonication. Cells were gathered by gentle centrifugation and washed with ice-cold 0.1 M potassium phosphate buffer, pH 7.0. After reconcentrating the cells, they were resuspended in the same buffer (10 ml per 1 g wet cells). Eight ml portions of this suspension were broken using a Braunsonic 1510 sonicator set at 100 W for 2 minutes(or until the solution was clear). Cooling was maintained using an icebath. The cell debris was removed by centrifugation(SS34 rotor, 5 krpm, 20 min) and the supernatant assayed for protein and ß-lactamase activity.

Enzyme assays. Activities were determined by measuring the change in absorbance with time at 240 nm of a solution containing 1 mM benzylpenicillin and 100 mM sodium phosphate, pH 7.0 at 30°C(5). These are the standard assay conditions used throughout this work. During purification, the inactive mutant ß-lactamase was assayed either by direct SDS-PAGE on 12 % acrylamide gels(6) and comparison with wild-type controls after silver staining (7) or by precipitating with rabbit anti-ß-lactamase antibodies (see immunoprecipitation below).

Generation of anti-ß-lactamase antibody. The initial innoculation utilized SDS-PAGE purified wild-type ß-lactamase. One mg of protein in an acrylamide gel slice (dialyzed to remove free acrylamide and SDS) was lyophilized, mashed in a mortar and pestle, and mixed with Freund's complete adjuvant. This material was injected into the popliteal lymph nodes of a rabbit. After 1 month, booster subcutaneous innoculations of 100 µg of purified ß-lactamase in incomplete Freund's adjuvant were followed by ear-bleeding one week later. The blood was allowed to clot for 2-4 hours, then the serum was collected by centrifugation. Serum samples were screened for presence of anti-ß-lactamase antibody by precipitin ring test assays.

Immunoprecipitation. Antigen(ca. 20 µg) and 15 µl rabbit anti-ß-lactamse serum were incubated on ice for 60 min in 100 µl total volume. Two hundred µl of reconstituted goat

antirabbit immunoglobin immunobeads(Biorad) was added to each sample and incubated on ice for an additional 30 min. The precipitate was collected(Microfuge, 1 min) and washed by resuspension three times in buffer[0.01 M Tris-HCl(pH 7.3), 0.15 M NaCl, 0.5% Triton-X100]. After removal of the final wash, 100 µl of elution buffer [0.063 M Tris-HCl(pH 6.8), 2% SDS, 2% fresh ß-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(6) and visualized by silver staining(7).

Immune blots. After electrophoresis on 12% SDS-polyacrylamide gels, protein was electrophoretically transferred to nitrocellulose (Schleicher & Schuell BA85) using a Bio-Rad Trans-blot cell following manufacturers instructions. The nitrocellulose was washed twice with 0.05% Tween-20 (Sigma), 10 mM sodium phosphate(pH 7.0), 0.9% sodium chloride, then reacted overnight with 1% bovine serum albumin and 1% normal goat serum in the same buffer. After washing three times in buffer, 50 ml of a 1:1000 dilution of rabbit anti-ß-lactamase was added and the membrane incubated at 37°C for 2.5 h. Bound antibody was visualized using an anti-rabbit immunopeptide system (Vectastain ABC kit, Vector Laboratories). This staining procedure involves adding biotinylated goat-anti-rabbit antibody followed by a complex composed of the multi-

valent biotin binding protein avidin and biotinylated horseradish peroxidase H. After washing away unbound proteins, the chromogenic peroxidase substrate 4-chloro-1-naphthol is then added with dilute hydrogen peroxide, resulting in a purple spot at sites where antibody has bound.

Ion-exchange column chromatography. Diethylaminoethyl cellulose (Whatman DE-52) was used in all columns. pH values are all measured at room temperature (22°C), even for columns that are run at lower temperatures. All solutions contained 0.02 % sodium azide as a preservative.

Gel-filtration column chromatography. All columns used either Ultragel AcA 54 (LKB) or Sephadex G-75 (Pharmacia).

Results and Discussion

All initial experiments designed to optimize expression and purification of pBR322 ß-lactamase were done using the wild-type enzyme unless otherwise specified.

Release of enzyme activity from cells. An osmotic shock procedure was used to simplify purification. This procedure produces a concentrate which is predominantly periplasmic proteins, thus reducing the number of cytoplasmic proteins, especially proteases, which would have to be removed during purification. Comparison of techniques for release of ß-lactamases from bacterial cells has indicated that osmotic shock preferentially releases plasmid-encoded lactamases in high yield(8,9).

By splitting a single batch of cells and comparing the efficacy of the osmotic shock procedure versus that of sonication, I found that the osmotic shock procedure consistently released 85-90 % of the amount of activity released by sonication. Since the enzyme yields were virtually identical and the specific activity of enzyme recovered using the osmotic shock procedure was much higher, all further isolations used this technique. As a further test for release, the cell pellet remaining after an osmotic shock procedure was sonicated. This released approximately 10 % of the amount of activity present in the supernatant.

Though some authors utilize lysozyme to degrade the cell wall during osmotic shock procedures, I found no difference in yield or purity of enzyme using lysozyme digestion (50 µg/ml in sucrose solution, 30 min at room temperature) as contrasted to the osmotic shock procedure without lysozyme treatment.

One change which did improve the efficacy of the osmotic shock procedure was increasing the volumes of sucrose solution and distilled water used. The amount of released activity varied with the volume of sucrose solution and distilled water, with the amount increasing with volume until a ratio of 20 ml per 1 g wet cells was reached. Solution volumes above this ratio resulted in no further increase in released activity.

The Tris-HCl wash and sucrose solutions were examined

for any loss of activity due to leaching in these steps. No activity was detected in these solutions in growths using the intrinsic pBR322 promoters. In growths using cells containing the tac promoter in which good induction was attained, some enzyme loss was detectable in these solutions. As this lost activity was generally small compared to the activity in the final osmotic shock solution, it was usually discarded. In the worst case of such activity loss, which was the best IPTG induction of the T71S mutant, 25 % of the total activity was found in the combined washes.

Growth curve. In order to determine the optimal fermentation time for maximizing enzyme yield, a growth curve was determined. At each time point, cell density was estimated by light scattering at 540 nm and enzyme activity was estimated after cell lysis using sonication. It was found that enzyme yield slightly lagged behind cell number until ten hours after the start of fermentation (at 37^{o}); after this time point, enzyme yield dropped dramatically.

Use of inducible promoter. Though the amp^{r} gene of pBR322 already contains two constitutive promoters(10), neither of these promoters has an especially strong ribosome binding affinity. To increase production of mutant beta lactamases, a plasmid was constructed placing the strong hybrid promoter tac(11) in front of the amp^{r} gene of pBR322. This plasmid(pKK) was transferred into the host cell D1210, which constitutively produces lac repressor($lacI^{q}$), which can

bind to the tac sequence. This plasmid-host combination was used for experiments maximizing IPTG induced ß-lactamase synthesis.

This plasmid was useful in expressing active ß-lactamase mutants. However, this construction interferes with the expression of the tet^r gene. This means a pKK plasmid containing an inactive ß-lactamase mutant would possess no selectable marker. To allow this promoter to be used for the inactive ß-lactamase mutants, the BamH1-HindIII fragment in the tet^r gene of pKK was replaced by a BamH1-HindII fragment from pGR417 coding for resistance to kanamycin(12). This gene is expressed in this construction. The EcoR1-Pvu1 fragment of the mutant bla genes were used to introduce the inactive mutants into this plasmid. These plasmids are identified by the prefix pJN.

Induction of ß-lactamase activity using the tac promoter. Even without the addition of IPTG, larger amounts of ß-lactamase were produced by plasmids containing the tac promoter, even in a $lacI_q$ strain(Table II-2). Addition of 0.1 mM IPTG resulted in a large increase in the amount of ß-lactamase produced. However, if the cells were exposed to this concentration of IPTG for longer than 20-30 minutes, yield of enzyme began to decrease.

Generation of specific anti-ß-lactamase antibody. Figure II-1 shows a western blot using immune rabbit serum to stain purified wild-type ß-lactamase, a whole cell extract

containing ß-lactamase, and the same E.coli host cells(LS1) without plasmid as a control. The antibody has a high affinity for wild-type ß-lactamase, although contaminating proteins in the crude extract and proteolytic fragments cross react to some extent.

Column chromatography of pBR322 ß-lactamase. The original procedure for ß-lactamase purification from periplasmic fluid(13) consisted of ion-exchange chromatography [DE-52,10 mM Tris-HCl(pH 6.75)]. In our hands, this procedure was not successful. Recovery of enzyme activity was never higher than 50 % from this step and the column flow rate was very slow, even when large diameter (10 cm) columns were used. Changing the pH of the buffer to any point between 6.5 and 7.5, or increasing the buffer concentration to 50 mM resulted in no improvement. This step was eventually discarded and replaced with the ammonium sulfate precipitation described below.

The next purification step was originally an ion-exchange gradient column(DE-52) using a linear gradient of pH 7.25 triethanolamine, 15 to 100 mM. In our hands, enzyme activity was eluted at the very end of this gradient. Accordingly, this gradient was replaced with a 25 to 200 mM gradient of the same buffer. Active enzyme was eluted in the middle of this gradient. All three of the active site mutants showed no change in elution position on this column.

These differences in ion-exchange behavior between the

pBR322 enzyme in our hands and the almost identical enzyme in the previously published procedure are almost certainly due to the known single amino acid difference between the two proteins. The pBR 322 (RTEM-1) enzyme possesses a Gln at position 37, while the RP1 (RTEM-2) enzyme has a Lys at this position(14,15). This difference, originally detected by comparison of the DNA sequence of the pBR322 enzyme with the protein sequence of the RP1 enzyme, has been confirmed by N-terminal sequencing of the purified pBR 322 protein.

The mutant ß-lactamases are temperature sensitive, but can be isolated. Initial attempts to isolate the active revertant enzyme T71S by growing cells at 37°C yielded little or no activity. Cells grown at 30°C produced detectable amounts of activity, even without the use of a strong promoter. Cells producing the wild-type ß-lactamase showed no signs of decreased yield of active enzyme until temperatures of 42-44°C were reached. A similar increase in the yield of the two inactive mutants S70T and S70T-T71S (as judged by SDS-PAGE quantification of ß-lactamase) was found at the lower growth temperature.

As shown in Chapters 3 and 4, the active revertant enzyme is still active at 37oC, but does denature with a half-life of 20-30 minutes. The periplasmic fluid of E.coli therefore is likely to contain proteases that amplify the thermal instability of this mutant. A number of protease inhibitors were added to the growth media in an attempt to

block such proteolysis. These inhibitors produced no increase in enzyme yield. Similarly, growth of the plasmid in the E.coli strain SG 4121, which is a lon- strain, resulted in no increase in activity. The lon locus encodes an ATP-dependent protease known to eliminate some defective proteins(16). This protease is apparently not the one involved in degrading these mutant β-lactamases.

Mutant ß-lactamase stability improves when the periplasmic fluid is separated from the host cells. However, the active revertant quickly loses activity if stored in periplasmic fluid. After storage at 4°C for one month, virtually all activity has vanished. The stability of this protein increases after ammonium sulfate precipitation(see below). This implies again that the protein is being degraded by a protease present in periplasmic fluid and that this protease is removed by the ammonium sulfate precipitation step. In contrast, the wild-type ß-lactamase is stable for at least three years when stored at 4°C in E.coli periplasmic fluid.

Ammonium sulfate precipitation. The initial ion-exchange column was replaced by an ammonium sulfate precipitation. The periplasmic extrudate was brought to 20 %(w/v) ammonium sulfate at room temperature and the precipitate was discarded. Sufficient solid ammonium sulfate was added to the supernatant to bring the concentration to 66% and the precipitate was collected. This precipitate contains 70 to 100 % of the original activity and the precipitation results

in a specific activity increase of 7 fold. The precipitates were easier to collect at room temperature than at cold room temperature (4°C). No pH adjustment was necessary. The stability of the mutant ß-lactamases improved dramatically after this step. The stabilization of ß-lactamase by sulfate ions has also been seen in physical studies of ß-lactamase denaturation(17).

Gel-filtration column chromatography. As a final purification step the enzyme was applied to 1 by 50 cm column of Ultragel AcA 54 and eluted with 20mM Tris-HCl(pH 7.0). The literature procedure included a further purification step for protein that was still impure at this stage. This step involved chromatography on DE-52 ion exchange cellulose in 10 mM potassium phosphate(pH 7.0). This DE-52 step only resulted in total loss of enzyme when repeated on the pBR 322 enzyme. Lots of pBR 322 enzyme that were not completely homogenous after passage over the AcA 54 column were concentrated and the AcA 54 step was repeated.

Reverse-phase liquid chromatography. ß-Lactamase samples were purified on a Vydac C4 column using a water-acetonitrile gradient with all solutions containing 0.1 % trifloroacetic acid(TFA). Though pure protein was obtained, the pBR 322 lactamase was denatured both by the acetonitrile alone as well as the TFA. This procedure was used as a check on the purity of samples used for kinetic analysis, as it often revealed contaminating proteins.

Summary of protein purification protocol. This is the final version of the growth and purification procedures used to generate the purified ß-lactamase enzyme characterized in the remainder of this work:

1. Fermentation of E.coli D1210 bearing plasmid containing mutant ß-lactamase gene with tac promoter at 30°C to late log phase.

2. IPTG (0.1mM) is added, followed by rapid cooling to 0-4°C.

3. Cells are gathered by centrifugation, washed, and then suspended in hyperosmotic sucrose with EDTA. After the cells are reconcentrated, they are suspended in ice-cold water and vigouously stirred. The cells are removed and sodium azide is added to the supernatant(=crude enzyme).

4. Solid ammonium sulfate(20 % w/v) is added to the crude enzyme and stirred at room temperature. The precipitate is discarded and additional solid ammonium sulfate(to 66-70 % w/v) is added, again followed by stirring at room temperature. The precipitate from this step is collected and dissolved in water. The residual ammonium sulfate is removed by dialysis and the enzyme solution is concentrated to a final volume of 25-30 ml.

5. The enzyme solution is applied to a 2.5 x 25 cm column of DE-52 equilibrated with 25 mM Triethanolamine-HCl (pH 7.25) and eluted with a 25 to 200 mM linear gradient of the same buffer.

6. The active fractions are concentrated and applied in a small volume (1-2 ml) to a 1 x 50 cm column of Ultragel AcA 54 and eluted with 20 mM Tris-HCl (pH 7.0). The high specific activity fractions are checked for homogeneity on 12 % PAGE-SDS. If impurities persist, step 6 is repeated.

Summary

It is possible to produce and purify active site mutant proteins of pBR322 ß-lactamase. All active site mutants examined behaved as temperature sensitive mutants and were unstable at the normal growth temperature of 37°C. The use of an IPTG induced tac promoter and a lower growth temperature increased production of the unstable T71S ß-lactamase fifty fold. The inactive mutants S70T and S70T-T71S were synthesized in detectable amounts, reacted with anti-ß-lactamase antibody, and behaved identically to the wild-type enzyme on SDS gels and on ion-exchange and gel filtration columns.

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Table II-1. The plasmids used in this work and their sources.

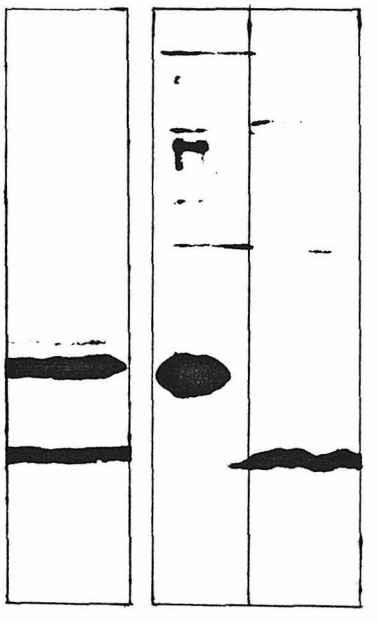
Name	Comment	Source
1.pBR322-22G9	S70T-T71S mutant	1-4 from G.
2.pBR322-997	S70T mutant	Dalbadie-McFarland
3.pBR322-R2	Active revertant from 22G9, shown to be T71S	
4.pBR322-GDM*	C77S mutant	
5.pKK	tac promoter source	J.Rossi
6.pARR	kanamycin resistance gene	L.Nanoff,Dupont

* This mutation was also obtained from Steve Schultz. The proteins obtained from the two plasmids were identical.

Table II-2. The yield of active T71S beta-lactamase as a function of promoter and IPTG induction.

Conditions	Total Yield (μkat/12 l)	Specific Activity of Extruded Protein ($\mu kat/A_{281}$)
pBR 322 amp promoters	8.07	.12
tac promoter, 30°, no induction	87.9	.14
tac promoter, 30°. IPTG induced	451	.12

Figure II-1. Photograph of peroxidase stained nitrocellulose membrane from immune blotting procedure. From left to right: whole cell extract of E.coli LS1 containing pBR322; purified beta-lactamase (.2 µg); LS1 cells containg no plasmid.



CHAPTER III

THE NATURE OF THE CATALYTIC DEFECT IN SOME ACTIVE SITE MUTANT BETA-LACTAMASES

Introduction

Two general mechanisms for the hydrolytic action of ß-lactamases have been proposed(1). These enzymes could act as general acid-base catalysts to facilitate the direct attack of water on the ß-lactam carbonyl. The Zn^{2+} dependent type B ß-lactamase uses this mechanism(2). Alternately, ß-lactamases may provide a nucleophile to attack the ß-lactam ring directly, leading to formation of an acyl-enzyme intermediate. Evidence for this second mechanism, involving the use of the highly conserved Ser70 residue in the formation of such an acyl-enzyme intermediate in the ring cleavage mediated by class A and class C ß-lactamases, has come from several sources. Initially, this residue was identified as beinh present in the active site by labeling with presumed mechanism-based inactivators such as 6-ß-bromopenicillanic acid(3). Poor substrates such as cloxacillin or cefoxitin have been used to identify a putative acyl-enzyme intermediate in a substrate cleavage reaction(4,5). In the case of the substrate cefoxitin it was possible to directly observe the formation of an ester, presumably the intermediate, by using FT-IR(5). It was also possible to trap a covalently linked enzyme-substrate intermediate using ^{14}C-cefoxitin(5). The alkaline lability of the enzyme-substrate complex formed between the S.aureus PC1 enzyme and a dansyl-cephalosporin derivative also suggests an intermediate ester(6).

Mutagenic replacement of this serine residue in the pBR322 enzyme with a cysteine residue yields a modified yet active enzyme(7). The replacement of the alcohol with a thiol results in a protein characterized by unaltered K_m values for benzylpenicillin and ampicillin with respect to the wild-type, but whose k_{cat} values are reduced to 1-2 % of wild-type values for these substrates. Thio-ß-lactamase also hydrolyzes a cephalosporin derivative(nitrocefin), but the K_m value for this substrate is so elevated that it is difficult to saturate the enzyme(8). This ability of thio-ßlactamase to retain hydrolytic activity for normal amide substrates is in marked contrast to the case in serine proteases, where replacement of the active site serine hydroxyl by a thiol results in the loss of amidase activity, whereas some degree of esterase activity remains(9).

One of the major problems that has hampered the study of the hydrolytic mechanism of ß-lactamase is the rapid rate at which good substrates are turned over. For example, benzylpenicillin(Penicillin G) is hydrolyzed by the RTEM enzyme with a k_{cat}/K_m of over 10^8 $M^{-1}s^{-1}$. For this reason, most detailed kinetic information about the class A ß-lactamases has been obtained using poor substrates, which are more slowly hydrolyzed. It is possible that the mechanism by which good substrates react may differ from the mechanism observed when poor substrates such as cefoxitin are examined. For example, the Bacillus cereus type I enzyme readily

hydrolyzes penicillins and cephalothin, but appears not to bind, and thus poorly cleaves, cefoxitin(10).

In spite of a vast number of protein modification experiments(reviewed in reference 11), evidence for the involvement of amino acids other than Ser70 in the hydrolyis of ß-lactams is sparse. Lys73 is a highly conserved residue in class A and C ß-lactamases and all other penicillin binding proteins. Site-saturation of the pBR322 enzyme at this position has revealed that this residue is essential for catalysis and cannot be replaced by any other naturally occurring amino acid(12). Chemical modification experiments using phenylpropynal have shown that the reaction of this compound with a nucleophile present in ß-lactamase inactivates this enzyme while generating a chromophore whose spectral properties are consistent with reaction with lysine or arginine(13). Lys73 is a logical candidate as the reactive group. In addition, mechanism-based inactivators such as penicillanic acid sulfones(14), clavulanic acid(15,16), and 6-acetylmethylenepenicillanic acid(17) appear to partition at the acyl-enzyme intermediate step, with a fraction of the intermediate reacting with an internal nucleophile to form an inactive modified enzyme. Low-resolution X-ray structures of ß-lactamase and other penicillin binding proteins place this lysine residue very close to the bound substrate, making this amino acid the only other irreplaceable residue in ß-lactamase(18).

A mechanism that accounts for most of the kinetic data available for the ß-lactamase promoted hydrolysis of penicillins is given in equation III-1:

$$\text{(III-1)} \qquad E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot S \xrightarrow{k_2} E\text{-}P \xrightarrow{k_3} E + P$$

The initial formation of the enzyme-substrate complex(E·S) appears to be very rapid. In the case of the Bacillus cereus ß-lactamase I there is evidence that the reaction with good substrates is diffusion controlled(19). By comparison, the hydrolysis of the sluggish substrate cephalothin (k_{cat}/K_m = 10^4 $M^{-1}s^{-1}$) shows no change with variations in viscosity (changed by adding sucrose), indicating that diffusion is not a rate limiting step for this substrate. Quantitative analysis of the "on-rate" constants(k_1) shows that this constant does not substantially differ for a wide variety of substrates. The variation in k_{cat} values for various substrates is primarily due to changes in the partition ratios, k_{-1}/k_2, for the various ES complexes. This variation appears to be due to differences in substrate-enzyme interaction, as the rate constants for the non-enzymatic second-order base-catalyzed ring opening reaction do not significantly differ for a wide variety of penicillins and cephalosporins (20,21). The substantial decrease in free energy upon cleavage of the ß-lactam ring in forming the acyl-enzyme intermediate makes it unlikely that this step is reversible.

After the acyl-enzyme intermediate, (E-P), is formed the simplifying assumption that deacylation is irreversible is also well justified. The K_m for penicillinate has been measured and is more than an order of magnitude larger than that observed for penicillins(22). As all kinetic observations desribed herein have been done under conditions where product concentration are small relative to substrate concentration, this back reaction can be safely ignored.

The mechanism for cephalosporin hydrolysis appears to be more complicated than that seen for penicillin hydrolysis. In the hydrolysis of cefoxitin by RTEM ß-lactamase a good leaving group(3'carbamoyloxy) can be released from the cephalosporin acyl-enzyme intermediate before deacylation takes place, leading to a partioning of the initial acyl-enzyme intermediate between hydrolysis, regenerating free enzyme, and the elimination reaction, yielding a second acyl-enzyme intermediate, which can then hydrolyze, also regenerating free enzyme(23). This branched pathway is illustrated in Figure III-1. In addition, observation of the hydrolysis of dansyl-cephalexin has revealed the existence of a kinetic intermediate prior to the formation of the acyl-enzyme intermediate(6). This may be a tetrahedral adduct formed prior to the acyl-enzyme.

Penicillin binding proteins and ß-lactamases can be viewed as following similar mechanistic pathways. Once an acyl-enzyme intermediate is formed, however, the two classes

of protein differ. While the ß-lactamases readily deacylate, generating a free penicillinate, the acyl-enzyme forms of the penicillin binding proteins have very long lifetimes. The penicillin is not released from a penicillin binding protein as penicillinate, but rather the thiazolidine ring fragments into at least two major components, for example, yielding the fragments N-phenylacetylglycine and N-formyl-D-penicillamine when benzylpenicillin is the original antibiotic(24).

The experiments desribed in this chapter are aimed at determining which step or steps in the catalytic hydrolysis ß-lactams are crippled in three purified mutant ß-lactamase. The enzymes studied include two with an inactive phenotype-(S70T-T70S and S70T) and an active revertant enzyme(T70S).

Materials and Methods

Materials. Sodium cefoxitin(Mefoxin) was a generous gift of Merck Sharp and Dohme. ^{35}S-benzylpenicillin was obtained from New England Nuclear Research Products. All other antibiotic substrates were from Sigma. All protein preparations used for the kinetic determinations was homogenous by SDSPAGE and purified according to the procedures given in Chapter 2. Protein concentrations were estimated by measuring the absorbance of stock solutions at 281 nm and using published conversion factors(5).

Determination of k_{cat} and K_m. All measurements were done at 30° C in 0.1 M potassium phosphate, pH 7.0, utilizing a Beck-

man Acta CIII UV-visible recording spectrophotometer with 1-cm path-length cells. For observations of cefoxitin cleavage at high absorbance values, a Beckman DU 7 spectrophotometer with a 1 mm path length cell was used. Hydrolytic cleavage of benzylpenicillin was observed at 240 nm, cleavage of cephalothin was observed at 260 nm, and cleavage of cefoxitin was observed at 261 nm. The ΔE values used were 500 $M^{-1}cm^{-1}$ for benzylpenicllin(25), 7700 $M^{-1}cm^{-1}$ for cephalothin(5), and 7775 $M^{-1}cm^{-1}$ for cefoxitin(5). Comparison of initial and final absorbances after complete reaction at these wavelenths confirmed the ΔE values.

Determination of inhibition constant for borate. The ability of sodium borate to inhibit the enzymatic cleavage of benzyl penicillin was determined at 30^{o}_{C} in 0.1 M potassium phosphate, pH 7.0. Substrate concentration was 0.1-1 mM and borate concentration was varied from 32-174 mM.

Determination of k_{cat} as a function of temperature. Purified enzyme was added to 1 mM benzylpenicillin in 0.1 M potassium phosphate,pH 7.0, preheated to the desired assay temperature. Activity was measured during the initial linear phase of the hydrolytic reaction.

Observation of pre-steady state burst. The measurement of the pre-steady state burst rate was made as previously described(4). To a 10.0 mm path-length cuvette was added a solution of enzyme in 0.1 M potassium phosphate buffer, pH 7.0. The reaction was initiated by addition of cefoxitin.

An optical quartz block was quickly inserted to shorten the path-length to 1.0 mm. The progress of the reaction was observed at 261 nm using a Beckman DU-7 spectrophotometer with the cuvette temperature maintained at 30° C. The change in absorbance due to mixing was determined by repeating the procedure in the absence of enzyme and was subtracted from the observed kinetic data. The rate constant(k_{obs}) and burst size(Π/e_o) were determined by applying the double Guggenheim method(26). For the inactive mutants, a similar procedure was used to measure possible acylation of enzyme, as this would result in opening of the ß-lactam ring of cefoxitin with a resulting change in absorbance at 261 nm.

Active site titration. The magnitude of the pre-steady state burst(Π/e_o) seen at 261 nm upon the addition of cefoxitin was measured as a function of enzyme concentration for both the wild-type and the active revertant ß-lactamase. The conditions used were the same as those described above.

Direct observation of deacylation rate. Purified enzyme (wild-type or active revertant, 0.19 nmole) was incubated at 30° C with cefoxitin(16.2 mM). An aliquot of this mixture was then added to 1 mM benzylpenicillin, 0.1 M potassium phosphate, pH 7.0, and the change in A_{240} observed at 30°C using a Beckman DU-7 spectrophotometer with 1-cm path length.

Isolation of acyl-enzyme. Mutant enzyme was incubated with ^{35}S-benzylpenicillin or ^{14}C-cefoxitin in 0.1 M phosphate

buffer, pH 7.0. After incubation, the mixture was applied to a Sephadex G-25 column(1 by 20 cm) and eluted with the same phosphate buffer. Twenty drop fractions were collected. Aliquots of each fraction were assayed for radioactivity by scintillation counting. Protein was estimated in each fraction by absorbance at 281 nm. Figure III-2 shows a typical column profile for such an experiment. This procedure was repeated with bovine serum albumin as a control.

Equilibrium dialysis. All solutions used were buffered by 0.1 M potassium phosphate, pH 7.0. Protein and substrate compartments were separated by a presoaked membrane(Specktraphor 1, molecular weight cutoff 6-8,000). Mutant ß-lactamases were present at 0.306 mg/ml(11.3 µM). Ligand was ^{35}S-benzylpenicillin(3.8 Ci/mmol) diluted with fresh unlabeled sodium benzylpenicillin to a specific activity of 38 mCi/mmol. Substrate concentrations ranged from 100 µM to 415 µM with duplicate runs done at each concentration. All experiments were done at a temperature of 30° C and the dialysis compartments were shaken during incubation. At the end of the incubation period, 20 µl was removed from each compartment and assayed for ^{35}S counts after dilution to 1 ml and addition of 10 ml scintillation fluid. Final volumes of each compartment were also measured. A control well containing only ligand on one side was run with each experiment as a check to insure that equilibrium had been reached. Also, the A_{281} of each ligand compartment was measured as a

control for protein leakage due to a damaged membrane. Control wells showed less than 8 % leakage of material absorbing at 281 nm after 24 hours shaking at 30° C.

Results and Discussion

Kinetic parameters of active revertant. Kinetic parameters were obtained from single reaction progress curves. Multiple initial substrate concentrations (ranging from 100 to 500 M for benzylpenicillin) were used as a controls for product inhibition. As this reaction is not readily reversible, it is possible to use any of the standard linear plots, such as Lineweaver-Burk, to determine k_{cat} and K_m even if the reaction extent is large, provided that $\overline{[S]}$ (the average substrate concentration for each time range) rather than $[S]_0$ is used as the horizontal axis variable(27). Table III-1 collects the appropriate kinetic parameters for the catalytically active T71S mutant for benzylpenicillin and cephalothin, while Table III-2 presents these data for cefoxitin. Also included for comparison are the values for the other known active site mutant thiolactamase S70C (8). The relative activities towards penicillin and cephalosporin are also shown. In the case of all of the substrates examined, no significant difference relative to the wild-type value was found for the K_m values for the active revertant, while all k_{cat} values for this enzyme were dramatically reduced. The k_{cat} values were approximately 15 % of the wild-type value

for benzylpenicillin and cephalothin and 8 % of wild-type for cefoxitin. The observation that the K_m values are unchanged and k_{cat} values are reduced is consistent with the active revertant enzyme binding substrate and acylating at the same rate as wild-type enzyme, but possessing a defect in its deacylation ability. As the cefoxitin acyl-enzyme intermediate is known to deacylate poorly even in the wild-type enzyme, this may explain the greater rate reduction (relative to wild-type) seen for cefoxitin hydrolysis by active revertant enzyme compared to the rate reductions seen for hydrolysis of other substrates by this active mutant enzyme. More direct evidence for the relative participation of changes in acylation and deacylation abilities are presented below. No increase in the rate of enzymatic hydrolysis of cefoxitin is seen upon addition of an exogenous nucleophile(hydroxylamine,48 mM) to either wildtype(4) or active revertant ß-lactamase. This suggests that the acylenzyme intermediate in the revertant ß-lactamase is still shielded from external nucleophiles.

The ratio of k_{cat}/K_m is the apparent second-order rate constant for the conversion of free enzyme plus substrate to free enzyme plus product. The $\log(k_{cat}/K_m)$ is proportional to the activation energy, $\Delta G_T^{\ddagger}$(28). The difference between transition state binding energies for two proteins assayed against the same substrate can be calculated from equation III-2(29):

(III-2) $$\Delta\Delta G_T^{\ddagger} = -RT \ln[(k_{cat}/K_m)_A / (k_{cat}/K_m)_B]$$

Using this equation, the change in the transition state binding energy for the enzymatic hydrolysis of benzylpenicillin and cephalothin by the T71S mutant of ß-lactamase can be estimated. The transition state binding energy for benzylpenicillin has been elevated by 945 calories relative to the wild-type enzyme, while the transition state for cephalothin hydrolysis has been raised by 1340 calories. This activation energy, $\Delta G_T^{\ddagger}$, is the sum of two factors-an algebraically positive term $\Delta G^{\ddagger}$, the activation energy of bond rearrangements, and an algebraically negative term, ΔG_S, the substrate binding energy. In the T71S ß-lactamase, either the activation energy term $\Delta G^{\ddagger}$ has been elevated relative to the wild-type enzyme, or the negative binding energy term ΔG_S has been made less negative. (Of course, a combination of both of these two changes may have occured). In the Michaelis-Menton mechanism, K_m is a function of ΔG_S. Since the K_m values for cleavage of ß-lactams by the T71S enzyme are unaltered, one can conclude that ΔG_S is unchanged and that the increase is in the activation energy for this reaction is due to an increase in $\Delta G^{\ddagger}$, the energy involved in the making and breaking of bonds necessary in reaching the transition state from the initial ES complex.

Purified active revertant enzyme is also capable of cleaving methicillin, ampicillin, and cephalosporin C, but a detailed kinetic analysis was not performed on these sub-

strates. This result is consistent with earlier phenotypic studies of this mutant, which showed that this mutant gave resistance to all penicillins tested, and gave slight resistance to cephalosporins.

Boric acid is one of the best known competitive inhibitors of RTEM ß-lactamase. The ability of boric acid to serve as a competitive inhibitor of benzylpenicillin was examined in the active revertant enzyme. The K_I for borate inhibition was unchanged from that seen in wild—type enzyme (Table III-3). This suggests that the hydroxyl groups of either a threonine or serine residue at position 71 interacts equally well with boric acid, or that the only interaction of this inhibitor is with the active site serine 70. Thiolactamase(-Ser70 to Cys) is not inhibited by borate(8), supporting the second possibility.

Activity as a function of temperature. The results of activity measurements made at elevated temperature are shown in Figure III-3. In the temperature range of 30 to 45°C the ratio of activity of the active revertant relative to wild type increases compared to the value of this ratio at 30°C. This activity increase is due to an increase in the value of k_{cat}. Above 45°C, it was impossible to accurately measure initial activity, as thermal denaturation of the mutant enzyme occured on the same time scale as the activity measurements. The altered stabilities of the mutant ß-lactamases are examined in more detail in Chapter 4.

Activity as a function of pH. No differences in activity as a function of pH were found between the active revertant enzyme and wild-type ß-lactamase in the pH range 4.0 to 8.0. Above this pH both enzymes rapidly denatured.

Active site titration. The reduction in k_{cat} seen for hydrolysis by the active revertant could be due either to a reduction of the intrinsic ability of each catalytic site to cleave a ß-lactam bond or it may be due to a decreased number of active sites in the revertant enzyme, with each one of these sites possessing normal catalytic ability(30). To distinguish between these possibilities, it is necessary to measure the number of functional catalytic sites independent of protein concentration. The number of active sites in ß-lactamase can be measured using the poor substrate cefoxitin. This substrate acylates rapidly relative to deacylation with an easily observed decrease in UV absorbance. Measurement of the size of the initial rapid burst and plotting this magnitude relative to amount of protein added allows determination of the number of active sites per unit of protein. Based on this procedure, the active revertant enzyme has nearly as many active sites per mg of protein as the wild-type enzyme possesses. The active revertant has 88+9 % of the number of active sites the wild-type enzyme has, far larger than the reduction in k_{cat} (8-15 % of wild-type, depending on the substrate). This reduction in k_{cat} thus cannot be solely due to a reduction in number of normal catalytic sites.

However, this procedure can not detect a reduction in number of sites capable of deacylation. As seen below, after sufficient time the transiently inactivated acyl enzyme regains its initial level of activity against benzylpenicillin, ruling out the inability of revertant enzyme active sites to deacylate as the cause for the diminished reaction rate.

Observation of pre-steady state burst. The active revertant enzyme shows no significant difference in burst size(n/e_o) or rate constant(k_{obs}) when compared with the wild-type enzyme(Figures III-4,5; Table III-2). This indicates that decrease in the acylation rate(k_2) is not the main cause for the lowered k_{cat} seen for the active revertant enzyme and suggests that the lowering of the steady-state hydrolysis rate is predominantly due to a decrease in deacylation rate (k_3).

Direct observation of deacylation rate. The deacylation rate for cefoxitin was also directly measured. By incubating enzyme at a cefoxitin concentration much higher than the K_m for this substrate, most of the enzyme was converted into the acyl-enzyme form. This transiently inactivated enzyme was then diluted into a larger volume of a good substrate(such as benzylpenicillin) at a high concentration relative to its K_m, resulting in the cefoxitin concentration being reduced below its K_m. As the enzyme deacylates, it is much more likely to react with the large excess of good substrate molecules

present. By observing the increase in ability to hydrolyze benzylpenicillin with time by a β-lactamase sample previously incubated with cefoxitin, the deacylation rates for wild-type and active revertant enzymes were determined. After sufficient incubation time, the cefoxitin treated enzyme (both wild-type and active revertant) reattained control levels of benzylpenicillin cleaving ability.

The deacylation rate for the active revertant enzyme is dramatically reduced relative to the wild-type enzyme(Table III-2). This reduction, to about 8 % of the wild-type value, corresponds well to the rate reduction seen for cefoxitin hydrolysis by this enzyme, adding further support to the hypothesis that the deacylation rate is the limiting step for hydrolyis of this substrate.

The observed deacylation rate reduction could be due to an inability of the nucleophile responsible for deacylation to properly interact with the acyl-enzyme intermediate. The methyl group missing in the Thr71 to Ser active revertant may provide an interaction that correctly orients this nucleophile, or it may generally stabilize the enzyme and prevent bulky substrates(such as cefoxitin) from severely distorting the enzyme conformation when the acyl-enzyme intermediate is formed. The ability of cefoxitin to induce structural perturbations in an RTEM ß-lactamase has been demonstrated using sensitivity to trypsin digestion as a probe(31)

The inactive ß-lactamase mutants do not form an acyl-en-

zyme intermediate. No evidence for formation of acyl-enzyme intermediate in ß-lactam hydrolysis by the two inactive mutant enzymes(S70T-T71S and S70T) was found in two different kinds of experiment. A typical spectrophotometric experiment, designed to measure formation of acyl-enzyme by measuring ring opening of cefoxitin(similar to the procedure described above to measure the presteady state burst) is shown in Figure III-6. The absorbance change at 261 nm was not significantly different than the change seen due to mixing upon adding buffer with no enzyme. Due to the size of this mixing artifact, it was impossible to rule out low levels of acyl-enzyme formation using this procedure alone. However, it was possible to assign an upper limit of 10 % of theoretical acylation, assuming all protein was folded in a native conformation and that denaturation had not taken place during purification or storage. It should be noted that the inactive mutants were presumed to be temperature-sensitive. They were produced at low temperature(30^{o}C) and were kept cold at all times during purification to minimize thermal and proteolytic degradation.

As a more sensitive probe of acyl-enzyme formation, the mutant proteins were reacted with radiolabelled substrate, and then enzyme-bound substrate was separated from unbound substrate by gel filtration. Using both a poor substrate (^{14}-C-cefoxitin) and a good substrate (^{35}-S-benzylpenicillin), no covalent enzyme-substrate intermediate formation was

detected beyond a low background of 0.02 % of theoretical. This background was similar in magnitude to the slight amount of non-specific binding seen for benzylpenicillin binding to a presumably nonspecific protein such as bovine serum albumin. This background value is presumably due to the high reactivity of the ß-lactam ring, which can randomly react with any surface nucleophile of a protein. These nonspecific covalent linkages are believed to be responsible for producing the epitopes involved in the allergic response to penicillin(32).

Incubation times in the radiolabeling experiments ranged from 30 minutes to 12 hours, with no difference in the extent of substrate binding detected. This result indicates that even very slow acylation is not occurring in the inactive mutant enzymes.

Ability of the inactive ß-lactamase mutants to bind substrate. Though neither of the inactive mutants were observed to form an acyl-enzyme intermediate, they were examined for ability to bind substrate non-covalently. It should be noted that even if these proteins show some affinity for a substrate such as benzylpenicillin, it cannot be assured that the substrate is binding at the correct location or in the proper configuration which would allow enzymatic catalysis as in an active protein. In addition, due to the speed with which the active proteins cleave a good substrate such as benzylpenicillin, it is difficult to estimate the

value for K_s (the substrate binding constant, k_{-1}/k_1) for these active proteins. Scatchard plots(33) of benzylpenicillin binding to the two inactive mutants showed binding sites with an average affinity of 100-200 M were present. The K_m for this substrate reacting with native wild-type enzyme is approximately 20-30 μM. If this K_m value is used as an estimate for the K_s value of the active enzyme, than the inactive mutants show about a six-fold decrease in binding ability. No significant difference in substrate binding was seen between the thr-thr and thr-ser enzymes.

Summary

The active revertant enzyme shows no differences in its substrate specificity or K_m relative to the wild-type enzyme. However, this enzyme does have a reduced k_{cat} value for all substrates, which is predominantly due to a decrease in the rate of deacylation. The inactive mutants(S70T-T71S and S70T) do not form an acyl-enzyme intermediate, but do bind substrate. Thus they are defective at the acylation step. These results show that only a primary nucleophile(either OH or SH)at position 70 of ß-lactamase allows formation of an acylenzyme intermediate. A secondary nucleophile, such as the hydroxyl of threonine, or a primary nucleophile in the adjacent position in the primary protein sequence, is not an adequate substitute.

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Table III-1. Comparison of Catalytic Parameters of Wild-Type and Active Revertant β-Lactamases

Penicillin G			
	K_m (μM)	k_{cat} (sec^{-1})	(k_{cat}/K_m) ($M^{-1}sec^{-1}$)
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.)	60 ± 10	20	3.3×10^5

Cephalosporins			
	K_m (μM)	k_{cat} (sec^{-1})	(k_{cat}/K_m) ($M^{-1}sec^{-1}$)
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.) (Cephaloridine, wild-type value in brackets)	1800 (1000)	2 (1700)	1.1×10^3 (1.7×10^6)

Penicillin G/Cephalosporin			
	K_m	k_{cat}	k_{cat}/K_m
Ser Thr (Wild-Type) (Penicillin G/Cephalothin)	0.10	8.7	84
Ser Ser (Penicillin G/ Cephalothin)	0.07	11.0	160
Cys Thr (Sigal et al.) (Penicillin G/Cephaloridine)	0.03 (0.05)	0.1 (1)	300

Table III-2. Comparison of catalytic parameters for the cleavage of cefoxitin by wild-type and active revertant β-lactamase. Literature values are from (5).

A. Steady-State Parameters

	Wild-type		T71S mutant
	observed	literature	
K_{cat} (s^{-1})	3.46×10^{-3}	4.0×10^{-3}	3.4×10^{-4}
K_m (mM)	0.70	0.65	0.78

B. Pre-Steady-State Burst

K_{obs} (s^{-1})	$1.28 \pm .12 \times 10^{-2}$	1.6×10^{-2}	$1.01 \pm .07 \times 10^{-2}$
π/ε_0	0.82	0.34 - 0.50	0.79

C. Deacylation Rate

K_3 (s^{-1})	2.9×10^{-3}	4.8×10^{-3}	2.8×10^{-4}

Table III-3. Constants for borate inhibition of benzylpenicillin hydrolysis by β-lactamases.

Protein	K_i
wild-type	1.02 ± .42 mM
active revertant (T71S)	0.93 ± .47 mM

Values are calculated assuming purely competitive inhibition and a benzylpenicillin K_m value of 24 μM.

Figure III-1

The reaction pathway used in the cleavage of cefoxitin (1) by RTEM beta-lactamase (EOH). The cefoxityl acyl-enzyme (4) can either deacylate, regenerating free enzyme followed by the breakdown of the unstable cephalosporanate (2), or the 3' leaving group (X^-) can be released before deacylation.

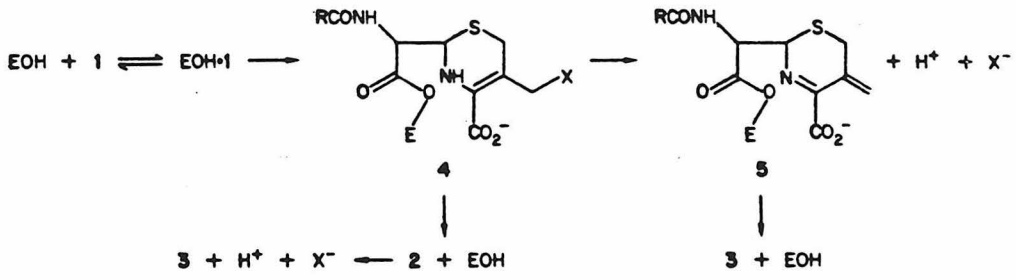

EOH + 1 ⇌ EOH•1
RCONH
S
NH
X
O
O
E
CO_2^-
4
RCONH
S
N
O
O
E
CO_2^-
+ H^+ + X^-
5
3 + H^+ + X^- ← 2 + EOH
3 + EOH

Figure III-2. Column profile showing elution of S70T-T71S mutant ß-lactamase from a gel filtration column after incubation with ^{35}S-labeled benzylpenicillin. Filled circles are cpm radioactivity, open circles are protein as measured by absorbance at 281 nm.

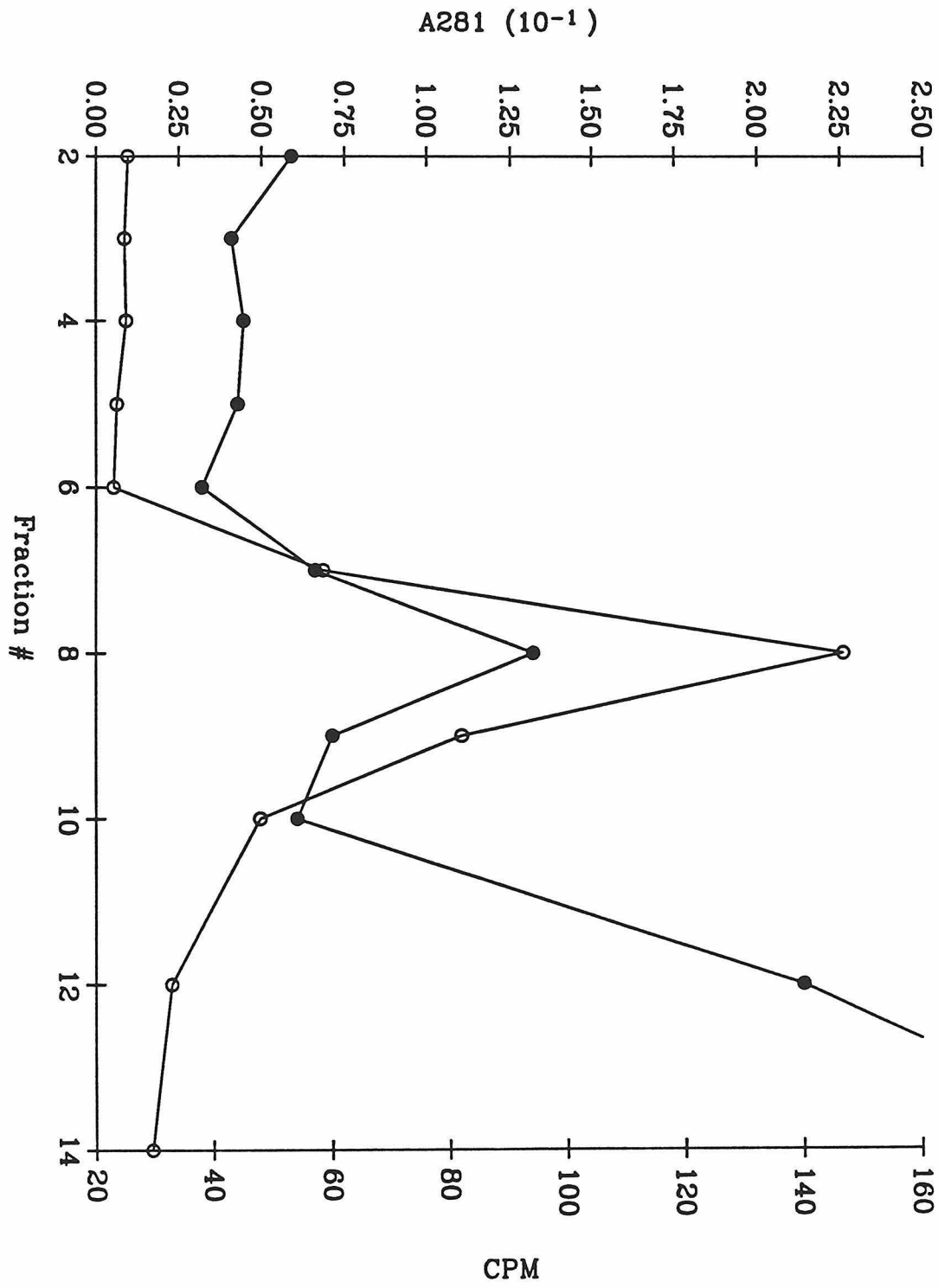

A281 (10^{-1})
0.00
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00
2.25
2.50
Fraction #
2
4
6
8
10
12
14
CPM
20
40
60
80
100
120
140
160

Figure III-3. The variation in the rate of benzylpenicillin hydrolysis with temperature for wild-type and T71S mutant beta-lactamase.

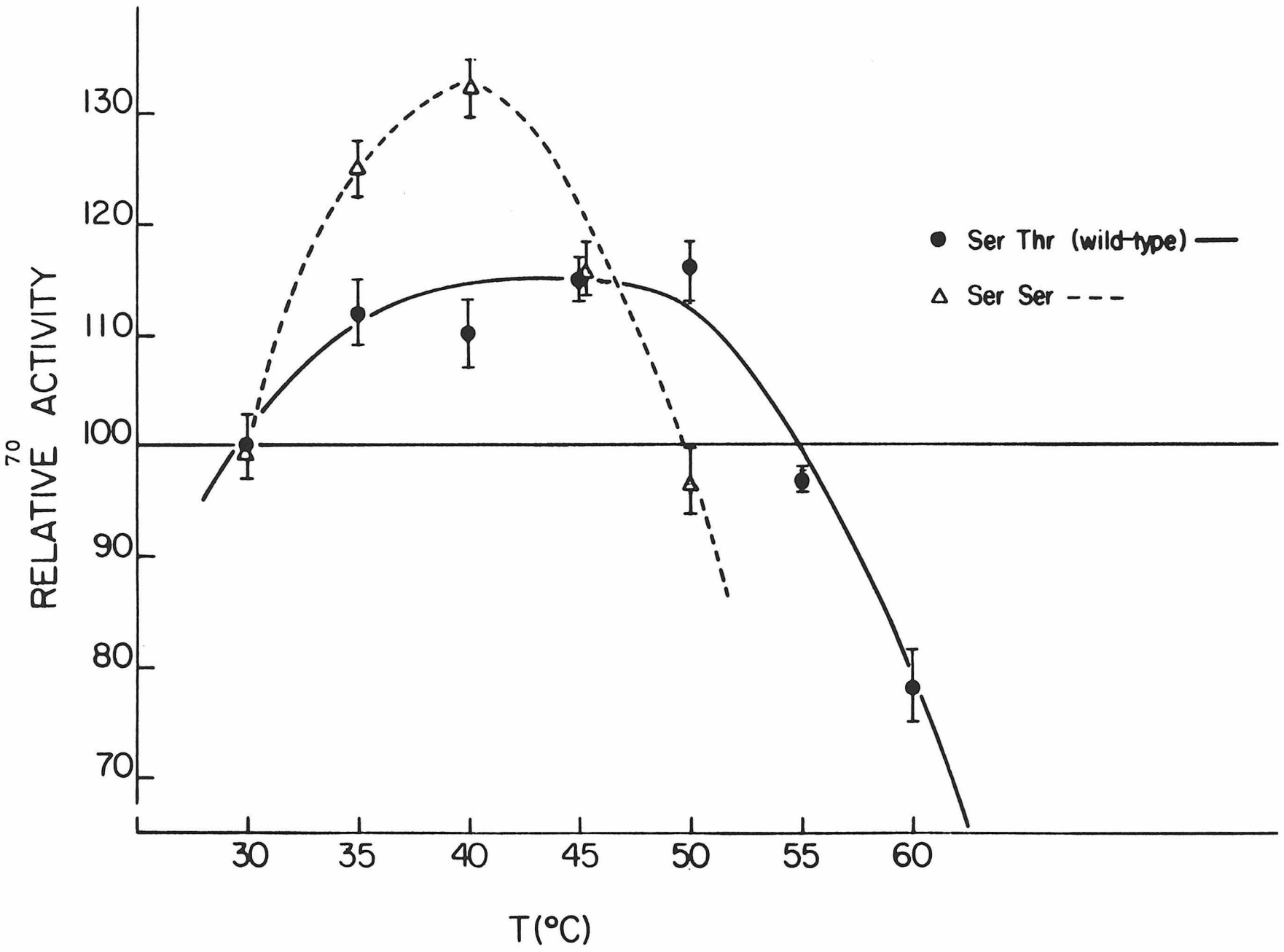

RELATIVE ACTIVITY
70
130
120
110
100
90
80
70
30
35
40
45
50
55
60
T(°C)
Ser Thr (wild-type)
Ser Ser

Figure III-4. Hydrolysis of cefoxitin catalyzed by the wild-type pBR 322 beta-lactamase. The arrow on the ordinate shows the size of the burst. The dashed line is the extrapolated steady-state rate.

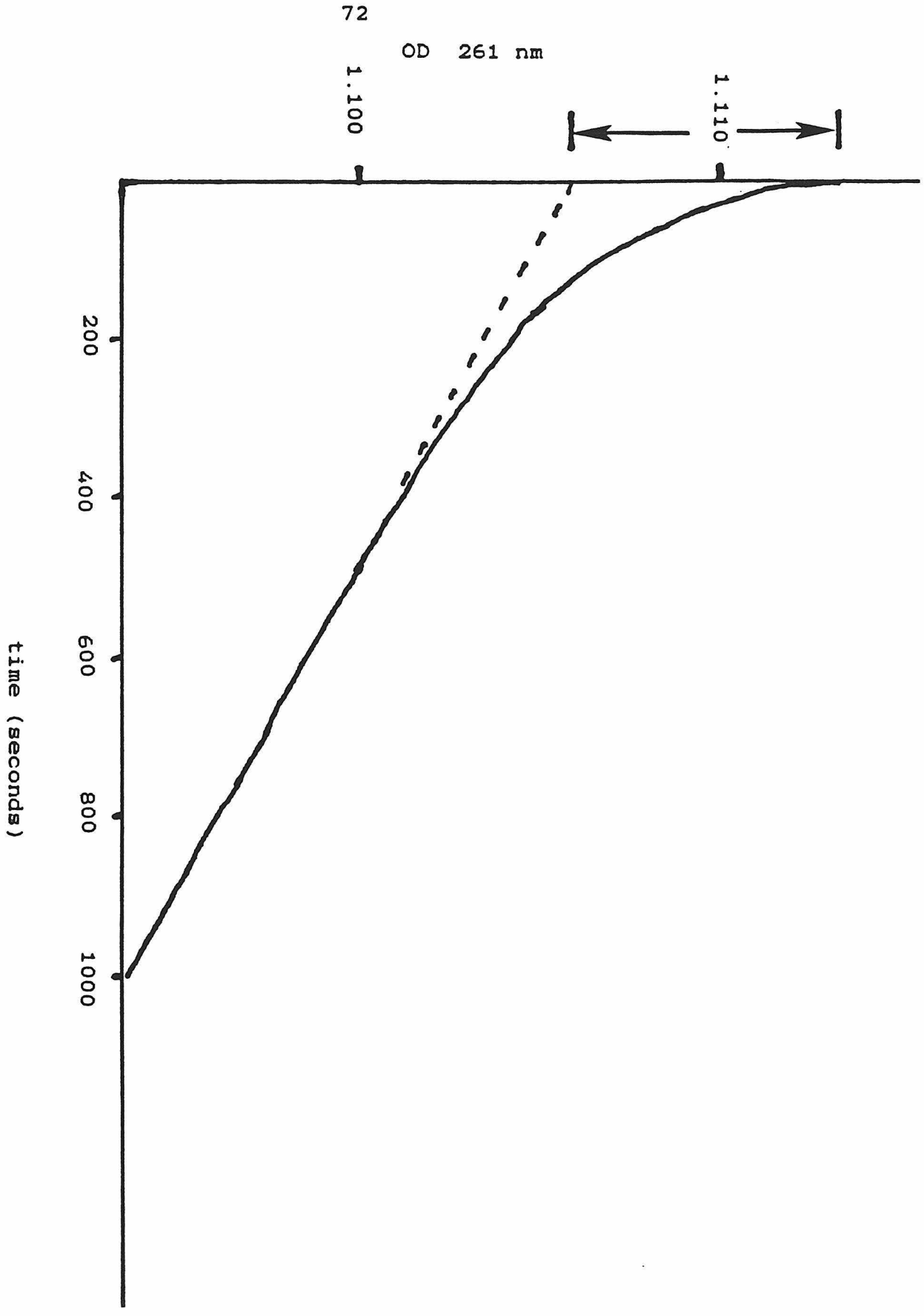

OD 261 nm
1.100
1.110
200
400
600
800
1000
time (seconds)

Figure III-5. Hydrolysis of cefoxitin catalyzed by the T71S active revertant beta-lactamase. The arrow on the ordinate shows the size of the pre-steady-state burst. The dashed line is the extrapolated steady-state rate.

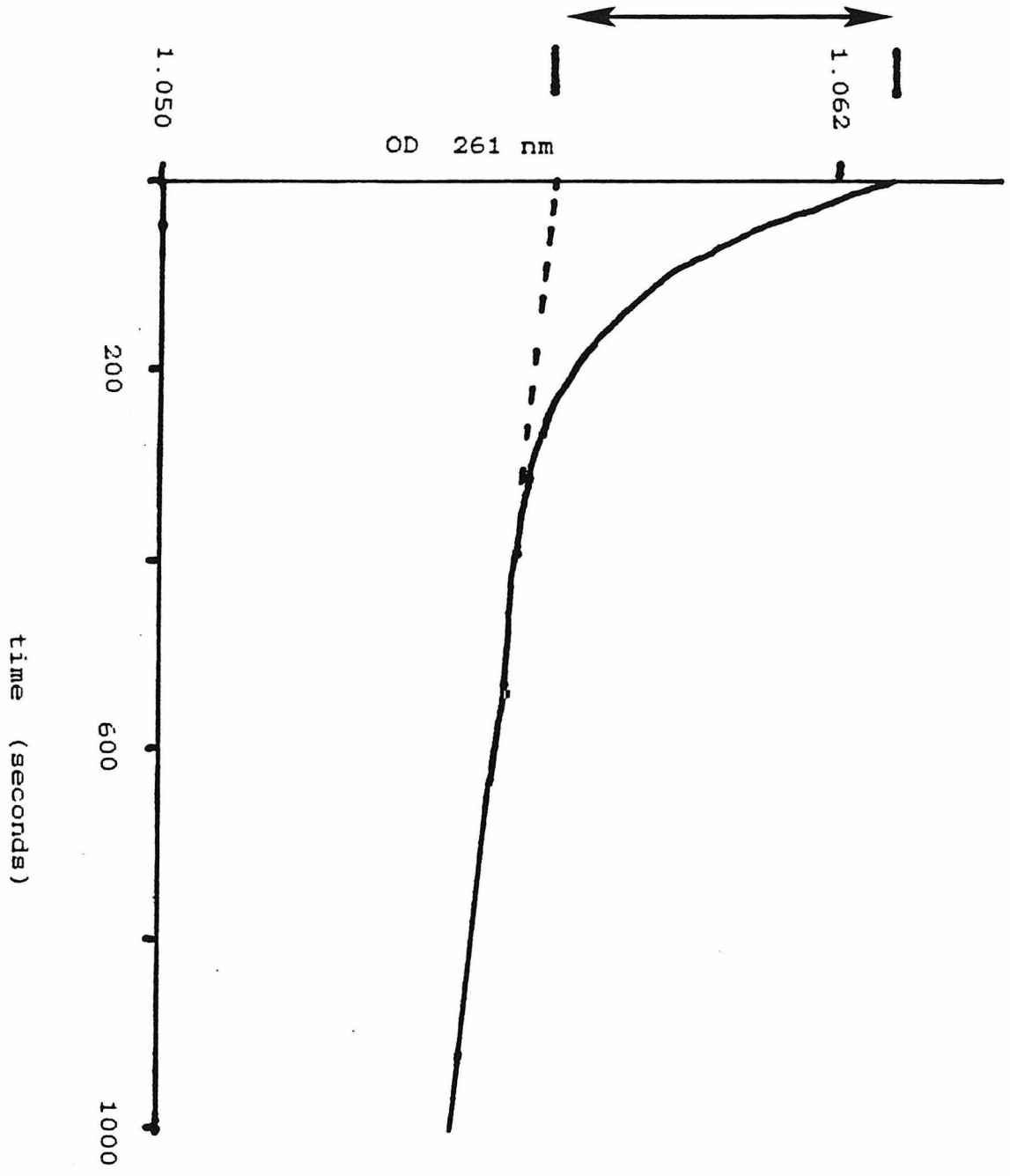
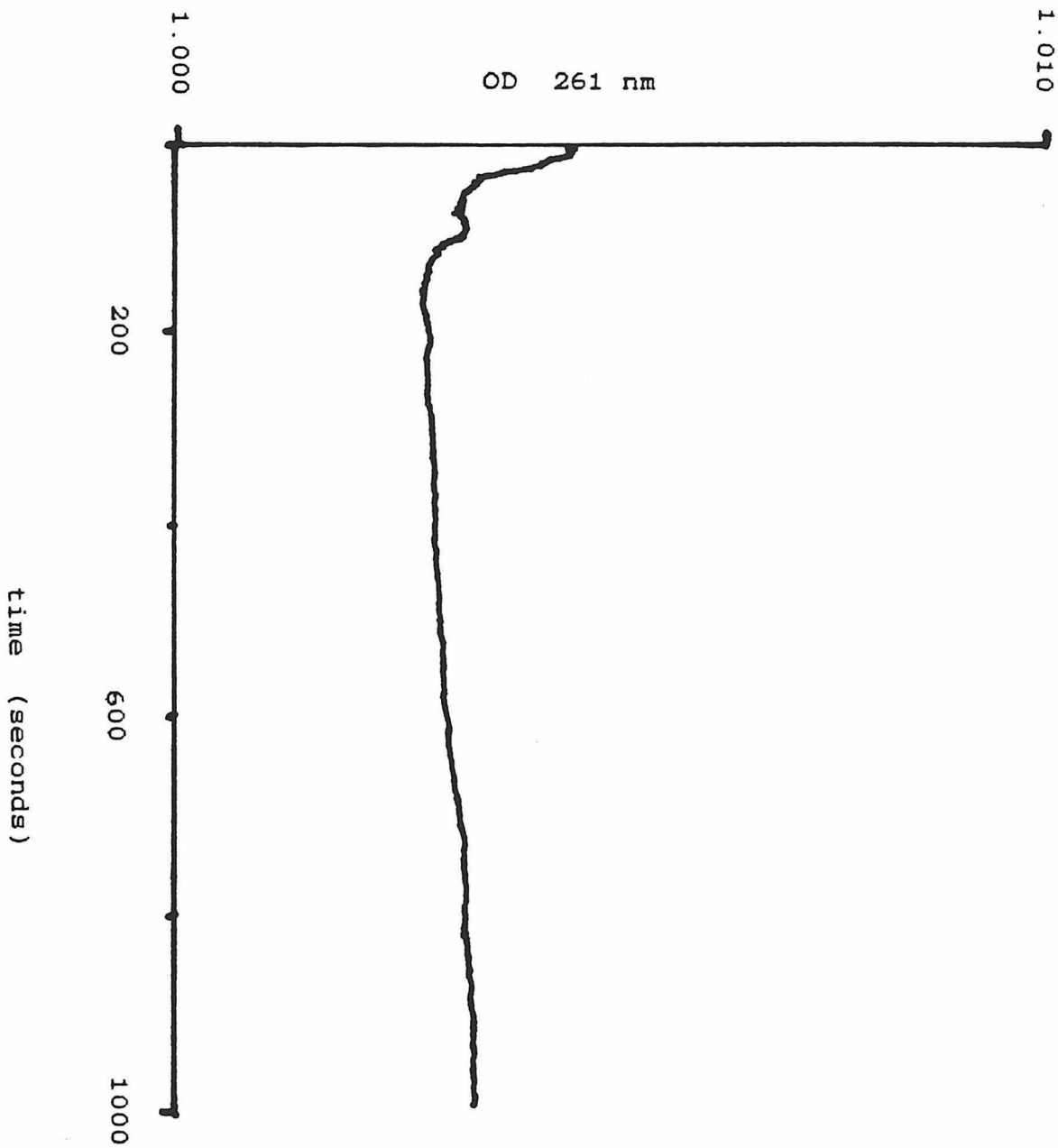

1.050
OD 261 nm
1.062
200
600
1000
time (seconds)

Figure III-6. Lack of hydrolysis of cefoxitin by the S70T-T71S double mutant of beta-lactamase. The change in OD in the first seconds is the same magnitude as that observed if no enzyme is present in the cuvette when substrate is added.


1.000
OD 261 nm
1.010
200
600
1000
time (seconds)

CHAPTER IV

INVESTIGATION OF THE STABILITY OF ACTIVE-SITE MUTANTS OF BETA-LACTAMASE

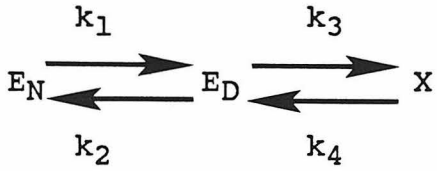
Introduction

The inability to isolate the active site mutants of beta-lactamase (S70T, T71S,S70T-T71S) at 37$^{o}_{C}$ suggested that these proteins were less stable than the wild-type enzyme. This temperature sensitive phenotype could arise in two ways. First, the enzyme may not be synthesized or secreted normally. Second, the enzyme may be synthesized normally, but may be rapidly denatured after synthesis and export to the periplasm. As reported previously, the pulse-chase studies used to measure the secretion of these mutants indicate that protein synthesis is normal for these mutants and suggests that these mutant proteins are denatured after secretion(1) The denaturation of a mutant protein can be due to thermal instability(2,3), instability due to the presence of chemical denaturants such as urea, and sensitivity to proteolysis(4). It should be noted that many mutant proteins are more stable than their wild-type counterparts(5-7). The inactivation of the active-site mutant beta lactamase by these processes was investigated and the results are presented in this chapter. In order to interpret these results, it is first necessary to review previous studies that have examined the denaturation of beta-lactamases and the stability of other specifically mutated proteins.

Plasmid encoded beta-lactamases have been the subjects of a number of studies aimed at understanding protein folding

and denaturation. In particular, the Staph.aureus PC1 and B.cereus enzymes have been studied by direct physical techniques, such as circular dichroism(8,9), proton NMR(10), ultraviolet and flouresent spectroscopy(11), and differential scanning calorimetry(12).

The following simple model has proven adequate to explain the conformational changes seen in beta-lactamases when they are perturbed by heat or chemical denaturants(13):



E_N = native state E_D = reversibly unfolded state

X = irreversibly denatured state, where k_4 is so slow as to be discounted

Proline isomerization has been ruled out as a step in the folding or unfolding of plasmid-encoded beta-lactamases(14).

It is important to note that lessons learned concerning the stability of one of the beta-lactamases may not be true for other beta-lactamases. For example, a threonine to isoleucine mutation in the PC1 enzyme causes a folding block that results in a completely inactive enzyme(15). The same mutation at the homologous position in the pBR 322 enzyme (residue 71) produces one of the most stable active mutations of this enzyme(16).

The effects of single amino acid changes on the folding and stability of a number of proteins have been studied. The

best characterized of these proteins is hemoglobin, in which 73 unstable variants have been isolated. The causes of the instability in these variant hemoglobins have been divided into nine subsets(17). The changes produced by single amino acid substitutions that result in unstable hemoglobin molecules include loss of a non-polar side chain, loss of a hydrogen bond, introduction of an interior dipole, and the replacement of a residue by a larger or smaller amino acid.

Serine and threonine only differ in that threonine possess an additional methyl group. This single amino acid substitution could produce any of the above-mentioned structural effects, either directly due to the presence of the methyl group or by the methyl group influencing the orientation of the hydroxyl group, causing changes in internal hydrogen bonding.

Another protein in which single amino acid substitutions have altered stability is the α subunit of tryptophan synthetase from E.coli. In this case, the stability of the enzyme correlates well with the hydrophobicity of the substituting amino acid residues(6,18).

When the amino acid sequences of three proteins(ferredoxin, lactate dehydrogenase, and D-glyceraldehyde 3-phosphate dehydrogenase) from many species of mesophilic and thermophylic organisms are compared the following substitutions are frequent(with the second member of each pair present in the thermophile):Gly to Ala, Ser to Ala, Ser to Thr,

Lys to Arg, and Asp to Glu(18).

Limited proteolysis has proven to be a useful technique for detecting conformational changes in proteins(19). The native form of many proteins is resistant to proteolysis and denaturants accelerate the protease reaction. For example, the heat-sensitive form of E.coli adenylate cyclase is readily inactivated by proteases at slightly elevated temperatures(4). This has led to the hypothesis the the unfolded form of a protein is the substrate for proteases, allowing proteolysis to serve as a probe for the conformational transition between native and unfolded states. In the case of ten different cytochrome c proteins, the proteolytic susceptibility of these proteins to four different proteases correlated well with the thermal stability of these proteins as measured by circular dicroism(20). A similar correlation between proteolytic sensitivity and denaturation as measured by circular dichroism is seen in mutants of the phage lambda repressor(21). As will be shown below, the wild-type pBr 322 beta-lactamase also shows a similar correlation in its thermal denaturation, as measured by circular dichroism, loss of enzymatic activity, and sensitivity to proteolysis.

Materials and Methods

Materials. The protein preparations used were purified using the procedures in Chapter 2. Thermolysin (Protease

type X from Bacillus thermoproteolyticus rokko) and TPCK-(N-tosyl-L-phenylalanine chloromethyl ketone)treated trypsin were from Sigma.

Thermal stability. Purified ß-lactamase (0.1 mg/ml in 0.1 M potssium 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 ß-lactamase solution (0.6 mg of ß-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 a final concntration of 0.01 M and cooling on ice. Aliquots were assayed for remaining ß-lactamase activity at 30°C. This procedure was repeated using TPCK-treated trypsin in place of thermolysin. Half-lives for activity loss were estimated by plotting log(residual activity/initial activity) versus time.

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 hours later. Cells were pelleted in a Microfuge, resuspended in 50 µl of gel-loading buffer (0.063 M Tris-HCl, pH 6.8; 2% SDS; 5% v/v ß-mercaptoethanol),

and boiled 10 min. Aliquots of supernatant (20 µl) were analyzed by immune blotting (p.21). Resistance to periplasmic proteases was estimated by incubating ß-lactamase in osmotic extrudate obtained from E.coli and assaying for residual activity at appropriate time points.

Stability in urea. The enzymatic activities towards benzylpenicillin of the wild-type enzyme and the T71S 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. The proteins were also assayed for residual activity after storage in 0.1 M potassium phosphate, pH 7.0 with urea added.

Circular Dichroism and Flouresence Spectroscopy. Purified protein was furnished to the laboratory of Dr. A.R. Fink, UC Santa Cruz.

Results

Thermal behavior. Table IV-1 summarizes the relative thermal stability of wild-type and active revertant (T71S) enzymes as measured by catalytic activity; whereas the parent enzyme is stable for periods longer than 30 min at 50°C, the T71S 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. This concentration dependence suggests that aggregation of thermally unfolded ß-lactamase

is a component of this irreversible denaturation. In addition, the chemically unfolded form of the S.aureus ß-lactamase(9) and the thermally denatured B.cereus I lactamase(22) also show concentration dependent irreversible aggregation. Similar concentration dependent thermal denaturation is seen for the lambda repressor(20). The thermally denatured lactamase shows no change in mobility on 12 % SDS-PAGE. Even at 4°C, purified T71S 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 inactivated over several weeks, again with no change in mobility on SDS-PAGE. This would appear to rule out autolysis as the cause for this loss of activity.

The circular dichroism of the wild-type enzyme as a function of temperature was observed; the enzyme shows a cooperative melting in the same temperature range in which enzymatic activity is lost (Figure IV-1). Circular dichroism changes at this wavelength (220 nm) are due to local changes in the backbone structure of the protein. This cooperative melting transition is consistent with the results obtained for the B.cereus I enzyme, where ultraviolet differnce spectra and cicular dichroism show large conformational changes during thermal denaturation in this temperature range(24). The active T71S mutant enzyme did not survive shipment to Santa Cruz(25).

The fluoresence spectum of the wild-type pBR 322 beta-lactamase isolated in this work shows that all fluorophores

are completely exposed to solvent at low temperature, in contrast to the B.cereus enzyme(25). Thus fluoresence changes cannot be used to monitor conformational changes in the E.coli enzyme.

Proteolytic stability. All of the active-site mutants show a marked increase in susceptibility to proteolytic degradation. Figure IV-2 shows this 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. This figure also illustrates that the two inactive mutants (S70T, S70T-T71S) are even less resistant to cellular proteases than the active T71S mutant. Proteins of lower molecular weight that react with antibody, and that are absent from control cells, are also present; these presumably are proteolytic fragments of ß-lactamase.

This increased rate of proteolysis for the active T71S mutant can also be seen when a chemically well-defined protease is used. For example, the T71S mutant enzyme is dramatically more sensitive to thermolysin digestion at both 37°C and 45°C (Table IV-2) than the wild-type ß-lactamase. The mutant protein was also more sensitive to digestion by TPCK-treated trypsin; however, it was difficult to estimate half-lives using trypsin due to the autolysis of the trypsin

during the assay.

A similar but less dramatic loss of enzyme activity is seen if extruded active revertant enzyme is incubated in E.coli periplasmic fluid. In contrast, wild-type enzyme shows no loss of activity after three years of storage in periplasmic fluid at 4oC.

Urea stability. The T71S revertant enzyme is also more sensitive to urea-induced denaturation than the wild-type enzyme. At 30oC and pH 7.0, the T71S 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 activity upon 50-fold dilution of urea, even after 48-h exposure at 37oC. (Note that these studies involve protein concentrations lower by a factor of at least 10 than those used in the studies of thermal stability.) The ready reversibility of this urea-induced denaturation compared to the irreversibility of thermal induced denaturation would seem to indicate that the denatured states produced by these two techniques differ dramatically.

Discussion

Though the mutation T71S does not drastically reduce catalytic activity, this change seriously destabilizes the three-dimensional structure of the protein. In the presence of proteases, as in the environment of the periplasmic space, the instability of the T71S mutant is significantly ampli-

fied. The other two active site mutants (S70T-T71S and S70T) are equally as sensitive to degradation by the intrinsic E.coli proteases. Possibly, the mutant proteins have a conformation resembling pre-β- lactamase, which also has reduced catalytic activity and is highly susceptible to proteolytic attack(26,27). In interesting contrast, the mutant S70C, which retains Thr71, has enhanced thermal stability and resistance to proteolysis.(28) This is consistent with the observation that enzymes from thermophilic organisms often have threonine residues in the place of serine residues found in the same enzymes in related mesophiles(18).

Summary

The inability to isolate three active site mutant beta-lactamases from E.coli following standard growth and purification procedures suggested that these enzymes possessed reduced stability. Purified protein of the active T71S mutant enzyme has reduced resistance to thermal denaturation. In addition, this enzyme is digested more rapidly than the wild-type enzyme both in vitro by chemically defined proteases such as thermolysin and trypsin as well as in vivo by E.coli proteases. The T71S enzyme is also denatured by lower urea concentrations than those required to denature the wild-type enzyme. The inactive S70T and S70T-T71S mutants are even more sensitive to proteolytic degradation by E.coli proteases than the active T71S mutant is.

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Table IV-1. Thermal Stability of Active β-Lactamases

	$t_{\frac{1}{2}}$	
	Wild-Type Ser Thr	Mutant Ser-Ser
40°C	-	9 min
45	-	5 min
50	>30 min	<1 min
55	13 min	
60	1-2 min	

Table IV-2. Resistance of Wild-Type and Revertant β-Lactamases to Thermolysin Digestion

Temperature	Wild-Type $t_{\frac{1}{2}}$	Revertant (ser-ser) $t_{\frac{1}{2}}$
37°C	50 min	3.5 min
44°C	18 min	<1 min

Figure IV-1. The thermal denaturation of wild-type pBR 322 β-lactamase detected by circular dichroism. Assay conditions are 3 μM enzyme, 0.1 M cacodylic acid, 1 M KCl, ph 7.0.

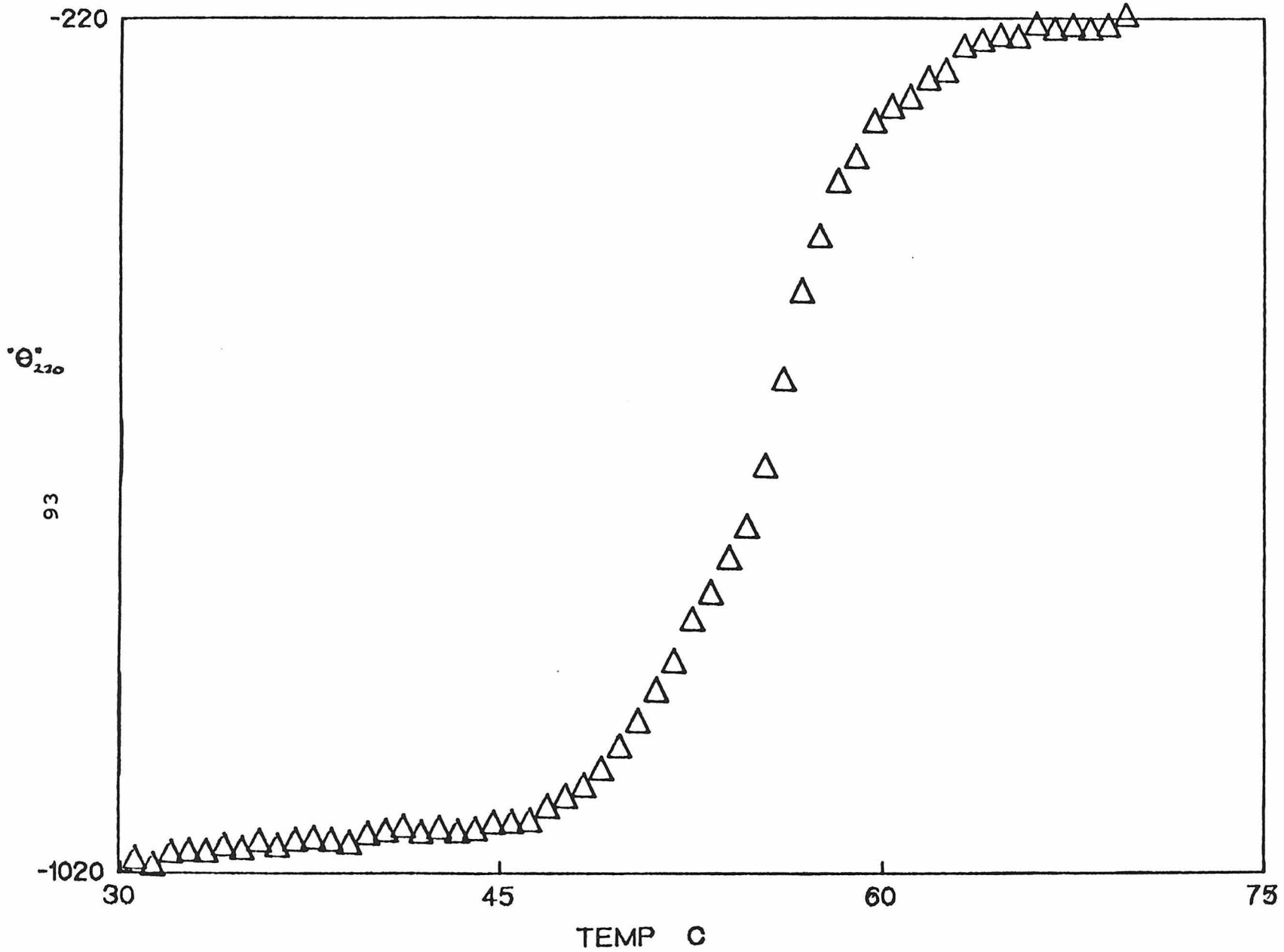
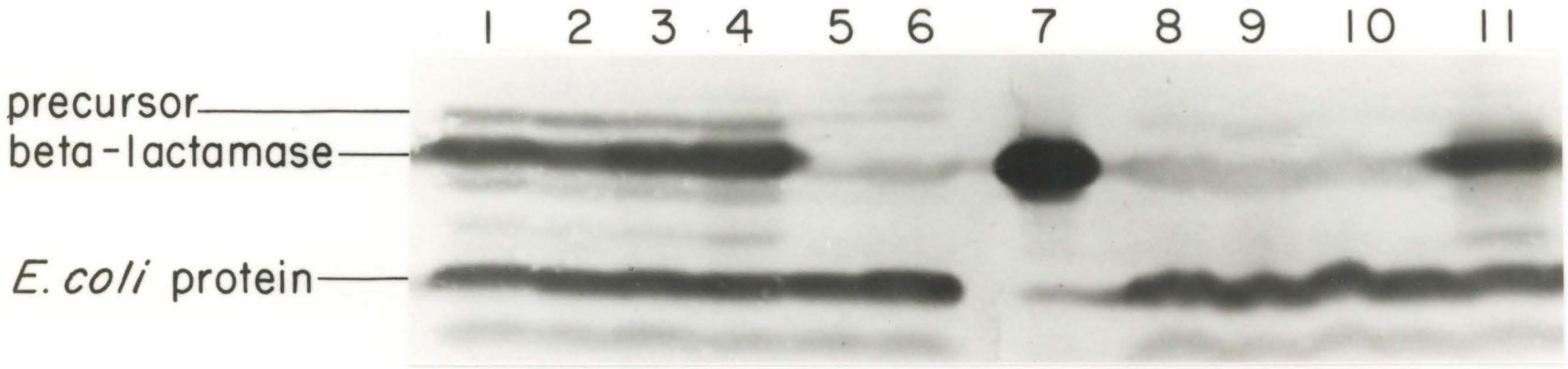

-220
-1020
θ_{110}
93
30
45
60
75
TEMP C

Figure IV-2. Photograph of peroxidase stained nitrocellulose membrane from immune blotting procedure showing beta-lactamase, its precursor, and an E.coli protein which also reacts with the antibody. Lanes 1-6, taken at late log phase; lane 7 is pure beta-lactamase control (0.14 μg); lanes 7-12, taken 12 hours later. Amino acids listed below are the two active site residues, 70 and 71: 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, furnished by S.Schultz); 6, LS1(control, no plasmid)


1 2 3 4 5 6 7 8 9 10 11
precursor
beta-lactamase
E. coli protein

CHAPTER V

INVESTIGATION OF THE ROLE PLAYED BY THE DISULFIDE BOND IN PBR 322 BETA-LACTAMASE

Introduction

The role of disulfide bonds in maintaining the structure of secreted proteins and stabilizing the conformation of globular proteins has received considerable attention(1-3). However, many proteins with naturally occuring disulfide bonds have shown the ability to assume their native conformation and retain activity without the formation of these disulfide bonds. This suggests that disulfide bonds that may enhance conformational stability are not absolutely required for correct folding or function.

The RTEM ß-lactamases encoded by the plasmids pBR322 contain a single disulfide bond formed between cysteine residues at positions 77 and 123(4,5). This disulfide bond is formed as the protein is secreted from the cell. The disulfide bond present in wild-type pBR322 ß-lactamase cannot be reduced without using extreme conditions which denature this protein. Site-directed mutagenesis was used to replace cysteine 77, one of the residues that participates in the disulfide bond, with a serine residue(6). Serine was chosen as the replacement amino acid due to its similarity in size to cysteine(7). This mutation resulted in a ß-lactamase that resembled the wild-type enzyme when host-cell phenotypes were compared(6). This chapter will discuss the catalytic properties and stability of the purified Cys77 to Ser mutant

β-lactamase.

Similar removal of the naturally occurring disulfide bond from interleukin 2 by oligonucleotide mediated replacement of a cysteine by either alanine or serine resulted in mutant proteins with considerably lower activity than the wild-type enzyme(8,9).

In addition to its use in studying the role of the disulfide bond in β-lactamase, the C77S mutant protein may also prove useful in solving the structure of this protein by X-ray diffraction. By replacing cysteine residue 77 with serine, the remaing sulfhydryl of cysteine 123 is left free. This forms a potential binding site for heavy metal atoms such as mercury. A sample of this mutant protein was purified and a mercury derivative was prepared for this purpose. This approach for generating a free sulfhydryl group could also be used for the attachment of other probes, such as nuclear magnetic resonance or fluorescence labels, to a specific region of a protein.

Materials and Methods

Production of purified Cys77 to Ser mutant protein. Plasmid pBR322 containing the C77S mutant β-lactamase gene was furnished by Dr. G. Dalbadie-McFarland. Protein was produced using the procedures described in Chapter II. Growth temperature was 30oC and ampicillin(20 mg/l) was used as the selecting antibiotic. The homogeneity of the protein

was determined by 12% SDS-PAGE followed by silver staining using standard procedures(10). Protein concentrations were determined using a previously determined extinction coefficient of 29,400 M-1cm-1 at 281 nm for pure β-lactamase(11).

Kinetic parameters of mutant protein. Catalytic parameters of purified Cys77 to Ser protein were determined as in Chapter III using benzylpenicillin and cephalothin as substrates.

Catalytic activity as a function of temperature. The activity of purified C77S mutant enzyme was measured at varying temperatures using benzylpenicillin as a substrate. All other conditions were identical to the standard assay conditions.

Thermal stability. Purified wild-type or mutant β-lactamase (0.1 mg/ml in 0.1 M potassium phosphate, pH 7.0) was incubated at the specified temperatures in a shaking water bath. Aliquots were removed periodically and assayed for activity at 30oC using benzylpenicillin as the substrate. Values for the log of residual activity were plotted against time at each given temperature to determine the half-life of the enzyme .

Catalytic activity as a function of urea concentration. The activity of purified β-lactamase to hydrolyze benzylpenicillin in the presence of varying concentrations of urea was measured at 30 oC with all other conditions identical to the standard assay conditions.

Reaction with mercurials. Purified β-lactamase (0.1 mg/ml) was treated with parahydroxy mercury benzoate or mercuric acetate (1 mM mercurial in 0.1 M potassium phosphate, pH=7.0) for 30 minutes at 30 oC. After this incubation, activity was determined using the standard assay.

Preparation of mercury derivative for X-ray diffraction. Homogenous C77S β-lactamase was rechromatographed on Ultragel AcA 54 to remove any proteolytic fragments which may have formed. The active protein eluted(6 mg) was concentrated to 1 ml and the buffer changed to 50 mM potassium phosphate by ultrafililtration(Amicon PM-10 membrane). The concentrated protein was brought to 1 mM in mercuric acetate and allowed to react at room temperature overnight. After dialysis against distilled water, this material was concentrated to a small volume by repeated lyophilization of small aliquots in an eppendorf tube. The dried derivatized protein was shipped with no further processing.

Results and Discussion

Production and purification of C77S mutant β-lactamase. No changes were necessary in the growth and purification procedures used to produce this mutant protein, although the growth temperature was maintained at 30oC to minimize possible thermal and proteolytic denaturation of this protein. No alterations in the behavior of this protein were observed during ammonium sulfate precipitation, ion-exchange chromato-

graphy, and gel-filtration chromatography. No evidence for formation of dimers that could have resulted from formation of intermolecular disulfide bonds between the free sulfhydryl groups of Cys 123 was observed. The presence of such dimers, if significant amounts had formed, should have been evident especially on the Ultragel-54 gel filtration column. The mobility of the purified C77S protein on 12 % SDS-PAGE was identical to the wild-type enzyme. The yield of the C77S mutant was the same as that obtained from the wild-type enzyme(approximately 0.4 mKat or 6 mg protein from an 11 l of culture).

Kinetic parameters. The kinetic parameters (kcat and Km) for the hydrolysis of benzylpenicillin and cephalothin by the C77S mutant protein are shown in Table V-1. At pH 7.0 and 30oC, these parameters are virtually identical to those measured for the wild-type enzyme. Likewise, the activities of these enzymes were indistiguishable at saturating substrate conditions ([benzylpenicillin]> 50 Km) for temperatures between 30oC and 40oC(Figure V-1). However, above this temperature, the activity of the C77S mutant becomes less than that of the wild-type enzyme, with denaturation taking place during the course of the assay.

Thermal stability. The half-life of the C77S mutant at elevated temperatures was directly examined (Table V-2). The C77S mutant protein is less stable to thermal denaturation than the wild-type enzyme, with the mutant enzyme losing

activity at temperatures 5-10o lower than the wild-type enzyme. However, the mutant protein lacking the disulfide bond is still far more resistant to thermal denaturation than the T71S mutant enzyme, which still retains the disulfide. The half-lives presented in Table V-2 reflect an irreversible denaturation of the enzyme; I have not been able to restore activity to wild-type or mutant enzymes that have been thermally denatured as described in these experiments.

Activity as a function of pH. The activity of the C77S mutant enzyme was also examined as a function of pH (Figure V-II). Between pH 6 and 8, the mutant enzyme are indistiguishable. Above pH 8, this mutant is less stable than the wild-type enzyme, while at low pH (below 6), the mutant appears slightly more active than the wild-type enzyme. This suggests that the loss of the proton from the free sulfhydryl group of Cys 123 may be involved in this loss of activity at higher pH. This is consistent with a similar loss of activity at elevated pH that has been seen for the T71C mutant of pBR 322 β-lactamase(19).

Resistance to proteases. The ability of the C77S to withstand E,coli periplasmic proteases was determined. At temperatures below 40o C, no difference in proteolytic sensitivity was seen. As shown in Figure V-3, however, at elevated temperatures the mutant protein lacking a disulfide is dramatically more sensitive to proteolysis.

Reaction with mercurials. No inhibition of enzymatic

activity of the C77S beta-lactamase was observed a incubation with 1 mM parahydroxymercuric benzoate or 1 mM mercuric acetate. This implies that attachment of divalent mercury to the free cys 123 residue does not reduce activity. This evidence further suggests that this region of the beta-lactamase is not intimately involved with the active site. Prolonged incubation of beta-lactamase, either mutant or wild-type, with these mercurial compounds results in gradual inactivation.

The mercuric acetate derivative was purified and sent to Dr. J. Knox to aid in the resolution of the diffraction structure of beta-lactamase. Unfortunately, this protein did not produce a usable crystal.

Summary

The C77S mutant β-lactamase was produced and purified to homogeneity. This protein was synthesized normally and showed no evidence for dimer formation. At temperatures below 40oC and in the pH range 5-8 it showed no differences in catalytic activity relative to the wild-type enzyme. Above 40oC or pH 8.0 this enzyme was less thermally stable than the wild-type enzyme. This mutant enzyme was also less resistant to proteolyis by intrinsic E.coli periplasmic proteases at high temperature.

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Table V-1 Kinetic Parameters for Hydrolysis of Benzylpenicillin

	k_{cat} s^{-1}	K_m (μm)
Cys 77→Ser	1950±200	24±12
Wild-type	2000±200	26±10

Table V-2 Half-Lives of Mutant β-Lactamases (in minutes)

Temperature (0°C)	Wild-type	Cys 77→Ser	Thr 71→Ser
40	ND	>700*	9
45	>1200*	40	5
50	500	22	<1
55	13	3.5	
60	1.5		

*At these temperatures the mutant enzymes retained at least 90% of their initial activity for the time indicated.

Figure V-1. Rate of benzylpenicillin hydrolysis as a function of temperature for the wild-type and C77S mutant β-lactamases.

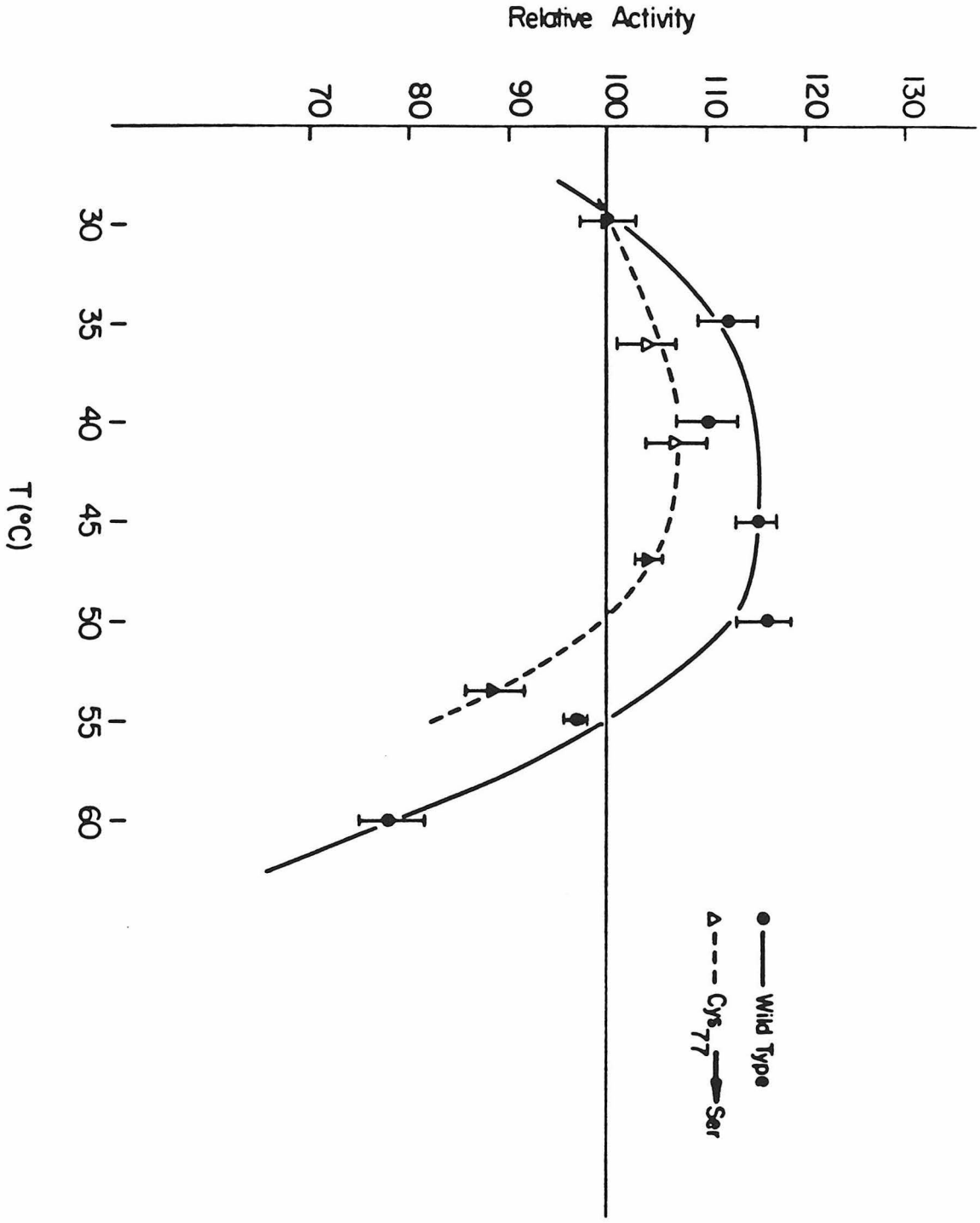

Relative Activity
70
80
90
100
110
120
130
30
35
40
45
50
55
60
T (°C)
Wild Type
Cys77 Ser

Figure V-2. Rate of hydrolysis of benzylpenicillin by wild-type (solid line, filled data points) and C77S mutant β-lactamase (open data points, dashed line) as a function of pH. The different shape data points represent different buffers.

All buffer concentrations are 0.1 M: O, potassium phosphate; ■, potassium acetate; ▲, potassium glycine.

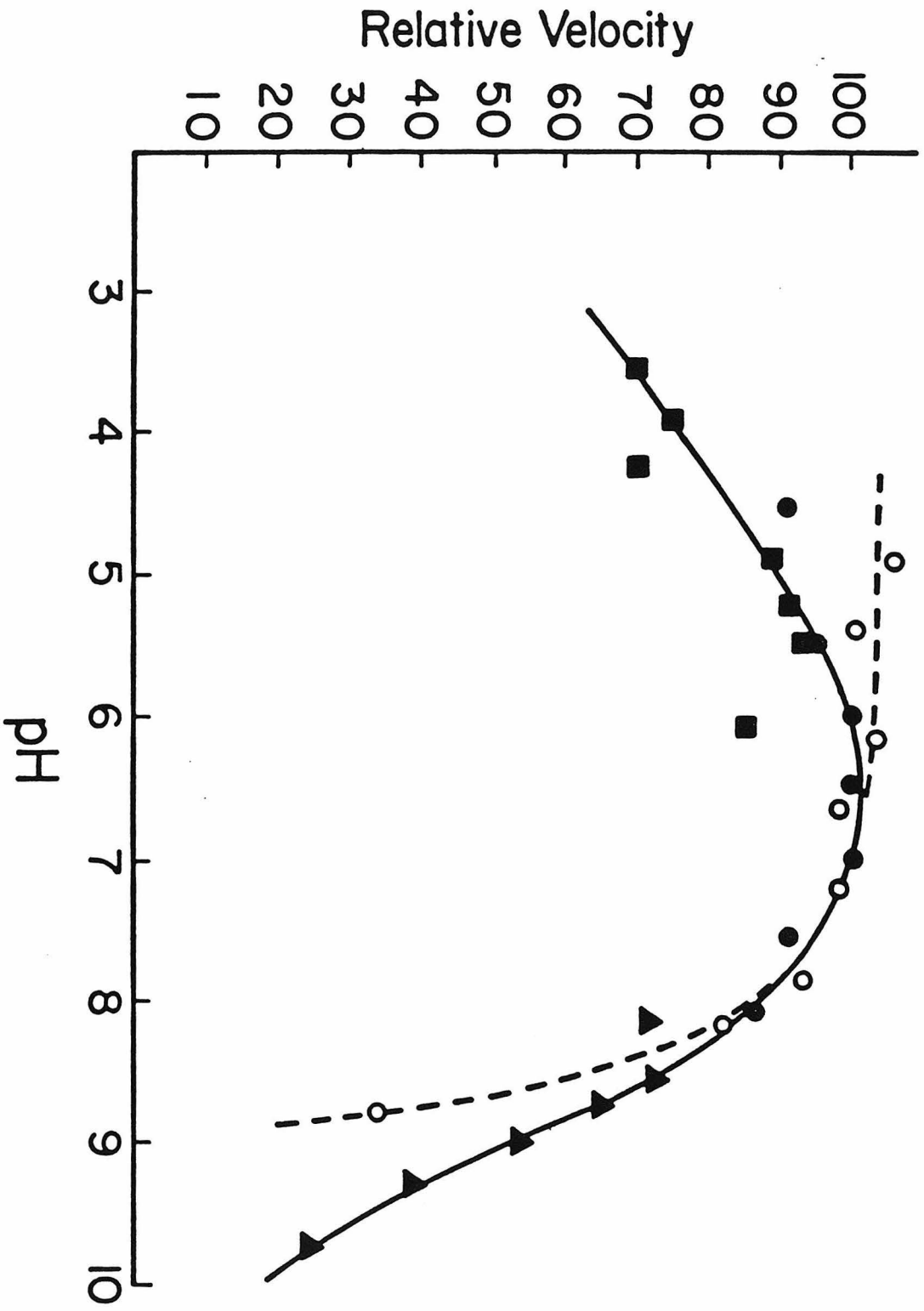
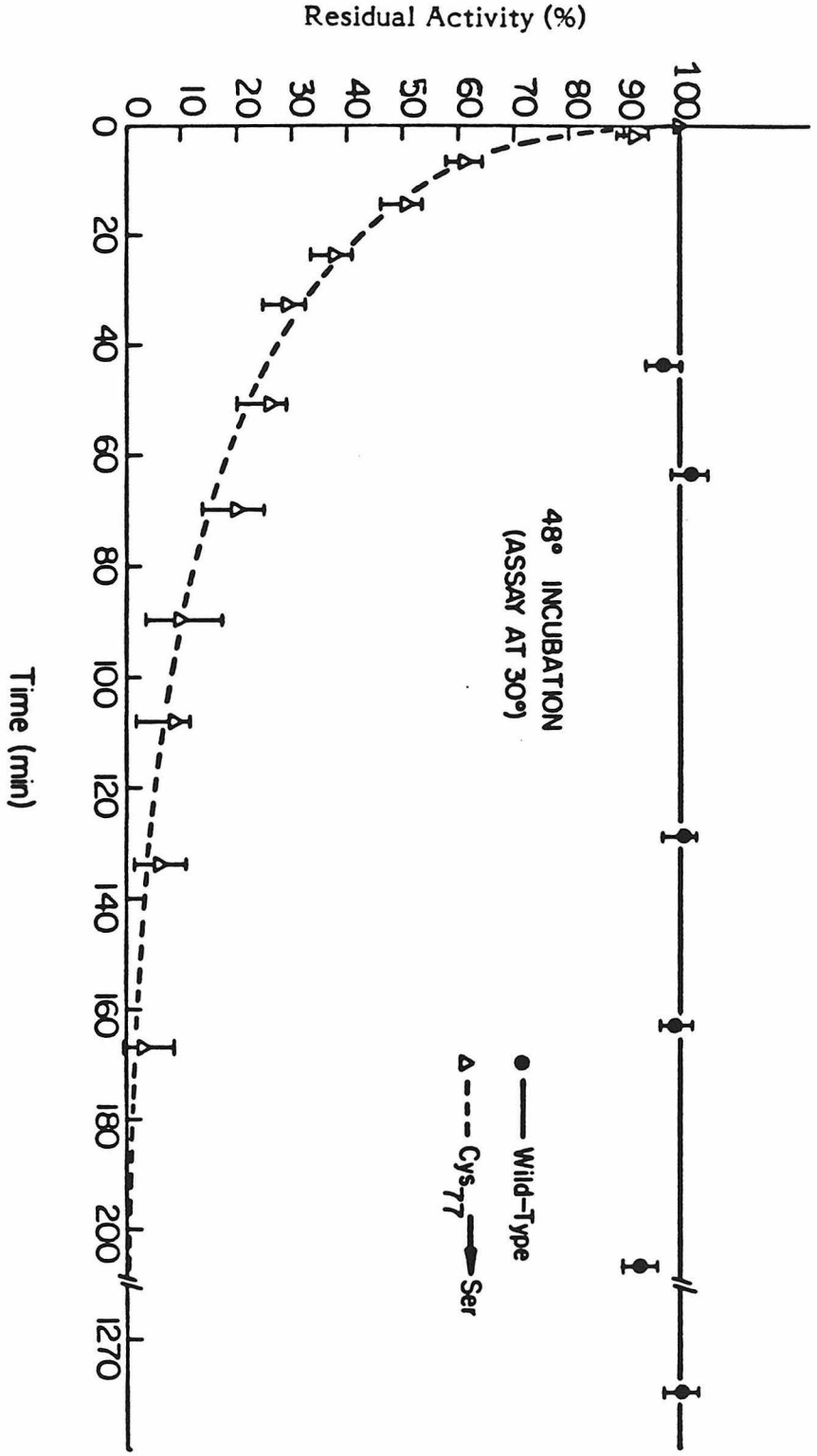

Relative Velocity
10
20
30
40
50
60
70
80
90
100
pH
3
4
5
6
7
8
9
10

Figure V-3. Loss of enzymatic activity seen upon incubation of C77S mutant ß-lactamase with E.coli periplasmic fluid at elevated temperature. No activity loss is seen for the wild-type enzyme under these conditions.


Residual Activity (%)
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
160
180
200
1270
Time (min)
48° INCUBATION
(ASSAY AT 30°)
Wild-Type
Cys77 Ser