Enzymatic Hydrolysis of the Amide Bond: Mutagenic Studies of the Mechanisms of α-Lytic Protease and β–Lactamase

Thesis by
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To

Harry and Bella Spears

and

Harry and Bebb Emerling

"A journey of a thousand miles must begin with a single step."

- Lao-tsu (c.604-c.531 B.C.)

".... no mater where you go, there you are."

- "Buckaroo Bonzaí" (1984)

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Abstract

The enzymatic hydrolysis of amide bonds was studied in two systems by site-specific mutagenic techniques. In the first study, I developed an expression system for the serine protease, α–lytic protease, from *Lysobacter enzymogenes* 496, using a previously constructed synthetic gene, which contained numerous unique restriction sites. Since the wild type enzyme is expressed as a zymogen, which is self-processed into the mature proteolytic enzyme, a unique chimeric expression system utilizing the concomitant expression of the pro-domain from the wild type enzyme was created. The system expresses the properly-folded, mature protease-domain of the enzyme, thus allowing for production of active-site mutants of α–lytic protease, that otherwise could not be obtained in the mature form.

The ability of the enzymatic machinery to enhance the nucleophilicity of a chemical group other than the active-site serine hydroxyl was investigated through the mutation of serine 195 to an alanine. The removal of the serine hydroxyl was hypothesized to provide sufficient volume in the active site to allow a water molecule to bind and possibly function as the attacking nucleophile in the hydrolysis. The structure of the enzyme would be minimally disturbed by the removal of the single atom. The mutant enzyme was assayed for activity on the serine protease inhibitor diethyl *p*-nitrophenyl phosphate. No enzymatic hydrolysis of the substrate was detected. Analysis of structural constraints of the enzyme suggests that a serine 195 glycine mutation might provide a more hydrophilic environment in the active site for the binding of a water molecule.

In the second study, I investigated the mechanism of RTEM-1 β-lactamase, an enzyme which hydrolyzes the amide bond of β-lactam antibiotics,

conferring antibiotic resistance to bacterial cells. Serine 130, a conserved active-site residue in the class A β-lactamases, has been proposed to be involved in positioning the conserved lysine 234 through a hydrogen bond interaction (Moews *et al.* (1990) *Proteins* **7** 156). The function of lysine 234 is known to be one solely of substrate binding (D. M. Long (1991) Ph.D. Thesis, California Institute of Technology). I performed site-saturation mutagenesis of serine 130, and the resulting 20 mutant enzymes were assayed for the ability to confer resistance to *E. coli* towards several β-lactam antibiotics. Four mutants (Ser 130 Gly, Ser 130 Thr, Ser 130 Asn, and Ser 130 Gln) conferred notable resistance to the penam antibiotics. These four mutants were purified to homogeneity, and the steady-state kinetic parameters for hydrolysis of benzylpenicillin were measured for each. The values of K_M for all four mutants were no more than ten-fold more than the wild type value. However, values of k_{cat} for the four mutants were decreased at least 1000-fold from that of the wild type, demonstrating clearly the involvement of serine 130 in catalysis. These results, along with an analogy to structural mutants of Thr 157 in T4 lysozyme, suggest how the four serine 130 mutants might maintain the native hydrogen bonding interactions of the serine with lysine 234, as well as participate in the catalytic mechanism.

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Abbreviations and Nomenclature

Amino acid residues are referred to by their single letter code or three letter abbreviations. Residue mutations are specified by the original residue name, followed by the residue position, followed by the substituted residue name(e.g., "Ser 195 Ala" or "S195A" for a subsitution of the serine residue at position 195 for an alanine).

CHAPTER 1

Introduction to Structure/Function Studies in Proteins

Introduction

The Fusion of Structure and Function within Proteins

The process of evolution has resulted in the creation of a myriad of macromolecules which constitute the physical framework for all the processes within an organism. The most diverse and abundant of these macromolecules are the proteins, which have, among numerous functions, developed into some of the most highly efficient catalysts, regulating and enhancing nearly all chemical processes in an organism. A protein is a linear polymer, constructed from the set of 20 different amino acid monomers, and as such is able to twist and fold upon itself in three dimensions. The functioning of a protein is determined by this folded three-dimensional structure, which, in turn, is almost exclusively dictated by the simple linear sequence of amino acids, referred to as the molecule's primary structure.

The folding of the polymeric backbone of a protein or enzyme places the various functional moieties of the amino acid side chains into unique structural arrangements that allow these functional groups to interact. These interactions are responsible for the various properties of structural organization and stability, as well as the catalysis and regulation that characterize the proteins and enzymes. Although the folding of the peptide chain positions residues in unique and felicitous arrangements within the protein, it is actually the interaction between these residues that prescribes and stabilizes the protein's folded structure.

The placement of the various amino acid functional groups within a protein creates unique microenvironments within the structure that can have the effect of altering the chemical properties of these functional groups, greatly increasing the diversity of functionalities observed in protein systems.

This effect is demonstrated by the perturbation of residue ionization constants in a number of proteins, such as the three unit decrease in the pK_a of the imidazole group of histidine 159 in papain versus the value of the free imidazole[1], and most notably by the increased nucleophilicity of the serine hydroxyl oxygen in the serine proteases and the β-lactamases, to be discussed in more detail in later chapters.

The chemical interactions between amino acid residues -- hydrogen bonds, salt bridges, aromatic stacking, and hydrophobic packing to name a few -- are well understood and characterized phenomena between small molecules and chemical groups. However, the sheer number of such interactions, and the subtle interplay between residues within the folded protein, belies all but a cursory understanding of the processes of protein folding, prediction of the folded structure from the primary sequence, or even the prediction of the catalytic mechanism or the functional roles of specific residues from a protein's known three-dimensional structure.

Structural information had previously led researchers to predict that aspartate 189 in the catalytic site of trypsin, a serine protease with a specificity for the positively-charged arginyl and lysyl side chains, was solely responsible for creating a negatively-charged binding pocket for the substrate's cationic side chain[2]. This hypothesis was tested through the substitution of the Asp residue with lysine, with the expectation that the resulting mutant enzyme would specifically cleave at aspartate and glutamate residues[3]. However, the mutant possessed an extremely reduced ability to hydrolyze peptides, and would cleave only at neutral residues. The previously held model that the functions of substrate binding and catalytic activity were defined by independent residues was clearly a simplification that was not always justified

As a further example, a number of researchers have attempted to increase the thermal and chemical stability of enzymes through the incorporation of disulfide bonds into the proteins' structures. The results of such studies have been mixed, with some mutant enzymes displaying a higher degree of stability than the corresponding wild type, while others displayed substantially decreased stability[4,5]. A clear guideline as to when such structural substitutions might produce more stable enzymes has yet to emerge. The functional roles of amino acids, particularly those within the active sites of enzymes, are not always easily predicted, even with X-ray structural information, and a given residue may in fact have several disparate functions.

It is a major goal of enzymology to understand the structure/function relationships which determine how polypeptide chains produce such complex and unique structures, and how various functional groups and their arrangements integrate in the performance of a catalytic mechanism.

Assaying Structure/Function Relationships in Proteins

A large array of experimental techniques has been applied to the study of the relationship between protein structure and function. Studies rely heavily on the ability to examine the protein of interest. Biophysical techniques such as nuclear magnetic resonance, circular dichroism, and X-ray and neutron diffraction, in conjunction with phenotypic and kinetic analyses, have provided detailed information on specific interactions within a number of protein systems. However, these techniques alone are insufficient to provide an understanding of many of the mechanistic relations in an enzymatic system, or to assess the degree to which various residues contribute to the unique structure of the protein. The investigation of such processes requires not only

the ability to inspect the system under study, but also the ability to generate specific changes within the protein so as to examine the various structural requirements that allow the system to function.

An analogy can be drawn to perturbation analysis in mathematical modeling of physical systems[6]. For systems that are too complex to model in complete detail, simpler models are constructed with additional parameters added piecewise. The "value" of each addition is investigated by perturbing the parameter and viewing its effect on the system as a whole. In a similar way, the mechanistic workings of an enzymatic reaction can be deduced through the substitution of individual residues and the examination of changes in the enzyme function.

Historically, protein structure/function studies have been somewhat limited in this aspect. Though the wild-type structures of homologous proteins can be compared, allowing inferences to be made about the roles of various conserved and semiconserved amino acids, obtaining known perturbations to a specific protein were generally restricted to either the isolation of naturally-occurring or randomly-induced mutations[7], or to the chemical modification of exposed residues in the isolated protein[8]. Such techniques have been used in the investigation of the functional moieties in hemoglobin through the isolation of a multitude of naturally occurring point mutations[9], and in the "chemical mutation" of a serine residue in trypsin to a cysteine, using organic chemistry techniques[10]. Though quite useful, these techniques are far short of a general methodology for producing explicit changes in a protein.

Site-Specific Mutagenesis of Proteins

The recent proliferation of gene manipulation techniques has provided a powerful tool for performing perturbation analyses on proteins. Though

generally referred to as "genetic engineering" and "protein engineering," the terms are somewhat misleading since an engineer usually designs a system to perform a desired task, whereas the protein researcher designs or modifies a protein system to investigate the task it performs, or more often the way in which it fails to perform. The use of the terms persists mostly as a reflection of the goal toward which the field is driving.

Possibly the single most important technique attributed as a feat of genetic engineering is actually an achievement of synthetic organic chemistry. The ability to synthesize single-stranded molecules of DNA, of specific sequences over a hundred bases long[11], has revolutionized the process of creating protein mutations. With the advent of these readily obtainable oligonucleotides, site-specific mutagenesis techniques could be performed on cloned genes to generate specific desired variants of the primary structure of the proteins they encode.

Two general methods of site-specific mutagenesis are currently used extensively in the production of protein variants. These techniques have been the subject of several reviews[12,13,14]. The first method, oligonucleotide-directed mutagenesis, is depicted in Figure 1. The gene of interest is cloned into the DNA of a single-stranded phage, and the phage DNA isolated to yield a single-stranded template of the gene. A synthetic oligonucleotide, complementary to the gene sequence around the mutation site and containing specific base mismatches for the mutation, is annealed to the gene, and DNA polymerase is used to fill in the complementary strand, creating a heteroduplex molecule. The heteroduplex DNA is used to transform bacteria cells and the progeny are screened for the incorporation of the mutations. Originally, this technique produced no more than 5% mutant progeny, primarily due to

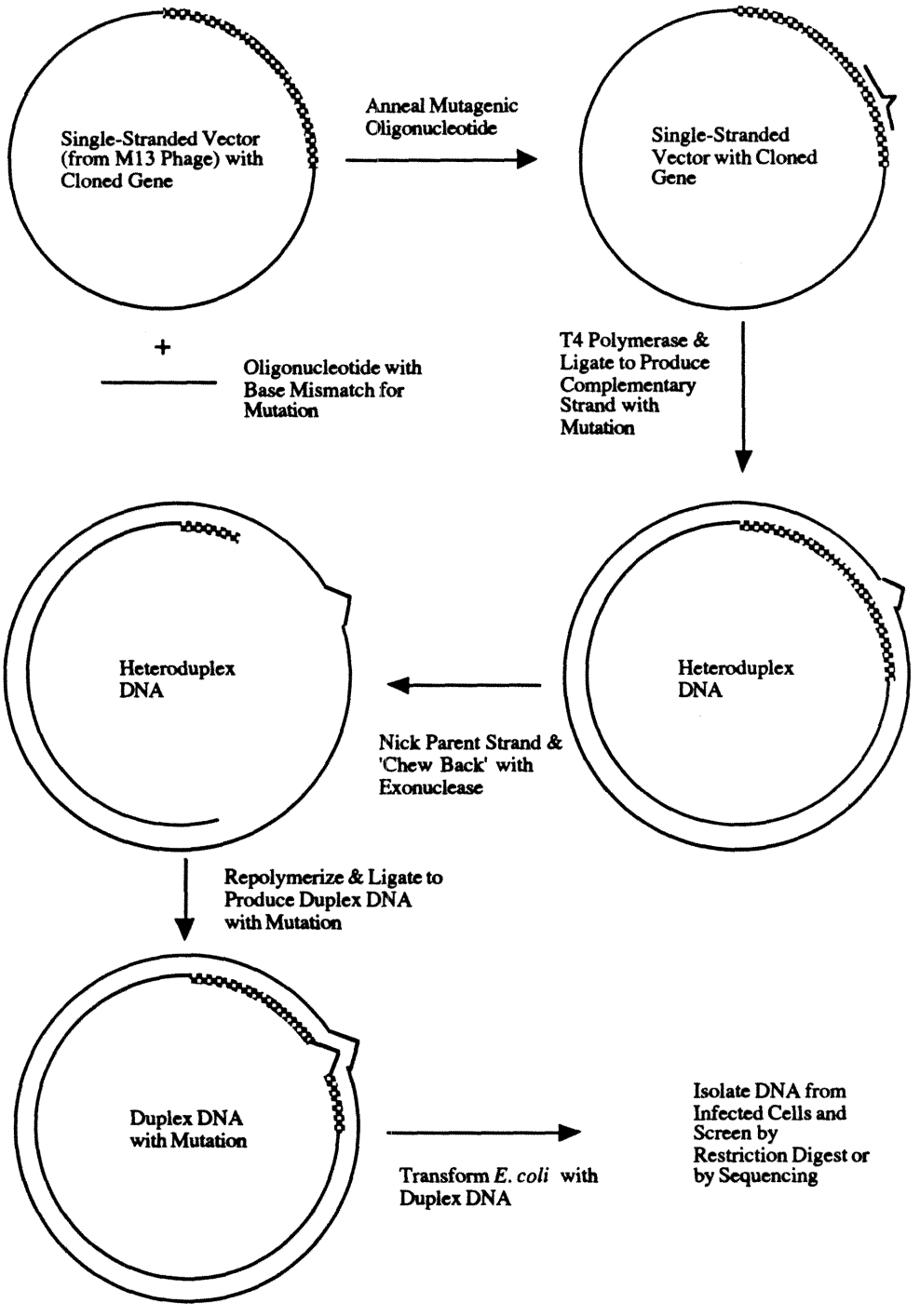

Figure 1. A schematic overview of oligonucleotide-directed mutagenesis.

mismatch repair of the DNA within the cell. Methods to select for propagation of the mutant strand, such as those developed by Eckstein[15] and Kunkle[16], are now capable of producing better than 90% mutant progeny.

Once the desired mutant has been obtained, the gene is generally cloned back into an expression plasmid for production of the mutant protein. Oligonucleotide-directed mutagenesis has been used in our laboratory to mutate active-site residues in the RTEM-1 β-lactamase[17], and to place histidine residues on the surface of yeast cytochrome c to allow for ruthenation of the protein, a technique used in the study of electron transfer rate in metalloproteins[18].

The second method, cassette mutagenesis, also makes critical use of synthetic oligonucleotides. In this case, however, two complementary oligonucleotide strands, coding for the mutation and the surrounding region of the gene, are annealed together to form a double-stranded DNA "cassette." The cassette is designed to possess cohesive overhanging ends for use in the subsequent ligation step (Figure 2). The cassette is then used to replace the corresponding region in the gene through the use of DNA endonuclease restriction enzymes and DNA ligase. As with oligonucleotide-directed mutagenesis, cells are transformed with the modified DNA and progeny are screened for the mutant gene. Cassette mutagenesis has numerous advantages over oligonucleotide-directed mutagenesis. Since both strands of the modified DNA contain the mutated sequence, cassette mutagenesis generally produces a greater percentage of mutant progeny. Additionally, cassette mutagenesis allows mutations to be created in a gene residing in an expression vector, eliminating the need to subclone the gene in and out of a mutagenesis vector, reducing the number of steps involved in the process. Lastly, the process does not involve a single-stranded DNA form, nor does it use an *in vitro* polymerization step, thus substantially decreasing the likelihood of unwanted secondary mutations being incorporated into the progeny gene. However, a

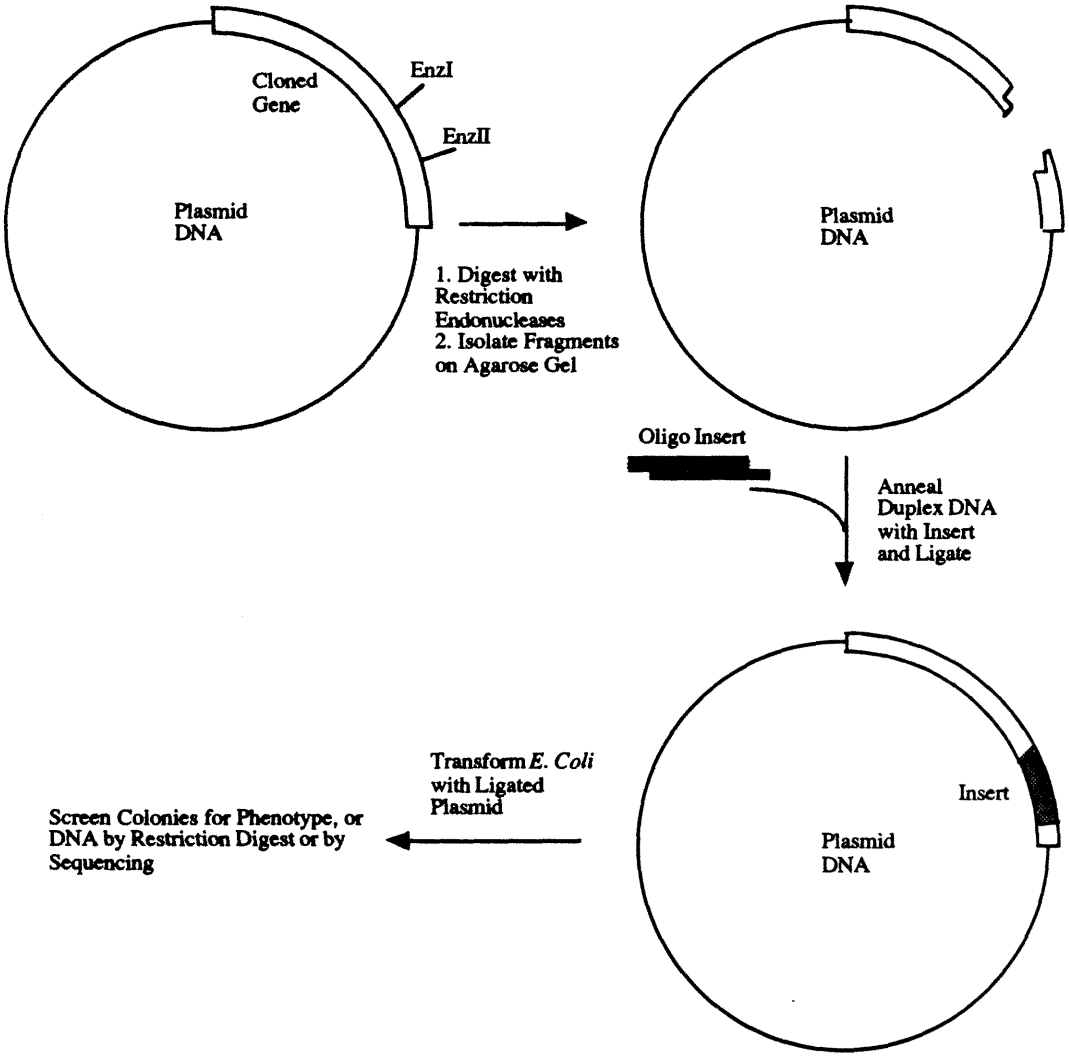

Figure 2. A schematic overview of cassette mutagenesis.

notable shortcoming of this method is the requirement for appropriately positioned restriction sites within the gene to allow for the excision of the wild type region and replacement with the synthetic cassette. Cloned genes from natural sources rarely possess restriction sites where the researcher desires, and oligonucleotide mutagenesis is often used to insert silent changes into a gene so as to create convenient restriction sites for subsequent cassette mutations.

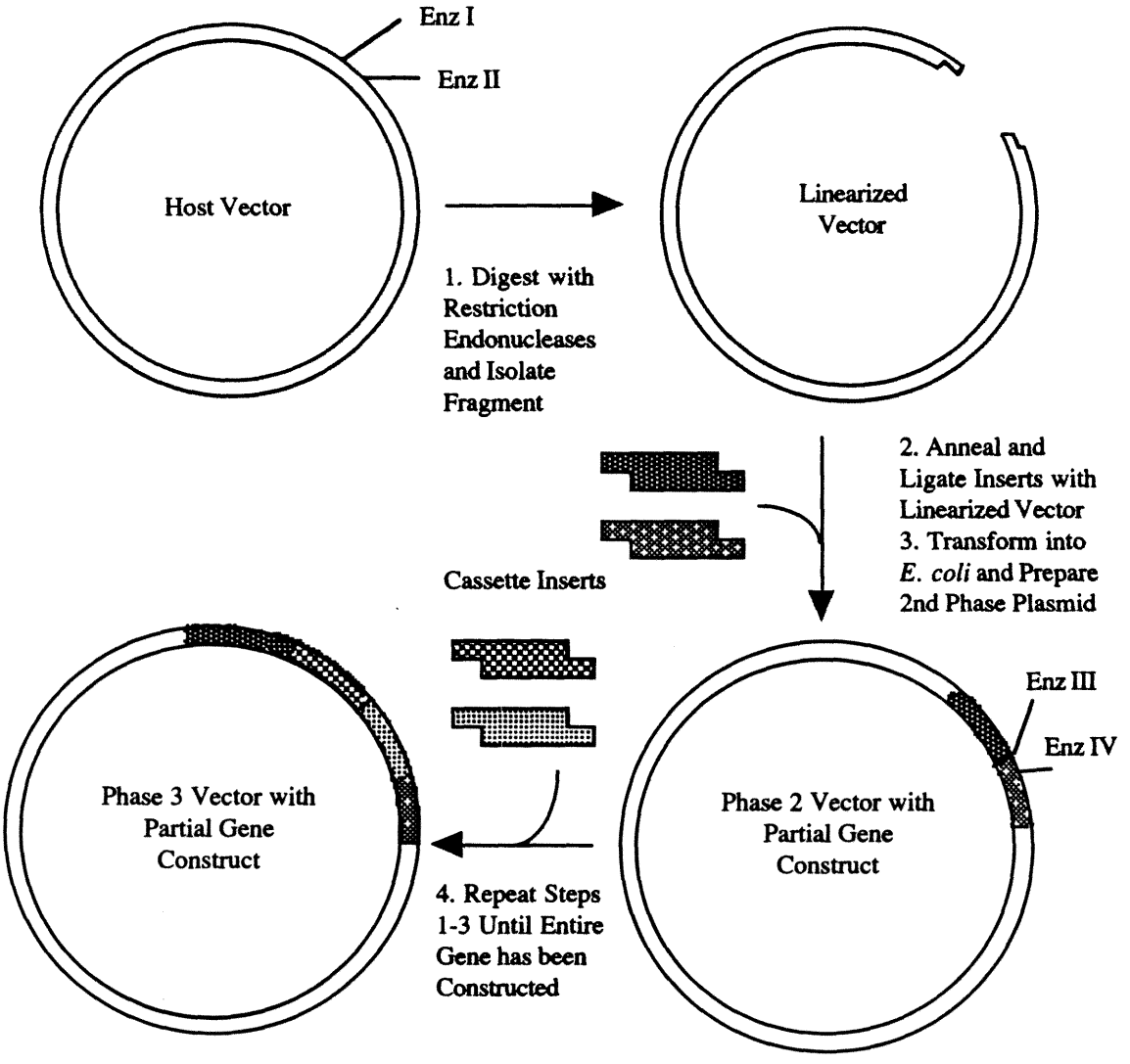

Figure 3. A general overview of the gene synthesis technique using cassette mutagenesis.

Cassette mutagenesis techniques have been used to create single- and multiple-residue substitutions in a number of protein systems, investigating the evolutionary relationships between proteins[19], catalysis mechanisms[20], and the folding and stability properties of proteins[21]. In addition, the method has been extended to the creation of chimeric genes and proteins through the exchange of entire coding domains between cloned genes, and to the complete synthetic generation of a gene using a stepwise insertion of cassettes (Figure 3). Examples of each of these techniques are seen in the experiments

of Wharton and Ptashne, where the DNA recognition helix of the 434 repressor was exchanged with the corresponding region from the P22 repressor, resulting in a mutant protein displaying the P22 repressor activity[22], and in the complete synthetic creation of the plastocyanin[23] and α-lytic protease[24] genes, performed in our laboratory.

Cassette mutagenesis has also been useful in site-saturation and specific residue random mutagenesis studies[25,26]. Using degenerate oligonucleotides (those containing multiple-base substitutions at specific positions), the effect of all 20 amino acids at a particular position in the protein can be investigated. Such experiments have been used in our laboratory to investigate the roles of residues Lys 234 and Ala 237 in the RTEM-1 β-lactamase, for example[27,28]. Site-saturation experiments are a more objective investigation of a residue's contribution to the catalytic and folding properties of an enzyme, because they avoid preconceived notions as to the function the residue plays.

A particularly pointed example of how a researcher's opinions can skew such an experiment is seen in the recently published work of Adachi *et al.*, where the researchers constructed four oligonucleotide-directed mutants of RTEM-1 β-lactamase, substituting the glutamate 166 residue with alanine, aspartate, glutamine, and asparagine[29]. Of the four mutants, only the Glu 166 Asp substitution displayed an ability to hydrolyze the β-lactam antibiotic. From these results the researchers concluded that the negatively charged carboxylate group is critical in the mechanism of β-lactam hydrolysis by the enzyme. However, site-saturation studies of glutamate 166, performed in our laboratory[28], identified two additional mutants, Glu 166 His and Glu 166 Tyr, that displayed notable activity. The level of activity for these additional mutants is very similar to that of the aspartate mutant. These results strongly

implicate the 166 residue as a basic and/or a hydrogen-bonding moiety, not requiring a negative charge as concluded in the study where only four substitutions were produced.

The Serine Hydrolases

The experiments described in this thesis relate to the study of structure/function relations in two members of a very broad class of enzymes called the serine hydrolases. These enzymes are all characterized by the hydrolysis of their substrates via the nucleophilic attack of an active-site serine residue, resulting in the formation of a covalent acyl-enzyme intermediate. Though the mechanisms of the enzymes that constitute this class are quite varied, including those for the two enzymes studied herein, a number of salient features are common to the class as whole. Along with hydrolysis proceeding through a two-step mechanism, all members of this class are believed to generate a tetrahedral transition state during formation of the intermediate species. Additionally, the enzymes all possess a critically positioned set of residues and/or secondary structural elements, neighboring the active-site serine, which enhance the nucleophilicity of the serine oxygen.

In Chapter 2, I describe the production of an expression system for the proteolytic enzyme α-lytic protease from a completely synthesized gene, and the investigation of the ability to produce organo-phosphatase activity in the enzyme through mutation of the active-site serine. Chapter 3 describes a site-saturation experiment performed on the conserved serine 130 residue in RTEM-1 β-lactamase and kinetic characterization of the active mutants. These experiments demonstrate that the role of Ser 130 in β-lactam hydrolysis is not one of simple substrate binding, as was previously proposed, but instead is clearly shown to be involved as a catalytic moiety in the hydrolysis.

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CHAPTER 2

Construction of a Complementation Expression System for a Synthetic Gene for α-Lytic Protease and Investigation of Organo-Phosphatase Activity in an Active-Site Mutant

Introduction

Serine Proteases

The serine proteases are an extensively studied family of enzymes responsible for the hydrolysis of the amide bond in peptides and proteins[1]. These enzymes are characterized by the presence of a uniquely reactive serine residue in the active site. Their existence is pervasive throughout biology: in the mammalian digestive enzymes, such as chymotrypsin and trypsin[2], as activators in the complement cascade[3], such as C5a, in the blood clotting component thrombin[4], and in the prokaryote degratory enzymes subtilisin and α–lytic protease[5,6]. Despite having diverse functions and environments, the serine proteases display a high degree of homology in both primary and tertiary structure, particularly in the region of the active site. They exist in systems requiring little specificity in substrate recognition, such as chymotrypsin[2], which will recognize any protein substrate with any one of three select amino acids at the cleavage site, as well as in highly restrictive recognition systems, such as those required for thrombin activation in the mammalian blood clotting cascade[4].

Two sub-families of the serine proteases have been elucidated, the trypsin sub-family and the subtilisin sub-family. In both sub-families, a catalytically essential arrangement of three residues has been characterized: the carboxylate of an aspartic acid, the imidazole of a histidine, and the hydroxyl of the aforementioned serine residue. The subtilisin sub-family is characterized by the sequence Thr-Ser-Met-Ala around the active-site serine, and the catalytic triad is numbered Asp 32, His 64, Ser 221. In contrast, the trypsin sub-family possesses the conserved primary sequence Gly-Asp-Ser-Gly-Gly around the active serine, and the triad is numbered Asp 102, His 57, Ser 195. In addition,

the conserved Asp 194 forms a salt bridge with the guanidinium group of Arg 138 in the bacterial enzymes[7], and with the amino terminus of Ile 16 in the mature mammalian enzymes. The trypsin and subtilisin sub-families represent an example of convergent evolution; the tertiary structures of the two classes differ quite substantially, yet the three-dimensional arrangement and utilization of the catalytic triad are the same.

The Catalytic Mechanism

The generally accepted mechanistic scheme for amide hydrolysis by the serine proteases is shown in Figure 1. The initial step involves the formation of the enzyme-substrate (ES) complex. Such a complex is maintained through hydrogen bonding, ionic, and Van der Waal's interactions[8]. The formation of the ES complex serves two important functions in the serine proteases: it provides the mechanism for the selection between various substrates, and allows for the proper orientation of the scissile bond of the substrate with respect to the catalytic residues in the active site. Alignment of the substrate within the enzyme is critical to the catalytic process, since reaction rates are directly affected by the orientation of the reacting species. Additionally, enzymatic catalysis uses the strategic placement of functional groups within the binding site to enhance reaction rates by stabilizing the high energy reaction transition states.

Upon the binding of substrate, nucleophilic attack of the Ser 195 hydroxyl on the carbonyl carbon of the scissile bond of the substrate results in the cleavage of the substrate amide bond, and the concomitant release of the amine product of the hydrolysis[1,6]. This reaction is postulated to proceed through a tetrahedral substrate-enzyme intermediate, and results in the formation of a covalent acyl-enzyme complex.

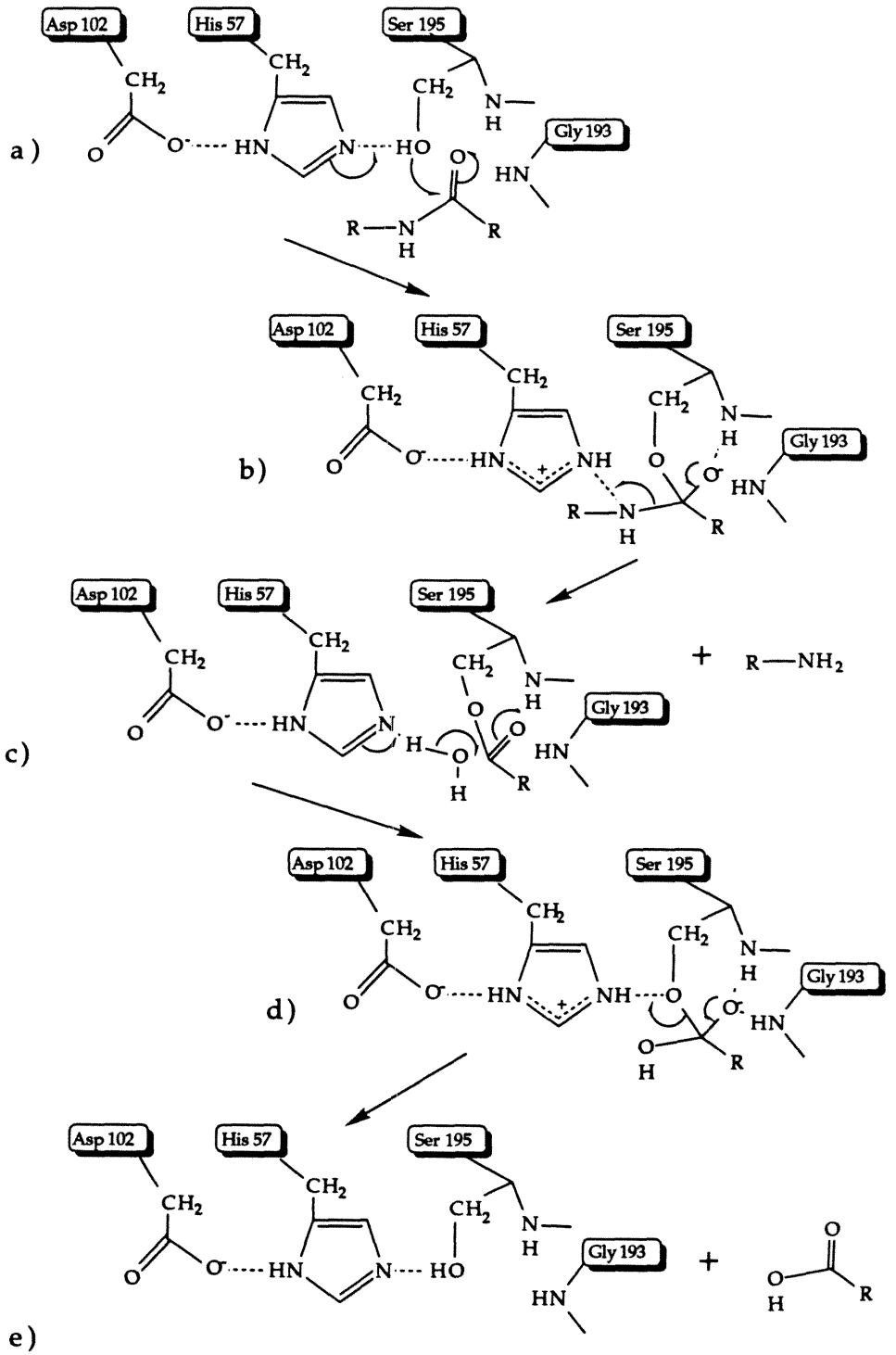

Figure 1. The mechanism of peptide hydrolysis by the serine proteases. A) Substrate binding. B) Tetrahedral transition state with the imidazole ring accepting the serine proton. Stabilization of the oxyanion transition state is provided by the amide N of residues Ser 195 and Gly 193. C) Acyl-enzyme complex with release of the amine product of hydrolysis. D) Tetrahedral transition state formed by attack of the acyl-enzyme complex by solvent water. E) Release of the carboxylate product of hydrolysis and regeneration of the free enzyme.

Two key features of the serine proteases play critical roles in the formation of the acyl-enzyme complex. First, the nucleophilicity of the serine hydroxyl, normally a rather ignoble nucleophile, is greatly enhanced by interaction with the histidine and aspartic acid residues in the conserve triad. The arrangement of these three residues allows the His-Asp dyad to accept and store the serine hydroxyl proton during the formation of the tetrahedral transition state. As the transition state collapses into the acyl-enzyme complex, the stored proton is donated to the amine leaving group. The functioning of the His-Asp dyad is thus one of general base catalysis. Neutron diffraction studies have shown the positive charge of the stored proton to exist on the histidine in the tetrahedral intermediate enzyme complex[9]; the aspartate residue apparently serves to orient the imidazole group and maintain it in the proper tautomeric form, with the proton on the N-1 nitrogen. It has further been suggested that the Asp-His dyad, in conjunction with the oxyanion hole (below), would induce a partial-charge separation on the carbonyl of the bound substrate, increasing the electrophilicity of the carbonyl carbon, and thus making it more susceptible to nucleophilic attack[10].

The second key catalytic feature is a conserved structure in the active site known as the oxyanion hole. In the trypsin sub-family, this structure consists of the backbone amide groups from residues Ser 195 and Gly 193. These groups are positioned in the active site so as to hydrogen bond to the carbonyl oxygen of the scissile bond, distorting the carbonyl towards a tetrahedral arrangement, and stabilizing the negative charge on the oxygen of the tetrahedral intermediate (Figure 1B). The oxyanion hole, in conjunction with the side chains of serine 195 and histidine 57, form an active-site pocket that preferentially binds the tetrahedral intermediate relative to the planar substrate

amide. The distortion of bound substrate towards the tetrahedral arrangement is an indispensable feature of protease activity[11].

Once the acyl-enzyme is formed and the generated amine group has dissociated from the enzyme, solvent water is capable of entering the vacated site in the catalytic pocket and hydrolyzing the acyl complex in a reaction that is essentially the reverse of the original nucleophilic attack. As in the acylation step above, the released proton from the attacking nucleophile (in this case water), is stored on the His 57 imidazole, and is returned to the active serine as the tetrahedral intermediate collapses to the carboxylate product. The carboxylate dissociates from the binding site (dissociation being enhanced by the trigonal arrangement of the carboxyl), regenerating the free enzyme and completing the catalytic reaction.

Phosphate Inactivation of the Serine Proteases

The steric and electrostatic complementarity of the serine protease active site for the high energy tetrahedral intermediates and transition states makes these enzymes very susceptible to inactivation by a family of compounds that mimic this transition state. Transition-state analogues, such as diisopropylfluorophosphate (DFP), have been shown to irreversibly inhibit the serine proteases[12], as well as many of the related serine hydrolases, by rapidly reacting with the active-site serine, forming a covalent tetrahedral adduct with the enzyme. Because the catalytic pocket preferentially binds the tetrahedral configuration, the organo-phospho-enzyme intermediate is essentially stable, with the complex hydrolyzing only at a very slow rate. These inhibitors are commonly used in protein purifications, as a means of disabling any non-specific protease activity that might destroy the protein being isolated[13], as well as in crystallographic studies of the transition state of serine proteases[14].

We have envisioned producing mutated proteases that would function as hydrolases of organo-phosphates. The rationale for converting a serine protease to a phosphatase involves altering the enzyme to avoid the accumulation of the enzyme-inhibitor intermediate, while still utilizing the enzymatic nucleophilic activation. Two approaches are possible: either by a mechanism that would increase the rate of hydrolysis of the covalent enzyme-phosphate complex, or by a mechanism where the covalent enzyme-phosphate intermediate is never produced, through the use of a nucleophile that is not covalently attached to the enzyme. In this latter scheme, the structural features of the enzyme that facilitate nucleophilic attack by the serine hydroxyl would be employed to enhance an unattached nucleophile, such as a bound water molecule. Once hydrolyzed, the phosphate inhibitor would freely dissociate from the enzyme. Replacement of Ser 195 with either alanine or glycine might allow a water molecule to be bound into the active site and oriented to allow for nucleophilic attack on organo-phosphate compounds.

α–Lytic Protease: A Serine Protease

α–Lytic protease (α–LP) is an extracellular degratory enzyme of the trypsin sub-family from the gram negative bacteria *Lysobacter enzymogenes* 496[15]. The mature enzyme is composed of 198 amino acids (MW ~19,900 daltons), and specifically hydrolyzes peptides possessing small residues, such as glycine, alanine, and valine, at the primary specificity position (the amino side of the scissile bond). The residues are numbered according to the chymotrypsin numbering scheme[10]. The structure for the enzyme has been determined to high resolution (1.7 Å) by X-ray crystallography (Figure 2), and shows the enzyme is composed of two domains, with the substrate binding site residing at the interface[16]. The enzyme exhibits unusual stability to proteolysis, extremes

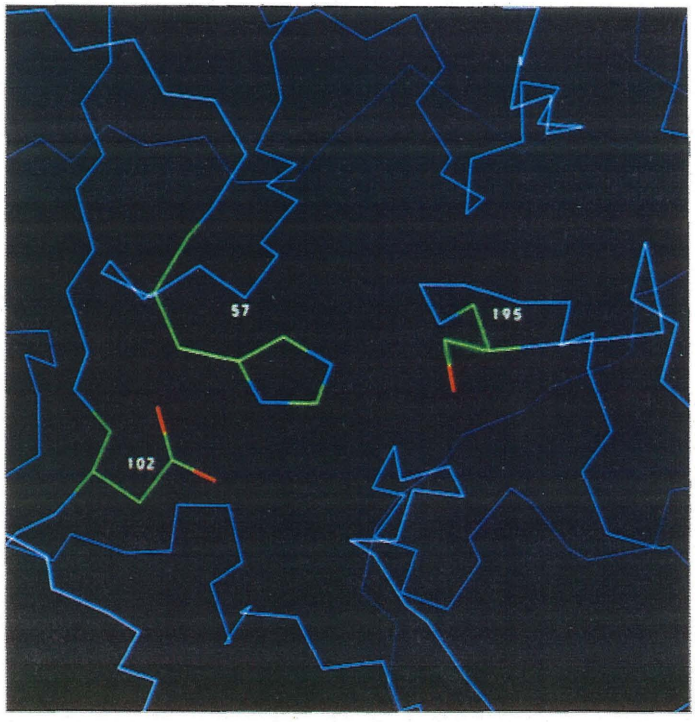

Figure 2. Three-dimensional structure of α-lytic protease from *Lysobacter enzymogenes* 496. Ribbon structure displays strands of α-helix and β-sheet. The Ser-His-Asp active-site triad is displayed in color.

of pH, and high temperatures[17]. Despite extensive research on α–lytic protease, both in our lab and elsewhere, the gene for α–LP was cloned only quite recently[18,19,20].

In order to provide a system for mutagenic structure/functions studies on this enzyme, our laboratory had synthesized a gene for α–lytic protease[21]. With only the protein sequence available, the gene was designed through the reverse translation of the primary protein sequence, resulting in a list of redundant codons. Selection of specific codons to replace the redundant ones was performed in two phases. The first phase selected codons based on the ability to place evenly-spaced, unique restriction sites in the gene. This provided a mechanism for constructing the gene in a step-wise fashion, as well as an elegant means of performing site-specific mutagenesis experiments through simple cassette replacements, as described in Chapter 1. The remaining codons were then chosen to optimize the gene for protein production by selecting specific codons shown to be preferred in the *E. coli* genome[22]. The sequence of the synthetic gene is shown in Figure 3. In order to prevent the possible expression of a potentially lethal protease in the cytoplasm of cells during the construction, the gene was designed with the substitution of alanine for the active-site serine. Once an expression system had been developed, the coding would be reverted back to the wild type sequence.

Recently, the wild type gene for α–lytic protease was cloned, and shown to be expressed as a 41 kD pre-pro-protein[23]. It was further demonstrated that the pro-region of the wild type enzyme was required for proper folding and activation of the mature α–LP enzyme. In order to take advantage of the features present in the synthetic α–LP gene, we chose to create a chimeric expression system for α–lytic protease, fusing the wild type pro-coding region of the gene in front of the synthetic mature region.

```
                  10                  20                  30                  40
                                                                          Mlu I
1   ATGGCAAACA  TCGTTGGCGG  TATCGAATAC  TCCATCAACA  ACGCGTCCCT  50
                                        Xho I       Sty I
50  GTGCTCTGTT  GGCTTCTCCG  TAACTCGAGG  TGCGACCAAG  GGCTTCGTTA  100
                                        Hpa I       Bssh II
101 CTGCTGGTCA  CTGTGGCACC  GTTAACGCGA  CTGCGCGCAT  CGGCGGTGCA  150
                                                Xma I
151 GTAGTAGGCA  CCTTCGCAGC  ACGTGTTTTC  CCGGGCAACG  ACCGTGCATG  200
      Hind III                                                      Sac I
201 GGTAAGCTTA  ACTTCCGCGC  AGACCCTGCT  GCCGCGTGTT  GCTAACGGGA  250

251 GCTCTTTCGT  AACTGTTCGT  GGTTCCACCG  AAGCAGCGGT  AGGCGCGGCT  300
                 Xma III     BstE II               Kpn I
301 GTTTGCCGTT  CCGGCCGTAC  TACCGGTTAC  CAGTGTGGTA  CCATCACTGC  350
                                        Nar I       Stu I
351 GAAAAACGTA  ACTGCTAACT  ACGCAGAAGG  CGCCGTTCGA  GGCCTGACCC  400
                Sph I
401 AGGGCAACGC  ATGCATGGGT  CGTGGCGACG  CTGGTGGCTC  TTGGATCACT  450
    Sac II
451 TCCGCGGGCC  AGGCACAGGG  TGTAATGTCT  GGTGGCAACG  TTCAGTCTAA  500
                    BamH I                                  Asu II
501 CGGCAACAAC  TGTGGGATCC  CGGCATCTCA  GCGTTCCTCT  CTGTTCGAAC  550

551 GTCTGCAGCC  GATCCTGTCC  CAGTACGGTC  TGTCCCTGGT  AACTGGTTAA  600
    Bgl II
601 AGATCT
```

Figure 3. DNA sequence of the synthetic gene for α-lytic protease[21].

The research presented in this chapter describes the construction of the chimeric gene, and expression of wild type α–LP enzyme and an active-site mutant, Ser 195 Ala. As expected, the active-site mutant was only found in the pro-enzymatic form, due to the inability of mutants to self-process into the mature enzyme. To produce mature, mutant enzymes, an expression system was devised to take advantage of recent findings showing that expression of the pro-region of the α–lytic gene as a separate peptide along with the protease peptide resulted in the proper folding and activation of the protease region[24]. Since this expression system does not require the covalent linkage of the two domains for activation of the enzyme, mutant enzyme can thus be produced in the mature form.

The two coding domains, the pro-region and the protease domain, were each constructed into independent genes, each with its own promoter and leader sequence, and placed into separate vectors. The two vectors were chosen to have compatible origins of replication, and unique selection markers, enabling the maintenance of both plasmids within a cell line. Expression was then induced from both genes simultaneously, allowing the pro-peptide to assist in the proper folding of the protease domain, yet providing for the separate isolation of the mature, inactive enzyme. The alanine mutant, thus isolated in the mature form, was assayed for the ability to catalytically hydrolyze organo-phosphate inhibitors.

Materials and Methods

Enzymes and Chemicals

Acrylamide and bis-acrylamide were purchased pre-mixed in 19:1 and 39.5:0.5 ratios, for protein and sequencing gels, respectively, from Boehringer Mannheim Biochemicals (BMB). Nitrocellulose and cellulose acetate membranes were purchased from Schleicher & Schuell, Inc. NuSieve GTG Low Melting Point (LMP) Agarose for DNA fragment isolation was supplied by FMC BioProducts. Molecular biology grade agarose, isopropyl-β-D-thioglactoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-glactoside (X-gal), and 10X ligase buffer were purchased from International Biotechnologies, Inc. Liquified phenol and chloroform were purchased from Fisher Scientific, Inc. Kanamycin sulfate was purchased from BMB; all other antibiotics were obtained from Sigma Chemical Company. Ultra-Pure urea and cesium chloride were purchased from Schwarz/Mann Biotech. Rabbit anti-serum to wild type α–lytic protease was obtained from Berkeley Antibody, Inc. The α–lytic protease substrates, N-acetyl-Ala-Ala-Ala-methyl ester and diethyl *p*-nitrophenyl phosphate, were obtained from Sigma Chemical Company. N-acetyl-Ala-Pro-Ala-*p*-nitroanilide was obtained from Star Biochemicals, Inc. Restriction enzyme Afl III was obtained from United States Biochemical, Inc. Restriction enzymes Not I, BspH I, and Dra III were purchased from New England BioLabs. All other restriction enzymes, T4 DNA ligase, DNase free RNase, calf intestinal alkaline phosphatase (CIAP), and T4 polynucleotide kinase were supplied by BMB. The water used was deionized and glass-distilled.

Bacterial Strains

Plasmid DNA were harbored in *Escherichia coli* strains LS1, XL1Blue^{tm}, or D1210. Culture medium for plasmid preparations was L broth (10 g tryptone, 5 g yeast extract, 10 g NaCl in 1 liter); L agar plates were made with the addition of 15 g bacto-agar per liter.

DNA

Plasmids pALP5 and pCTERM-2n3 were generous gifts of Professor David Agard at the University of California, San Fransisco. Plasmid pACYC184 was a gift from A. Gatenby at DuPont Inc. Plasmid pBR32LP, containing the synthetic α–lytic protease gene, was constructed in our laboratory by Diane Perez.

Ethanol precipitations of DNA were performed by adding a one-tenth volume of 3 M sodium acetate and a 2.5 volume of absolute ethanol to the DNA solution, and precipitating for 15 minutes on dry ice. Larger volumes were precipitated using a 0.8 volume of *i*-propanol in place of absolute ethanol. The samples were centrifuged at 14,000 RPM for 15 minutes at 4°C. Pelleted DNA was then washed twice with 100 µl of 70% ethanol and dried in vacuo. The DNA was resuspended in TE (10 mM Tris pH 8.0, 1 mM EDTA). All DNA concentrations were estimated from UV absorbance at 260 nm, and purity estimated from the A_{260}/A_{280} ratio.

Synthetic oligonucleotides were synthesized by the Caltech Microchemical Facility using phosphoramidite chemistry[25] on the Applied Biosystems automated DNA synthesizer, model 380A. Sixty nanomoles from a 0.2 µmole synthesis were purified through preparative polyacrylamide gel electrophoresis (20%, 0.1 X 20 X 40 cm gels; 500 V, 12 hours) in TBE buffer (89 mM boric acid, 89 mM Tris base, 0.2 mM EDTA). DNA was visualized through UV shadowing onto a fluorescent indicator silica TLC plate. Appropriate bands

were excised from the gel, and the DNA extracted from the crushed gel into 1 ml of 2 M NaCl in TE overnight at 37°C. Samples were desalted using two sequential G-25 Sephadex spin columns (2.5 ml, TE equilibrated, resin bed; 2,500 RPM, 10 minutes).

Plasmid Preparations

Plasmid DNA was purified from *E. coli* growths by alkaline lysis[26]. Mini-preps were performed by growing 2 ml of culture to saturation. The cells were pelleted in a microfuge at room temperature (RT) for five minutes. Cells were resuspended in 100 µl Solution I (50 mM Glucose, 10 mM EDTA, 1 mg/ml lysozyme, 25 mM Tris; pH 8.0) and incubated at RT for five minutes. Five microliters of DNase free RNase was added at this step to remove excess RNA. Fresh Solution II (0.2 M NaOH, 1% SDS) was added (200 µl), followed by incubation for five minutes at 0°C. The pH was neutralized with 150 µl of cold (4°C) Solution III (1% formic acid, 60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid in 100 ml) and the mixture centrifuged for 10 minutes at 4°C to precipitate the cellular debris. The supernatant was removed and extracted twice with 100 µl of a 1:1 ratio of TE saturated phenol and chloroform. The DNA was then ethanol precipitated. Large-scale preparations were further purified through ultracentrifugation in cesium chloride (1 g/ml), ethidium bromide (50 µg/ml) gradients (45,000 RPM, 20 hours)[13].

Transformations and Plating

Cells were made competent for transformation by plasmid DNA through an adapted Hanahan procedure[27]. *E. coli* cells were incubated at 37°C to an OD_{550} of ~0.3 in 50 ml SOB media (20 g tryptone, 5 g yeast extract, 0.6 g NaCl, 0.2 g KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ in 1 liter). Two tubes of cell culture (25 ml) were placed on ice for 15 minutes and then centrifuged at 4°C for five

minutes at 4000 RPM. The pellets were drained, resuspended in 8 ml transformation buffer I (12 g RbCl, 9.9 g $MnCl_2 \cdot H_2O$, 1.5 g $CaCl_2 \cdot H_2O$, 150 g glycerol, 30 ml 1 M KOAc pH 7.5 in 1 liter; adjusted to pH 5.8 with 0.2 M acetic acid), and allowed to incubate on ice for 15 minutes. Cell suspensions were centrifuged (4°C, 4000 RPM, five minutes), drained, and resuspended 2 ml in transformation buffer II (1.2 g RbCl 11 g $CaCl_2 \cdot H_2O$, 150 g glycerol, 20 ml 0.5 M MOPS pH 6.8 in 1 liter). Competent cells were transformed within two hours of preparation or frozen in liquid nitrogen and stored at -70°C. Frozen cells were used after thawing on ice for 10 minutes.

Transformations were performed by gently mixing plasmid DNA (5 to 25 ng) with 300 µl competent cells in a 1.5 ml eppendorf tube. Samples were allowed to incubate on ice for 40 minutes, followed by heat shocking at 42°C for 90 seconds. Cells were then allowed to incubate on ice for an additional five minutes, before adding 800 µl SOC media (SOB media with an additional 10 mM of glucose) and incubating tubes for one hour at 37°C. Tubes were gently centrifuged (~1000 RPM for 30 seconds), and approximately 900 µl of the supernatant discarded. Pellets were then resuspended in the remaining supernatant and plated onto L-agar containing appropriate antibiotics. Plates were incubated at 37°C overnight.

Restriction Digest and Fragment Isolation

Restriction digests were typically performed on 5 µg of plasmid DNA, using 4-12 units of restriction enzyme in a reaction volume of 15 µl, incubated at 37°C for one to two hours. Fragments were separated on 1.0% - 1.2% agarose gels containing ~5 ng/ml ethidium bromide in TAE buffer (4.8 g Tris base, 1.14 ml glacial acetic acid, 2 ml 0.5 M EDTA in 1 liter) at 100 V for two to four hours. DNA fragments were visualized by UV irradiation. DNA

fragments were isolated from preparative gels made from NuSieve GTG LMP agarose electrophoresed at 30 V overnight at 4°C. Gel slices containing DNA fragments were placed in 1.5 ml eppendorf tubes, melted at 60°C for 15 minutes, and an equal volume of TE saturated phenol was added to each tube. The samples were thoroughly mixed, and allowed to incubate on ice for 30 minutes. The phases were separated by centrifugation at 14,000 RPM for five minutes. The aqueous (upper) phase was retained, extracted twice with phenol/chloroform, followed by the precipitation of the DNA with ethanol. Dried DNA pellets were taken up in 25 µl TE.

Terminal phosphates were removed from whole restriction digest or isolated DNA fragments by adding 5-20 µl of calf intestine alkaline phosphatase diluted to 0.1 units per ml in CIAP dilution buffer (50 mM Tris, 0.1 mM EDTA; pH 8.0) to DNA samples and incubating at 37°C for one hour. Samples were then incubated for 30 minutes at 75°C, twice phenol/chloroform extracted, followed by precipitation with ethanol. Typically, DNA pellets were redissolved in 25 µl TE and used in ligation reactions directly, without further isolation.

Kinasing and Annealing Synthetic Oligonucleotides

To 12 pmoles each of purified complementary oligonucleotides were added 2.5 µl 10X kinase buffer (100 mM Tris, 10 mM MgCl2), 2.5 µl 100 mM ATP, 1.8 µl 100 mM dithiotheritol (DTT), and two to five units of T4 polynucleotide kinase in a total volume of 25 µl. The reaction was incubated for 45 minutes at 37°C. The kinase was denatured by placing the samples in boiling water for five minutes. Typically, two liters of boiling water were used, and the kinased oligonucleotides were left to equilibrate gradually to RT in the large vessel, allowing the complementary oligonucleotides to anneal.

Plasmid Sequencing

Plasmid DNA was sequenced by the dideoxy method[28] for denatured, double-stranded DNA[29]. The sequencing primers were synthesized to be 16-18 bases in length and bind approximately 100 bases upstream of the region to be sequenced. Plasmid DNA (5 µg) was mixed with 25 pmoles of sequencing primer in total volume of 10 µl of TE. The plasmid was denatured and the primer annealed to the DNA by boiling for five minutes, followed by flash freezing in a dry ice/ethanol bath. Chain extension reactions were performed with slight variations on the protocol described in the SEQUENASE^{tm} kit from United States Biochemical[30]. The annealed primer-DNA complex was incubated with 0.5 µl labeling mix, 2 µl 100 mM DTT, 2 µl sequenase reaction buffer, 1 µl [α–^{35}S]-dATP (1000 Ci/mmole), and 2 µl of a one-eighth dilution of Sequenase enzyme, for three minutes at RT. The reaction mixture was then partitioned (3 µl) into each of the four dideoxynucleotide termination mixes (2.5 µl), mixed, and incubated for 10 minutes at 37°C. Reactions were stopped by the addition of 4 µl of Maxam-Gilbert loading buffer[31], and incubated at 100°C for two to five minutes. Samples were loaded onto 5-8% polyacrylamide/TBE gels, run at 50 mA for three hours, and autoradiographed 12-48 hours using Kodak XAR film. Figure 4 displays a typical sequencing gel.

Protein Expression

For expression of the α–lytic protease genes, the PhoA promoter was induced through phosphate depletion in a modified MOPS medium[32,33] (40 mM morpholinepropanesulfonic acid, 4 mM Tricine pH 7.4, 10 µM $FeSO_4$, 9.5 mM NH_4Cl, 2.8 mM K_2SO_4, 0.5 µM $CaCl_2$, 0.5 mM $MgCl_2$, 50 mM NaCl, 3 nM $(NH_4)_6Mo_7O_{24}$, 0.4 µM H_3BO_3, 30 nM $CoCl_2$, 10 nM $CuSO_4$, 80 nM $MnCl_2$, 10 nM $ZnSO_4$). Starter cultures of cells harboring α–LP expression

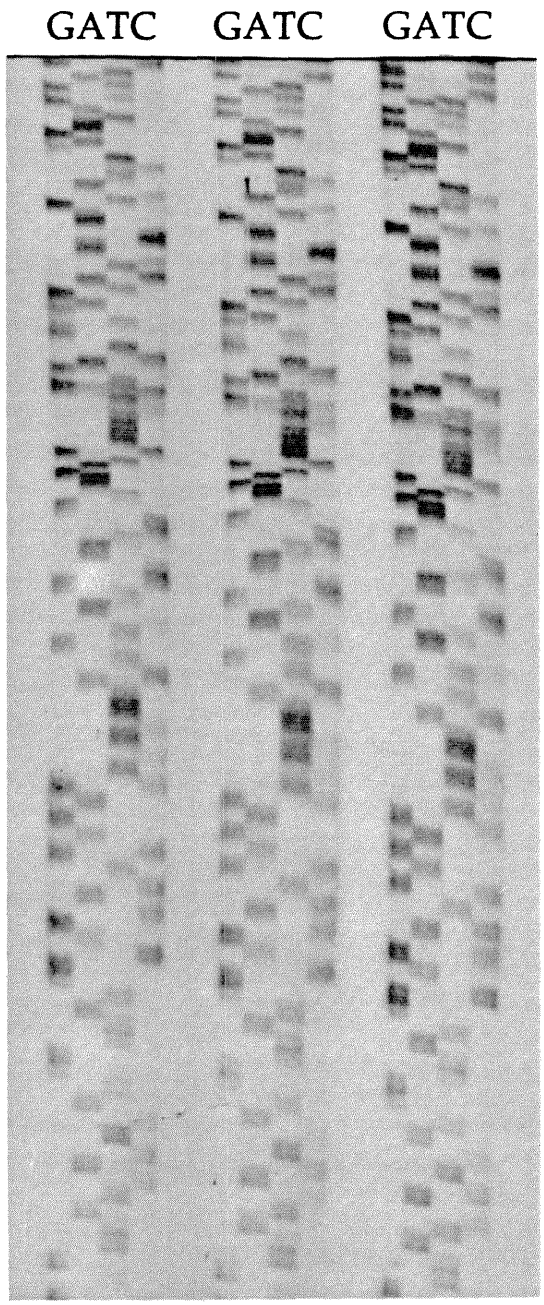

Figure 4. A representative sequencing gel. Double-stranded dideoxy sequencing was used. Sequences shown are of samples from the pALP32P2M construct.

plasmids were grown in L broth (2-4 ml), containing appropriate antibiotics, overnight at 37°C. The cells were centrifuged down, the supernatant removed, and the cell pellets resuspended in an equivalent volume of MOPS media. This wash was performed a second time, and the cell suspension was then used to inoculate MOPS media (50-500 ml) supplemented with 0.4% glucose, and 0.15% yeast extract, as a source of phosphate, containing the appropriate antibiotics. Cultures were grown at 25°C, on an orbital shaker, for three to five days. Expression was monitored for production of α–lytic protease through Western blot analysis.

Gel Electrophoresis of Proteins and Western Staining

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots were performed to determine presence and purity of α–lytic protease in samples. Western blots also provided a method of visualizing processing of the pro-α–LP. Protein samples (5-20 µl) containing between 0.1 µg and 10 µg of protein were added to an equal volume of loading buffer (10% v/v glycerol, 5% v/v 2-mercaptoethanol, 3% w/v SDS, 62.5 mM Tris-HCl pH 6.8, 0.05% w/v bromophenol blue) and boiled for five minutes. For cell samples, 100 µl of cell growths were centrifuged down, the supernatant removed, and the cells resuspended in 30 µl of loading buffer and boiled for five minutes. Samples were loaded on a 15 cm 12-15% acrylamide gel with a 4% stack. Gels were run at a constant current of 10 mA for ~12 hours in Tris-glycine SDS buffer (12 g Tris base, 57.6 glycine, 3.2 g SDS in 4 liters). Bands were visualized in the acrylamide gel by Brilliant Blue G colloidal staining[34]. For Western stains, protein bands were transferred from the acrylamide gel onto a nitrocellulose membrane, using an electroblotting cell, in a modified tris-glycine buffer (without SDS and containing a 20% v/v of methanol) at a constant cur-

rent of 100 mA for one to two hours. α–Lytic protease was visualized, following binding of rabbit anti-α–lytic protease antiserum, with the Vectastain^{tm} ABC immuno-peroxidase system[35].

Membrane-Bound Protease Activity Assay

Expression of active α–lytic protease was visualized through a process of binding the excreted enzyme to a membrane followed by the treating of the membrane to a colorametric assay for α–LP activity. A sterile (autoclaved) nitrocellulose membrane was placed onto a MOPS expression media (above) agar plate. An L-shaped pasteur pipet was used to remove any trapped air between the membrane and the agar. A sterile cellulose acetate membrane was placed on top of the nitrocellulose and allowed to become wetted with the moisture in the agar. This inert membrane functions as a support for colony growth.

Cell cultures were then spread onto the cellulose acetate membrane. Alternatively, colonies were carefully picked (so as not to puncture the membranes), onto the cellulose acetate membrane. Plates were incubated at RT for two to three days, in which time proteins extruded from the cells bound to the nitrocellulose membrane.

The nitrocellulose (lower) membrane was removed and soaked in 2 mM NaOH for three minutes. The membrane was transferred to a fresh solution of 2.5 mg of α–LP substrate (N-acetyl-Ala-Ala-Ala-methyl Ester) in 5 ml of 1 mM phenol red pH 8-9, and swirled for three to five minutes. The membrane was removed and allowed to drip dry. Colonies with appreciable activity were displayed as yellow spots on the red background within three to seven minutes.

Organo-Phosphatase Activity Assay

The organo-phosphatase activity of α–lytic protease mutants was assayed on a Hewlett-Packard 8452A diode array spectrophotometer using a 1 cm quartz cell. The spectrophotometer was equipped with an 89090A peltier thermostated cell holder; the temperature was maintained to ±0.05°C. The cell compartment was also stirred with a magnetic stir bar throughout the assay.

Crude preparations of the α–LP mutant enzymes were used for the assay. Enzyme was expressed as above, and the growth media was centrifuged at 10,000 RPM for 20 minutes to pellet the cells from the media. The supernatant was removed and dialyzed in 8,000 kD MWCO dialysis tubing against H_2O at 4°C, to a dilution of $1{:}1x10^9$. Samples were lyophilized and stored at -20°C. Immediately prior to assaying the enzyme, approximately 1 mg of lyophilized sample was resuspended to a concentration of 10 mg per ml in cold (4°C) 50 mM Tris pH 7.75. Phosphatase activity was assayed using diethyl *p*-nitrophenyl phosphate (DNP) as a substrate. This substrate is a very high sensitivity indicator of the hydrolysis reaction[6], with a $\Delta\varepsilon$ (the difference between the molar absorptivities of the product, *p*-nitrophenol, and the DNP substrate), of 14,750 M^{-1} cm^{-1}.

The protocol for the assay was as follows: 2.0 ml of buffer (10% v/v acetonitrile in 50 mM Tris, pH 7.75) was placed into the sample cuvette, and the temperature allowed to equilibrate for five minutes. The absorbance baseline was then measured, and the spectrophotometer was set to record at 15-second intervals, the difference between absorbances at 402 nm, the peak in the *p*-nitrophenol spectrum, and a reference wavelength of 600 nm, where both the substrate and the product were transparent. An aliquot of the substrate,

diluted in acetonitrile, was added to the cuvette and the reaction recorded until complete mixing had occurred and the rate of spontaneous hydrolysis was determined, ~1,000 seconds. An aliquot of the enzyme solution was then added, and recording continued until a constant rate of hydrolysis was again observed, generally on the order of 5,000 to 10,000 seconds. The absorbance traces were analyzed using standard linear regression techniques.

Cassette Mutagenesis: Creation of an Sty I Site in the Synthetic α–Lytic Gene

A unique Sty I restriction site was placed in the synthetic α–lytic protease gene through a two-fragment cassette replacement (Figure 5). Synthetic complementary oligonucleotides, encompassing nucleotides 72 through 130 of the mature portion of α–lytic protease, were designed with an Sty I restriction site at position 83. The oligonucleotides were kinased and annealed as above. The pBR32LP plasmid (0.04 pmoles), containing the synthetic α–LP gene (S195A), was digested simultaneously with the two restriction enzymes, Xho I and BssH I, removing a 59 base-pair fragment from the beginning of the α–LP gene. The reaction mixture was subsequently phosphatased. The synthetic cassette (0.8 pmoles) was added to the cleaved pBR32LP, along with 2.5 µl of 10X ligation buffer and 2 units of T4 DNA ligase. The final volume was brought to 25 µl. Control reactions were performed with the absence of the insert DNA to monitor the reformation of the parent plasmid. The ligation reactions were carried at 16°C for 16 hours, followed by transformation of competent *E. coli* LS1 cells with 0.5 µl of the reaction mixtures. Cells were plated onto ampicillin (100 mg/l) L agar plates. Plasmids were screened for the presence of the Sty I cleavage site. The plasmid was labeled pBR32LP-STY.

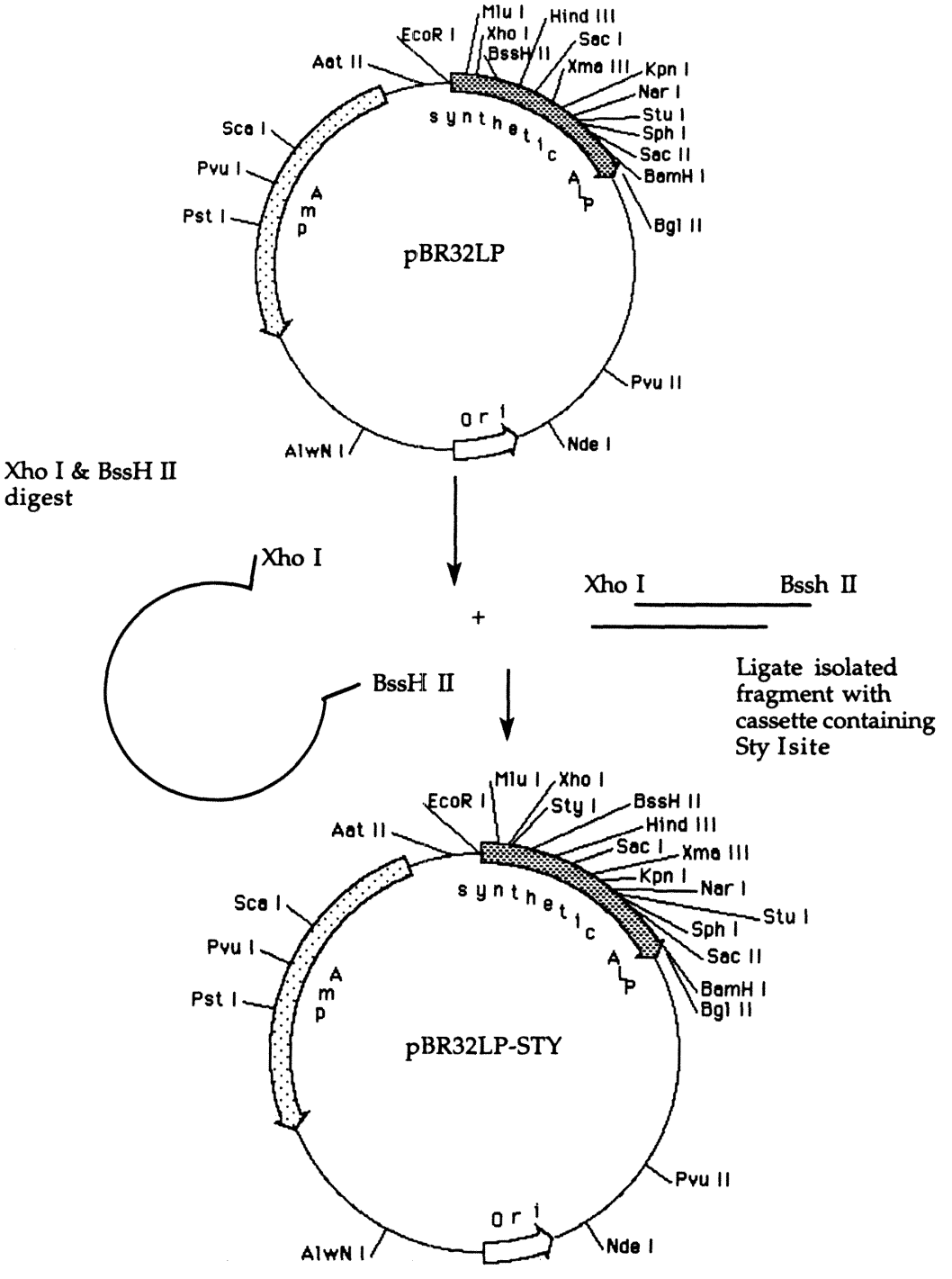

Figure 5. Scheme for insertion of Sty I restriction site into plasmid pBR32LP, using cassette mutagenesis.

Construction of Chimera α–Lytic Protease Expression System

The chimera α–lytic protease gene was constructed through the replacement of the majority of the mature coding portion of the wild type pro-α–lytic protease gene (encompassing nucleotides 83 through 597) with the corresponding segment from the synthetic α–LP gene. A three-fragment ligation was performed, as outlined in Figure 6. From the pALP5 plasmid, the 941 base-pair Sph I/Sty I fragment and the 3096 base-pair Sph I/BamH I fragment were isolated through LMP agarose electrophoresis. These two fragments (0.07 pmoles each) were combined with the isolated Bgl II/Sty I 515 base-pair from pBR32LP-STY (0.1 pmoles), and incubated with 2 units of T4 DNA ligase and 2.5 µl of 10X ligase buffer in a final volume of 25 µl at 16°C for 16 hours. Control reactions were run on the three possible pairings of fragments to test for contamination by either of the parent plasmids. Ligation mixtures (10 µl) were used to transform competent *E. coli* LS1 cells. Transformation reactions were plated onto ampicillin (100 mg/l) L agar plates, and colonies were screened by restriction mapping for the presence of a one Hind III site, two BamH I sites, and three Sty I sites. Plasmids with the appropriate restriction pattern were further screened by sequencing.

Cassette Mutagenesis: Reversion of pALPE-S195A to Wild Type pALPE

In order to remove the serine 195 alanine mutation present in the synthetic portion of the chimeric gene, a three-fragment cassette mutation was performed (Figure 7). A 42 base-pair synthetic cassette coding for the wild type serine 195 was kinased and annealed, and 0.1 pmoles were added to a mixture of the Not I/Sph I 1174 base-pair (0.05 pmoles) and the Not I/Sac II 3334 base-pair (0.05 pmoles) fragments from pALPE-S195A. The mixture was incubated with 2 units of T4 DNA ligase and 2.5 µl of 10X ligase buffer in a final volume

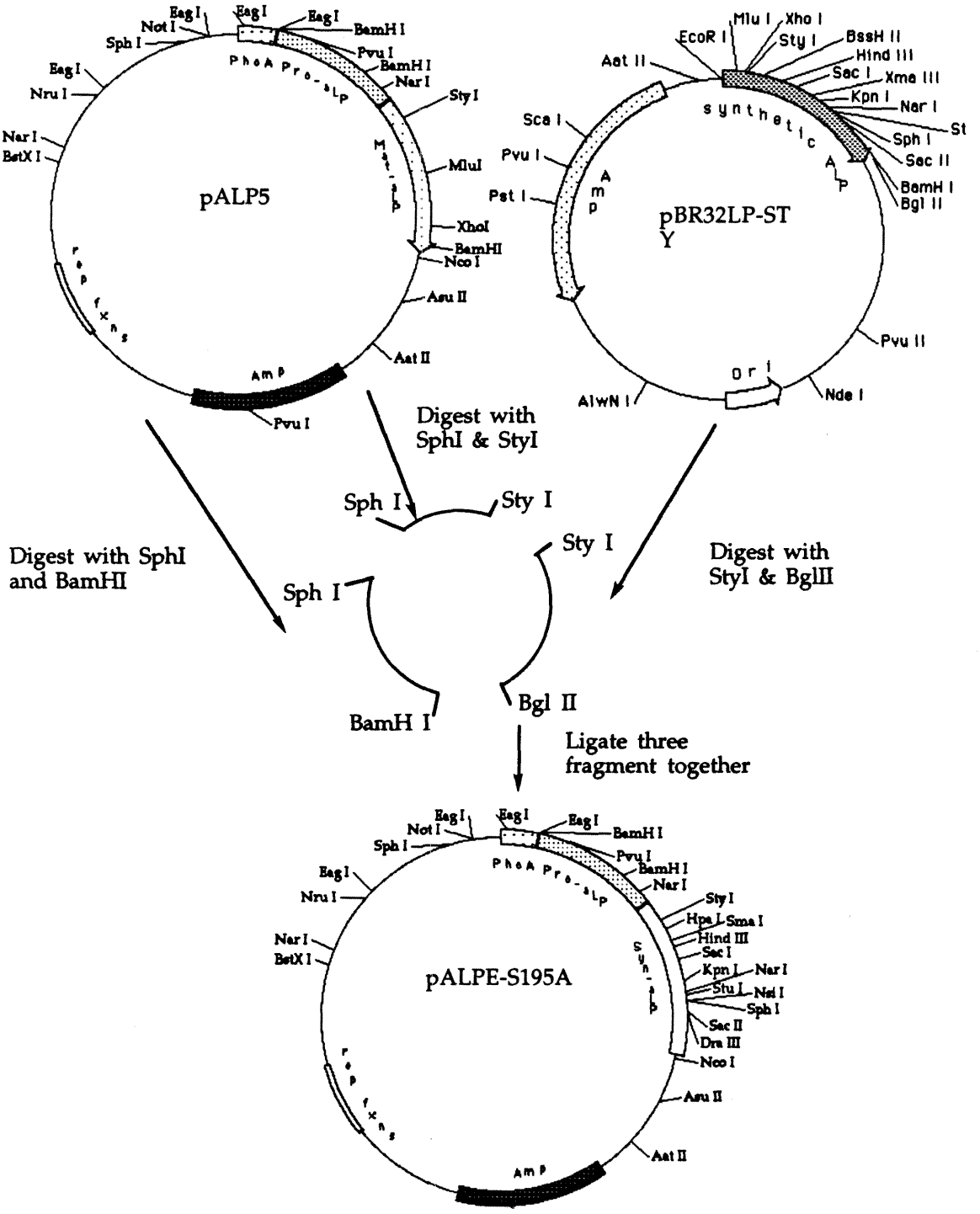

Figure 6. Scheme for construction of the pALPE-S195A plasmid by inserting the synthetic α-lytic protease gene into the pALP5 pro-α-lytic protease expression vector.

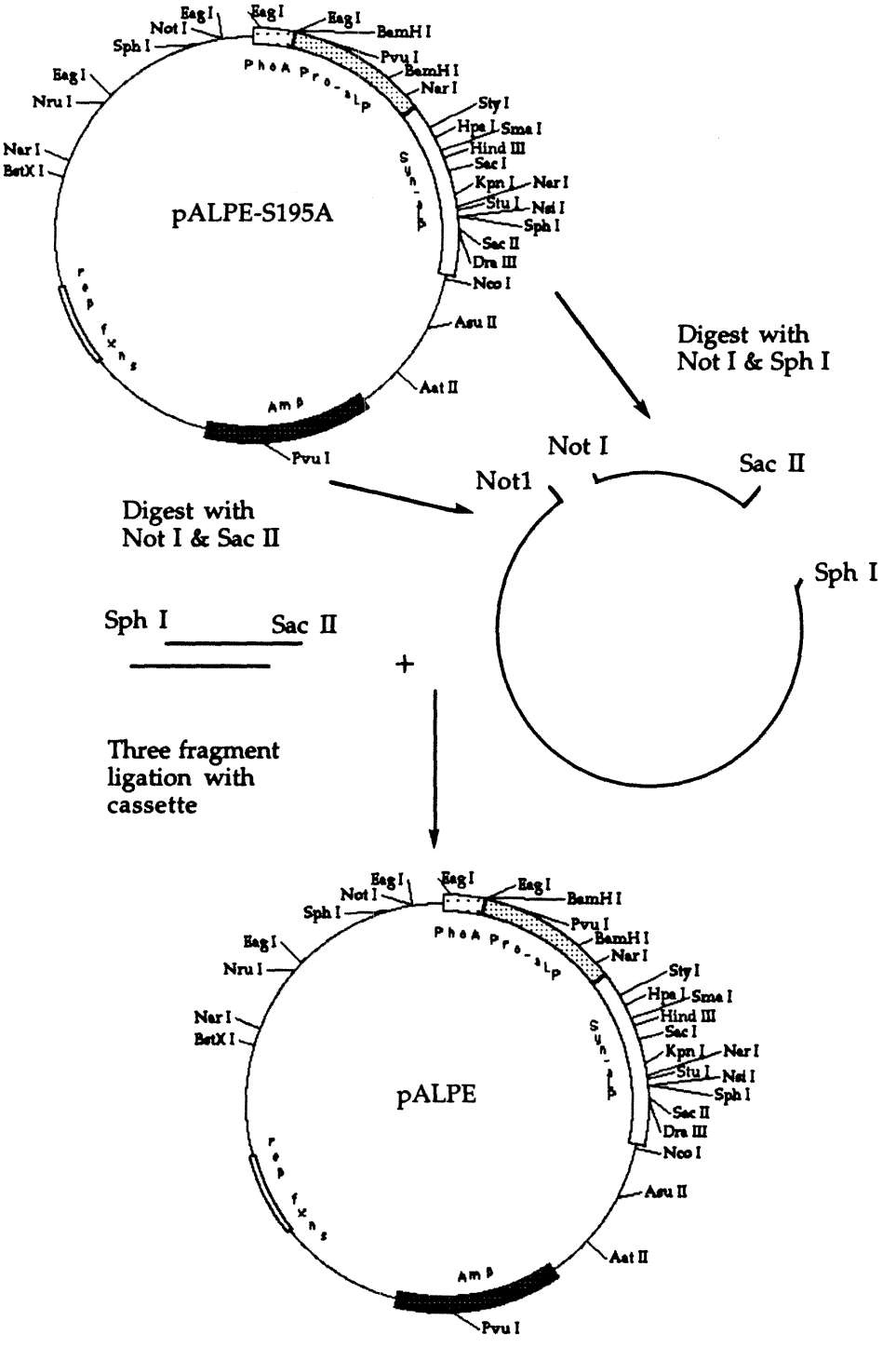

Figure 7. Scheme for reversion of the pALPE-S195A α–lytic protease expression vector to the wild type sequence, using cassette mutagenesis.

of 25 µl at 16°C for 16 hours. Control reactions were run without the insert to test for contamination by the parent plasmid. Competent *E. coli* LS1 cells were transformed with 10 µl of the ligation mixtures and plated onto ampicillin (100 mg/l) L agar plates. Colonies were screened by sequencing.

Construction of a PhoA Promoter Carrying Vector (pBR322-PhoA)

The PhoA promoter and leader coding sequence were isolated from the pALP5 plasmid and inserted into a carrier vector (pBR322) for use in the complementation expression system construction as outlined in Figure 8. pBR322 (0.08 pmoles) was digested with restriction enzymes BamH I and Sph I, and was subsequently phosphatased, phenol/chloroform extracted, and ethanol precipitated. The DNA was resuspended in TE and added to the BamH I/Sph I 353 base-pair fragment from pALP5 (0.9 pmoles). The ligation reaction was carried out with 2 units of T4 DNA ligase and 2 µl of 10X ligase buffer in a final volume of 20 µl at 16°C for 16 hours. Five µl of the ligation mixture were used to transform competent *E. coli* LS1 cells, which were plated onto ampicillin (100 mg/l) L agar plates. Over 100 colonies were picked and screened for sensitivity to growth on tetracycline. Forty-three colonies did not grow on tetracycline indicating presence of the PhoA insert in the *Tet* gene of pBR322.

Construction of a Protease Domain Expression Vector (pALP32P1M)

A gene for expressing only the mature coding region of the α–lytic protein was created through a three-fragment ligation of the gene fragment from pALPE and a synthetic linker into pBR322-PhoA vector (Figure 9). The pBR322-PhoA plasmid (0.4 pmoles) was linearized with BamH I and phosphatased. To this fragment was added the Bgl II/Mlu I 540 base-pair fragment from pBR32LP-STY (0.5 pmoles), along with a synthetic Bgl II/Mlu I linker

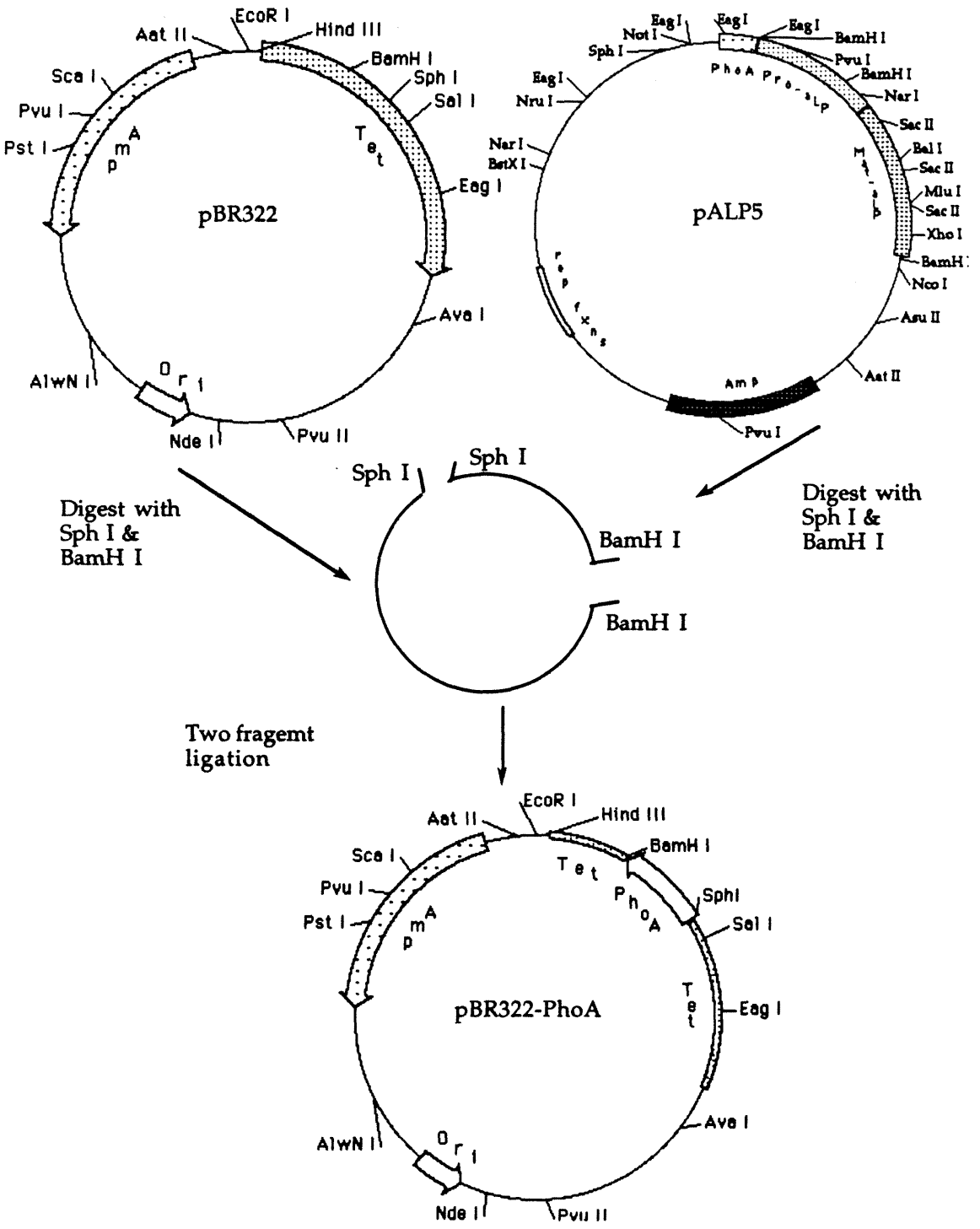

Figure 8. Scheme for insertion of the PhoA promoter and signal sequence into the plasmid pBR322.

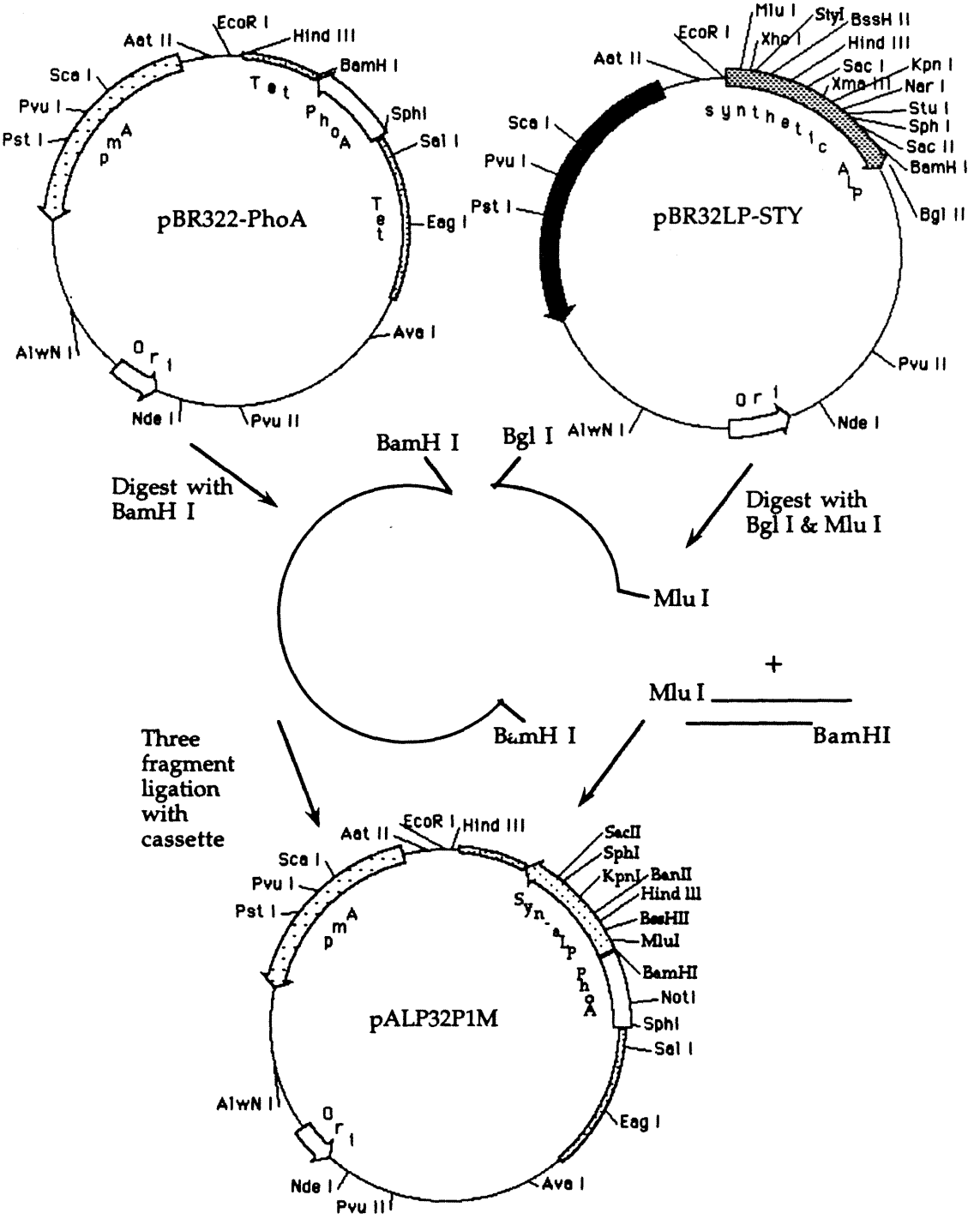

Figure 9. Scheme for construction of a plasmid for expression of only the mature α-lytic protease domain.

(0.5 pmoles) coding for the first 13 residues of the α–LP mature region gene. The ligation reaction was carried out with 2 units of T4 DNA ligase and 2.5 µl 10X ligase buffer in a final volume of 25 µl at 16°C for 16 hours. Five µl of the ligation mixture was used to transform competent *E. coli* D1210 cells and the transformation reactions plated on ampicillin L agar plates. Colonies were screened by restriction digest for the presence of a Dra III cleavage site. Dra III susceptible plasmids were then further screened for proper orientation of the linker and insert with respect to the PhoA promoter through restriction mapping.

Construction of Pro-Coding Region Expression Vectors (pALPKSP1P and pALP184P2P)

A vector for expressing the pro-region of α–lytic protease, separate from the mature portion, was constructed through a three-fragment ligation of the PhoA promoter/leader and pro-regions of the wild type α–LP gene, along with a synthetic linker, into the pBLUESCRIPT^{tm} KS+ sequencing/expression plasmid (Figure 10). pALP5 (0.05 pmoles) was digested with Nru I/Nar I and the 1,212 base-pair fragment was isolated. A standard 25 µl ligation reaction was carried out on a mixture of this isolated fragment, a Nar I/Not I synthetic linker (0.5 pmoles) coding for the last nine residues of the pro region, and a Not I linearized and phosphatased pBLUESCRIPT^{tm} (0.05 pmoles). 10 µl of the ligation mixture was transformed into competent *E. coli* XL1Blue cells and plated on ampicillin (100 mg/l), X-gal/IPTG (80 µg/ml / 10 mM) L agar plates. White colonies (indicating the presence of an insert in the β-glactosidase gene of pBLUESCRIPT^{tm}) were initially screened for resistance to Mlu I digestion. Resistant plasmids were further screened by restriction mapping.

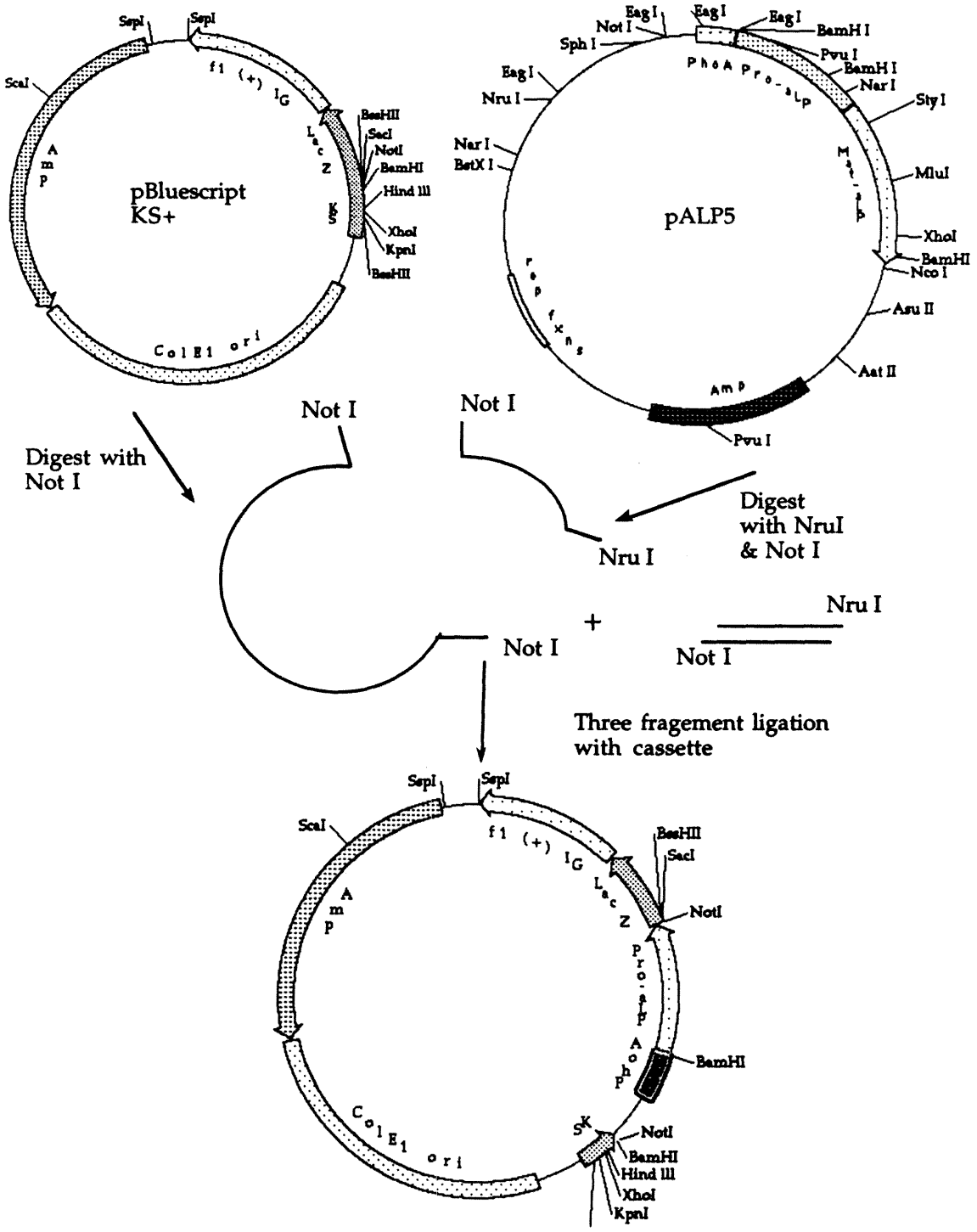

Figure 10. Scheme for construction of a plasmid for expression of only the pro-domain of α-lytic protease.

Results and Discussion

Construction of Chimeric Expression Vector

With the cloning of the wild type α–LP gene, and its subsequent expression using the promoter and leader sequence from the *E. coli* PhoA gene by the Agard laboratory[23], the clear choice for expression of the synthetic gene was to substitute the protease coding portion of the wild type gene with the synthetic gene. Using cassette mutagenesis, a unique Sty I restriction site was added to the synthetic α–lytic protease gene by introducing a single silent mutation at the 88th base in the gene. This Sty I site and an existing Bgl II site down stream of the synthetic gene were used to excise the majority of the gene. The excised fragment was then used as a cassette replacement for the corresponding region of the wild type α–lytic protease gene in the PhoA, pro-α–LP expression vector, pALP5. Figure 6 depicts the three-fragment ligation scheme employed to generate the chimera. The presence of a second Sty I site in the pALP5 vector precluded using a simple two-fragment replacement scheme. (The Bgl II and BamH I restriction sites possess complementary cohesive ends, allowing the two sites to be ligated together, yet removing both sites in the product plasmid.) This chimeric gene contained only 14 of the 16 unique restriction sites present in the original synthetic gene, due to the loss of the two sites upstream of the Sty I site.

Two colonies from the ampicillin resistant transformants were isolated by restriction mapping analysis. DNA sequencing of the two plasmids confirmed the proper construction of the chimeric gene. One of these plasmids was used to construct a separate vector wherein the Ser 195 Ala substitution that existed in the original synthetic gene was reverted back to serine. These constructions were labeled pALPE and pALPE-S195A, respectively.

α–Lytic protease was expressed through the induction of the PhoA promoter by the depletion of phosphate in the media. The pro-enzyme is translocated into the periplasm of the cell, where it self-cleaves into the mature enzymatic form. Growths of wild type α–lytic protease accumulate large amounts of the enzyme in the media, apparently from leakage of the enzyme through the periplasmic membrane[33].

Activity was screened for using the membrane-bound protease activity assay[36]. *E. coli* cells harboring either the pALPE or pALPE-S195A plasmids were grown on the membrane-agar plates previously described, over a two-day period, allowing expressed enzyme excreted into the extracellular space to become bound to the nitrocellulose membrane. Bound The α–lytic protease activity of the bound enzyme was assayed by immersing membranes in a solution of the protease substrate, N-acetyl-Ala-Ala-Ala-methyl ester, and phenol red; the localized increase in acidity from cleavage of the substrate produces a colorimetric change in the vicinity of colonies producing active enzyme. As expected, the assay displayed clearly active α–lytic protease expressed from cells containing the pALPE (wild type coding gene) plasmid. However, cells harboring the pALPE-S195A plasmid, as well as control cells without either of the α–lytic protease vectors, displayed no activity.

To determine whether expression of the mutant α–lytic protease enzyme was taking place and to what extent the enzyme was being properly translocated and folded, Western analyses were performed on supernatants from four day growths in MOPS media. Cells harboring the wild type coding plasmid revealed a ~20kD band, indicative of the mature enzyme, and traces of a ~40kD band, indicating presence of small amounts of the pre-enzyme precursor, in supernatant samples (Figure 11). However, cells expressing the Ser 195 Ala gene (pALPE-S195A) displayed only the ~40kD band in the supernatant

samples. The lack of the 20kD band in Western stains of pALPE-S195A harboring cells demonstrates that the Ser 195 Ala active-site mutant is incapable of self-processing, resulting in its expression only as the pro-enzyme.

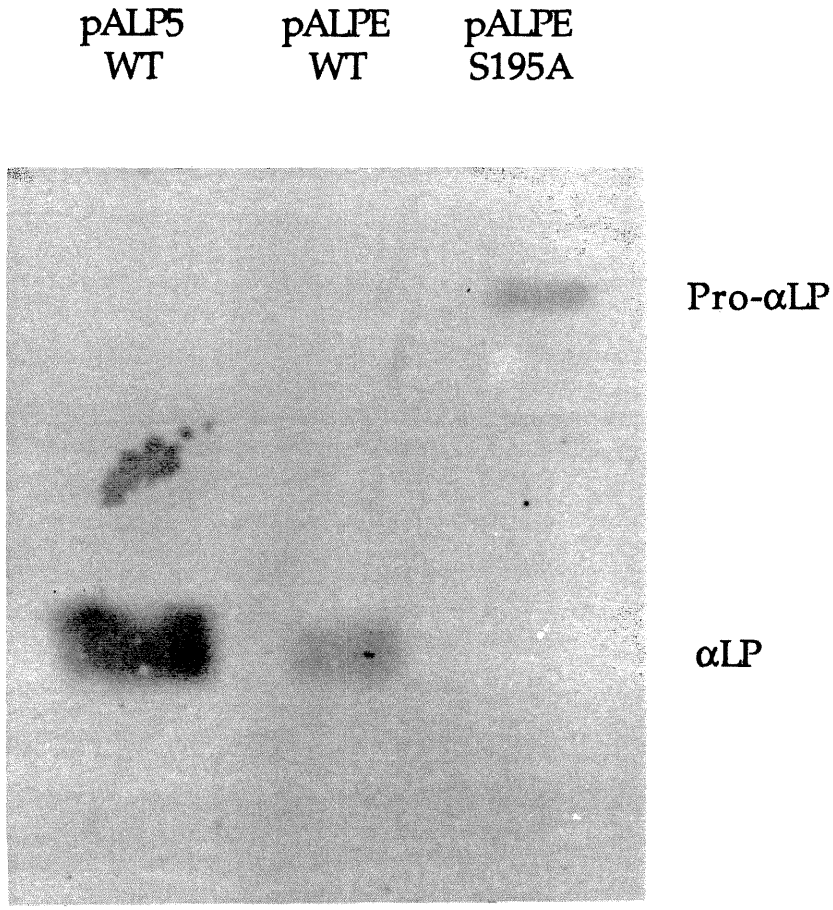

Figure 11. Western blot of α-lytic protease samples, using antisera to wild type α-LP. Lane 1 is wild type α-LP expressed from the pALP5 vector. Lane 2 is wild type α-LP expressed from the pALPE vector. Lane 3 is the Ser 195 Ala mutant of α-LP expressed from the pALPE-S195A vector. Trace amounts of the ~40kD pro-enzyme can be seen in the two wild type samples. However, mature enzyme is not displayed in the mutant sample, indicating that the mutant enzyme cannot self-process.

The Complementation Expression System

Initial experiments with the wild type α–lytic protease gene demonstrated that expression of the protease portion of the gene alone resulted in the production of an inactive, and presumedly improperly folded, α–LP enzyme[17].

By separating the pro-coding and protease-coding regions of the wild type gene, and placing each region, each with its own leader sequence, under the control of an independent promoter, J. Silen *et al.* demonstrated that, though the proper folding (activation) of α–lytic protease required the presence of the pro-region of the protein, it was not necessary that the pro-peptide be covalently attached to the protease portion of the enzyme for activation, as it is in the naturally occurring zymogen. The α–LP pro-region thus functions as both an inhibitor of protease activity while in the cytosol of the cell, and as a chaperonin[37] for the protease domain in arranging the unfolded protease into a state felicitous for activity.

I chose to create a similar expression system as a means of expressing properly folded mutants of the α–lytic protease domain. The "complementation" expression system was designed to consist of the two domains of the α–LP gene cloned into and expressed from separate plasmids. Plasmids from compatible groups were chosen, allowing the two vectors to co-exist in the same cell line. The PhoA promoter and leader sequence from the recombinant α–lytic protease expression vector were chosen as the upstream sequences for both sub-genes, since they had been shown to produce high yields in the expression and translocation of the wild type enzyme.

Construction of the two sub-genes from the wild type gene and the synthetic gene was performed in four steps. Initially, the PhoA promoter and leader coding sequence were isolated from the pALP5 vector and cloned into pBR322, interrupting the *Tet* gene. Transformants were screened for sensitivity to tetracycline, indicating the presence of the insert in the *Tet* gene. A three-fragment ligation of the synthetic gene, from pBR32LP-STYI, a synthetic oligonucleotide linker, and the pBR322-PhoA plasmid was employed to construct a vector (pALP32P1M) for expression of the α–lytic protease domain

alone. The use of the original synthetic α–LP gene meant that the pALP32P1M construct contained the full complement of restriction sites in the synthetic gene. The product plasmids were screened by restriction mapping.

The construction of a vector for expression of the pro-domain alone was performed through a three-fragment ligation of the PhoA promoter/leader and pro-region of the wild type α–LP gene from pALP5, along with a synthetic linker, into the pBLUESCRIPT^{tm} plasmid. Plasmids were again screened by restriction mapping.

A plasmid (pCTERM-2n3) containing a modified α–lytic pro-coding region, already isolated from the mature coding region, became available from the Agard laboratory. A three amino acid change made in the pro-coding domain had been shown to enhance the specific activity of α–lytic protease isolated from a complementation system using this modified pro-peptide[38]. It is believed that the modified pro-protein is better able to properly fold the mature α–LP enzyme, thus producing a higher percentage of active enzyme in the protein preparations. I chose to reconstruct the pro-peptide expression vector using this modified gene.

As stated earlier, the maintenance of the two sub-genes on separate plasmids in a single cell required that the two plasmids have compatible origins of replication. Accordingly, the pro-coding domain gene from the pCTERM-2N3 plasmid was cloned into the pACYC184 plasmid. This plasmid contains the p15A origin of replication and the chloramphenicol resistance gene, allowing it to co-exist and be maintained with vectors possessing the ColE1 origin and ampicillin resistance, such as pALP32P1M. With the two sub-genes in place on compatible vectors, an expression cell line was created through a double transformation and selection of colonies resistant to both

ampicillin and chloramphenicol. Agarose electrophoresis of plasmid DNA isolated from ampicillin/chloramphenicol resistant colonies displays the two distinct vectors harbored in such cell lines (Figure 12). These double-vector cell lines will be referred to as "the complementation expression system" throughout the remainder of the chapter.

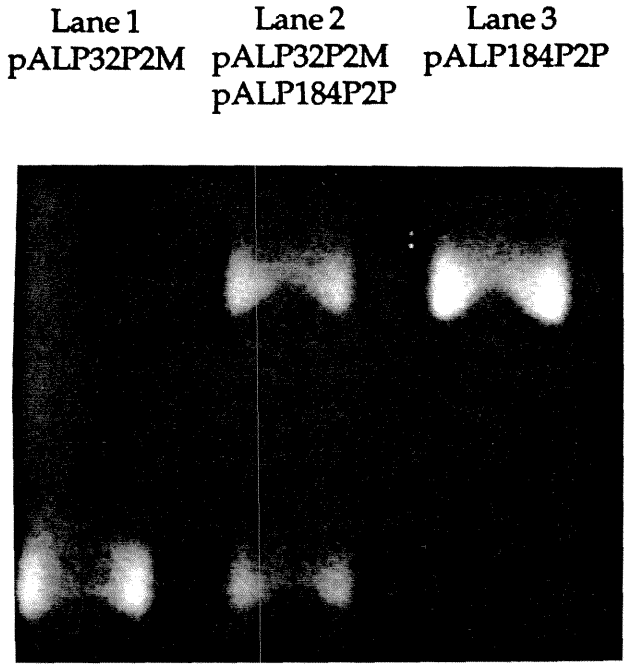

Figure 12. Agarose gel of plasmids prepared from the complementation α-lytic protease expression cell line. Lane 1 is a sample from cells harboring only the mature α-LP expression vector. Lane 3 is a sample from cells harboring only the pro-domain expression vector. Lane 2 is a sample form cells harboring both expression vectors.

Expression and Characterization of α–Lytic Protease Mutants

Mutant α–LP enzymes were expressed from cells harboring the "complementation" expression plasmids. With both the pro-domain and the

protease domain genes under PhoA control, expression was induced by phosphate depletion of cells grown in MOPS media. A quick and crude isolation scheme took advantage of the excretion of the enzyme into the growth media, by centrifuging the cells out of solution, followed by the removal of the low molecular weight constituents of the media by dialysis. The remaining solution, containing the enzyme, was lyophilyzed to dryness.

Western blots of lyophilized samples (Figure 13) displayed a ~20 kD band for both expression of the wild type enzyme and the Ser 195 Ala mutant, indicating that the protease domain is being produced and exported. Since the protease domain is expressed separately, the existence of Western staining material in the growth media is not proof that the enzyme is properly folded. However, expression of the protease domain in the absence of the pro-peptide results in very little of the 20 kD peptide being detected in the media. Presumedly, the improperly folded protease domain is not stable to other proteolytic enzymes produced by the cells.

The activity of the excreted enzyme was used to assess the success of the complementation system in producing properly folded α–LP. Using the membrane-bound activity assay, colonies were assayed for excreted activity on the substrate N-acetyl-Ala-Ala-Ala-methyl ester. After three days of growth at room temperature, wild type α–LP expressed from the complementation system displayed an activity equivalent to that obtained from the wild type pALPE vector. However, there was no activity expressed from the complementation cell line containing the alanine mutant, or from either the wild type or the Ser 195 Ala mutant protease domain expressed alone. These results clearly show that, in the case of the wild type protease domain, the complementation expression system produces properly folded active enzyme,

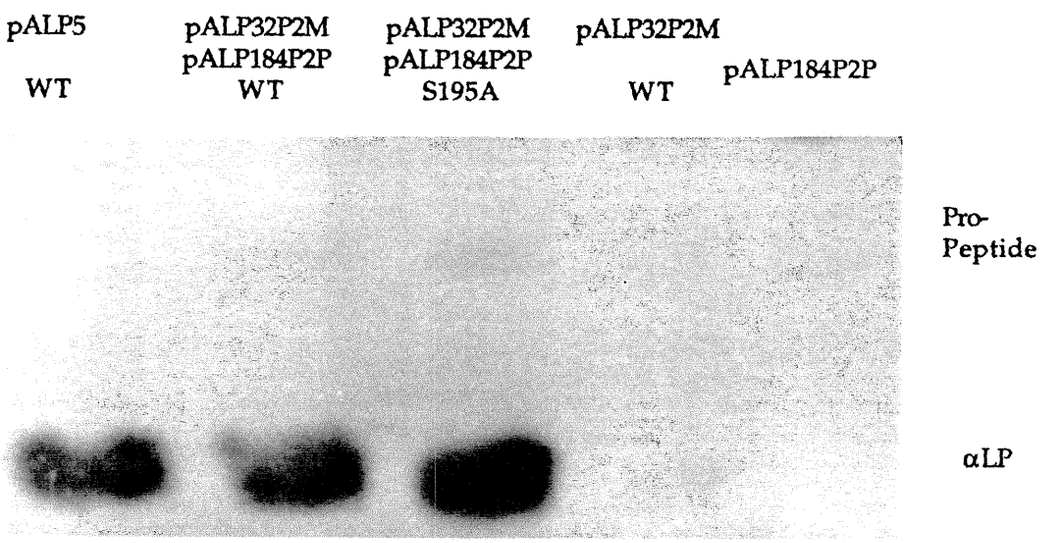

Figure 13. Western blots of supernatant samples from α-lytic protease complementation expression cell lines. Lane 1 is wild type α-LP from the pALP5 vector. Lane 2 is wild type α-LP from complementations cells harboring the wild type protease domain and the pro-domain expression vectors. Lane 3 is the S195A mutant α-LP from complementation cells harboring the mutant protease domain and the pro-domain expression vectors. Lane 4 is from cells harboring only the protease domain expression vector. Lane 5 is from cells harboring only the pro-domain expression vector.

and as such can be assumed to produce properly folded mutants of α–lytic protease.

Assaying Phosphatase Activity

The use of phosphate esters, such as diethyl *p*-nitrophenyl phosphate (DNP), as substrates for proteolytic enzymes exhibit biphasic kinetics. This kinetic behavior is a result of the rapid acylation of the enzyme with the sub-

strate, followed by a slow deacylation. For DNP, these kinetics are manifest as a burst of *p*-nitrophenol, from the acylation, proportional to the enzyme concentration and the degree of enzyme saturation by the substrate. After the initial burst, enzyme active sites are occupied by the phosphate-enzyme complex, and the rate of *p*-nitrophenol production becomes proportional to the rate of enzyme deacylation (turnover). For the wild type α–lytic protease, the deacylation rate is nearly zero, with the half-life of the complex being on the order of hours[6]. In addition to the biphasic enzymatic kinetics, diethyl *p*-nitrophenyl phosphate undergoes a continuous second order hydrolysis reaction with hydroxide, and as such, reaction profile plots of wild type α–LP display a steady linear increase in *p*-nitrophenol concentration, both prior to the addition of enzyme, and after the initial burst acylation of the enzyme (Figure 14).

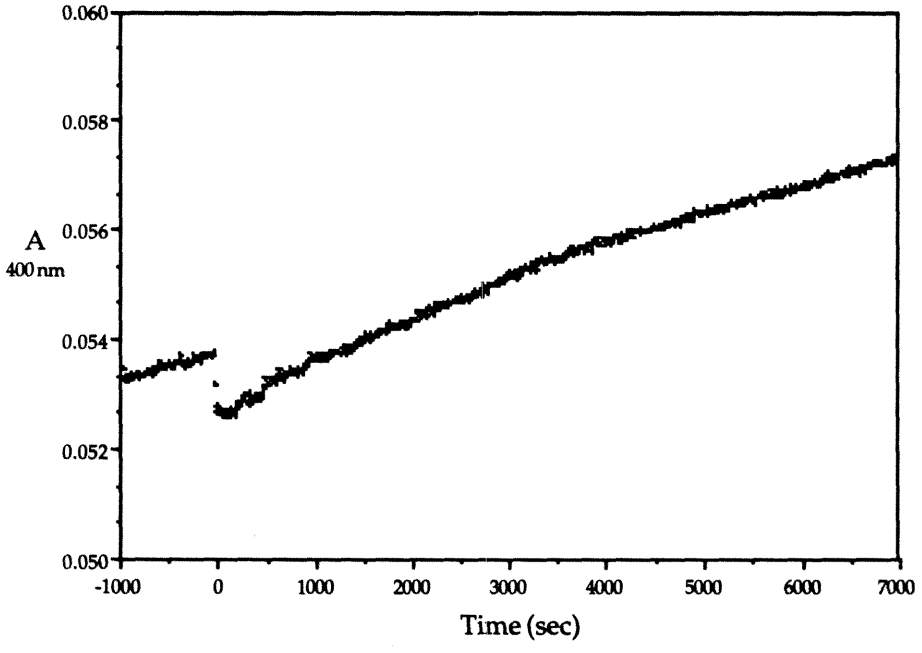

Figure 14. Typical reaction profile of α-lytic protease and diethyl *p*-nitrophenyl phosphate. E_o = ~2x10-6 M; S_o = 91 μM; 10% (v/v) acetonitrile, 50 mM Tris, pH 7.75, 25°C. The reaction rate can be seen to be non-linear upon addition of enzyme and return to the linear hydroxide mediated hydrolysis rate of the inhibitor after the added enzyme has been acylated. The absorbance decrease at time zero is a dilution effect observed upon addition of the enzyme.

If one assumes that the dissociation of the product from the serine 195 mutants is faster than the rate of hydrolysis of the substrate, then organophosphatase activity would be expected to be seen as an increase in the rate of *p*-nitrophenol production, without the presence of the initial biphasic burst, since the mutants would be incapable of forming a covalent complex with the substrate. A typical plot of the Ser 195 Ala mutant (Figure 15) does in fact demonstrate a general lack of the burst kinetic phase. However, linear regression analysis of the pre-enzymatic and enzymatic reactions displayed no significant increase in the rate of DNP hydrolysis, with the measured rates being within 10% of each other. Reaction profiles were determined on various concentrations of DNP and mutant enzyme and displayed similar plots. Many of the reactions displayed a slight change in hydrolysis rate upon addition of

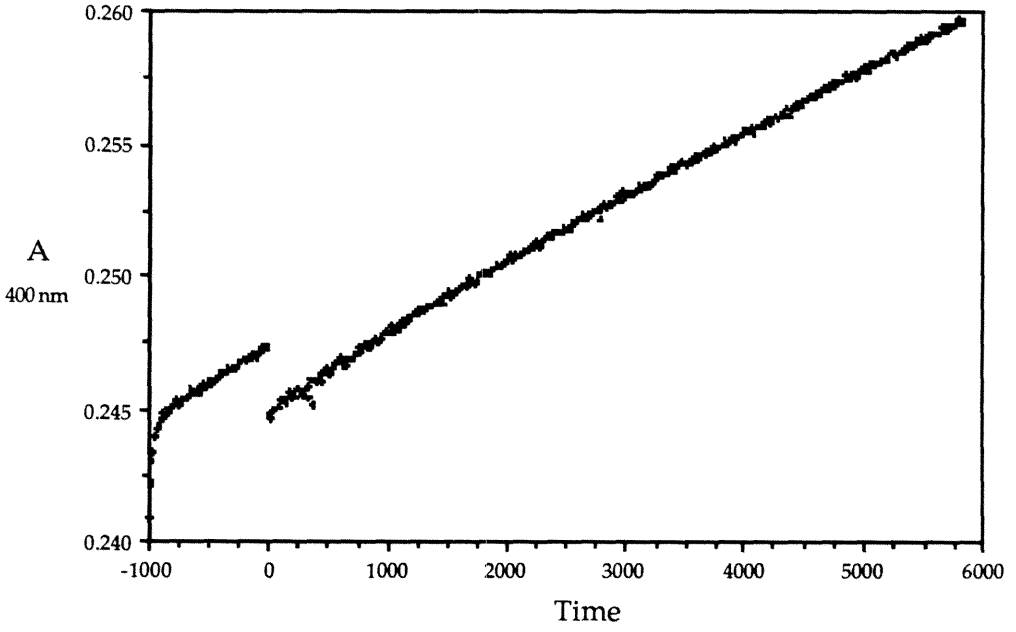

Figure 15. Typical reaction profile of the Ser 195 Ala mutant α-lytic protease and diethyl *p*-nitrophenyl phosphate. E_o = ~5x10-6 M; S_o = 430 μM; 10% (v/v) acetonitrile, 50 mM Tris, pH 7.75, 25° C. The reaction rate can be seen to maintain a nearly linear non-enzymatic hydrolysis rate of the substrate upon addition of the mutant enzyme. The slight non-linearity seen in the reaction profile is an artifact that does not vary with substrate or enzyme concentration.

enzyme, however this change was always less than a ~10% variation from the background rate and did not vary predictably with substrate or enzyme concentration, indicating that it was an artifact of the assay, possibly due to slow or incomplete mixing.

The lack of detection of organo-phosphatase activity in the alanine mutant is not entirely unexpected. Though the replacement of the active-site serine with an alanine would provide a sufficient volume in the active site to accommodate a water molecule for use as the "activated" nucleophile (Figure 16), the methyl side chain of the alanine might tend to exclude the highly polar water molecule from close association in the pocket[8]. Mutagenesis experiments on subtilisin, involving the substitution of the active-site serine 221 with an alanine[39], have demonstrated a measured decrease in the catalytic hydrolysis rate of peptide ester substrates on the order of 10^{-6}. An equivalent decrease in the rate of nucleophilic attack on the DNP substrate, with respect to wild type α–LP, would be below the detection limits of the experimental methods employed.

The Ser 195 Ala substitution of α–LP was chosen as the first attempt at producing phosphatase activity because it made the minimum perturbation in the enzyme, changing only a single atom. This was important since more radical changes might result in the inability of the enzyme to properly fold. However, it must be noted that the Ser 195 Gly mutant has the potential of expressing a greater organo-phosphatase activity than that detected for the alanine mutant. A Ser 195 Gly mutant not only provides plenty of space in the active site for a water molecule, but the lack of the aliphatic side chain would provide an environment substantially more hydrophilic than alanine, and as such, increases the likelihood of water binding into the catalytic pocket.

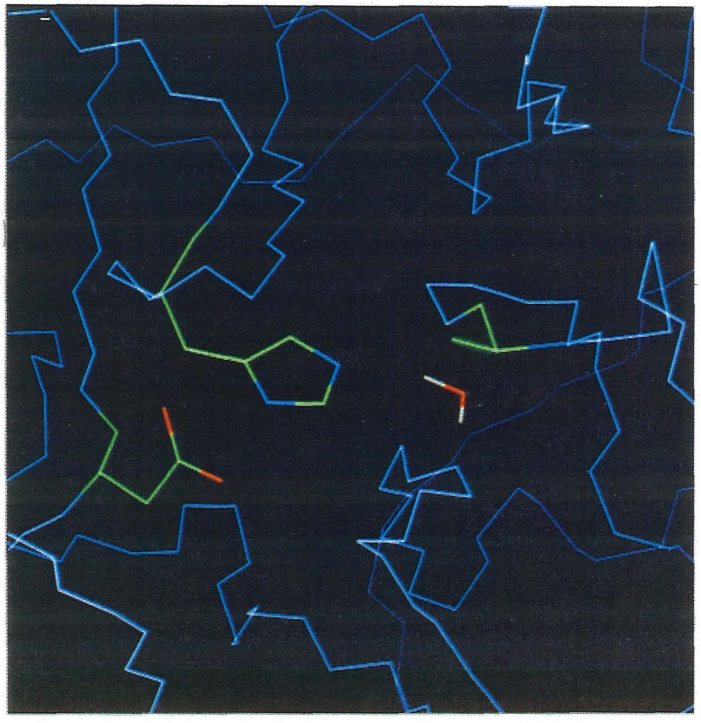

Figure 16. Active site of the Ser 195 Ala mutant α-lytic protease. A water molecule is shown occupying the volume freed by removal of the serine hydroxyl.

Conclusion

In order to further study the structure/function relationships of a protease system, I set out to construct an expression system for the synthetic α–lytic protease gene constructed in our laboratory. The cloning of the wild type α–lytic gene by another laboratory demonstrated the necessity of a 166 amino acid pro-region for the proper folding and activation of the protease. Since the mature form of α–LP was produced through the auto-catalytic cleavage of the pro-sequence from the protease domain of the enzyme, it was expected that the first structure/function mutants of interest, those involving active-site residues, might be unable to self-process and would thus be obtainable only in the pro-enzymatic form. This was indeed the case for the first mutant expressed from the synthetic gene, the Ser 195 Ala substitution.

The discovery that the pro-peptide of α–lytic protease was capable of assisting in the proper folding of the protease domain, when the two proteins were not covalently attached, presented an eloquent solution to the dilemma of expressing mature mutant α–LP enzymes. Using the synthetic α–lytic protease gene, the pro-coding region from the wild type gene, and the PhoA promoter and leader sequence used in the original wild type α–LP expression system, I constructed a "complementation" expression system. The system consisted of both a protease domain gene and a pro-coding region gene on two separate plasmids, each controlled by a PhoA promoter and containing a PhoA leader sequence, and produced, in the case of the wild type sequence, active α–lytic protease.

A mutant consisting of an alanine substitution at the active-site serine 195 position was produced using the complementation expression system. The mutant enzyme was shown to possess no measurable protease activity. The

mutant was also investigated for the ability to catalytically hydrolyze the organo-phosphate protease inhibitor diethyl *p*-nitrophenyl phosphate. We hypothesize that such activity could be expected to ensue from the steric and electrostatic enhancement of the nucleophilicity of a water molecule potentially bound in place of the missing serine hydroxyl. Though no such activity was detected, there is the possibility that the mutant may possess an activity below the detectable limits of the current assay. In addition, the somewhat hydrophobic side chain of the alanine may prevent a water from being so bound, and the more hydrophilic nature of a glycine substitution in the active site might be required for such a catalytic mechanism to proceed.

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CHAPTER 3

The Role of Serine 130 in RTEM-1 β-Lactamase Studied by Site-Saturation Mutagenesis

Introduction

Penicillin and the Penicillin Binding Proteins

In 1929, Alexander Fleming discovered the existence of a compound produced by certain fungi that inhibited the growth of bacteria in media on which the fungi were growing[1]. Penicillin and the other related cephem and penam antibiotics (Figure 1) have since been shown to block bacterial cell wall biosynthesis by inhibiting the crosslinking of cell wall peptidoglycans by the D-Ala-D-Ala carboxypeptidases and transferases[2]. These enzymes are responsible for the cleavage of the terminal D-alanine from the D-Ala-D-Ala moiety, through a nucleophilic attack of the active-site serine on the amide bond of the substrate. This results in the formation of an acyl-enzyme intermediate with the penultimate D-Ala residue. This intermediate is subsequently degraded, either through hydrolysis with water, or through attack by the terminal amino group of glycine from a crosslinking peptide on an adjacent peptidoglycan strand, resulting in the covalent attachment of the two strands.

It has been proposed by Tipper and Strominger that the β-lactam antibiotics structurally mimic the D-Ala-D-Ala dipeptide[3] and are thus able to function as substrates for the D-Ala-D-Ala carboxypeptidases and transferases (Figure 2). Once the substrate has bound to the active site, the enzyme rapidly cleaves the β-lactam ring forming the acyl-enzyme complex (Figure 3). However, unlike the D-Ala-D-Ala substrates, the rate of deacylation, via hydrolysis of the covalent bond, is greatly reduced, due in part to the attachment of the imino "leaving" group, through the "backside" of the β-lactam ring, to the remaining portion of the antibiotic, thus presumably blocking access of a water molecule to the acyl bond. With this covalently bound

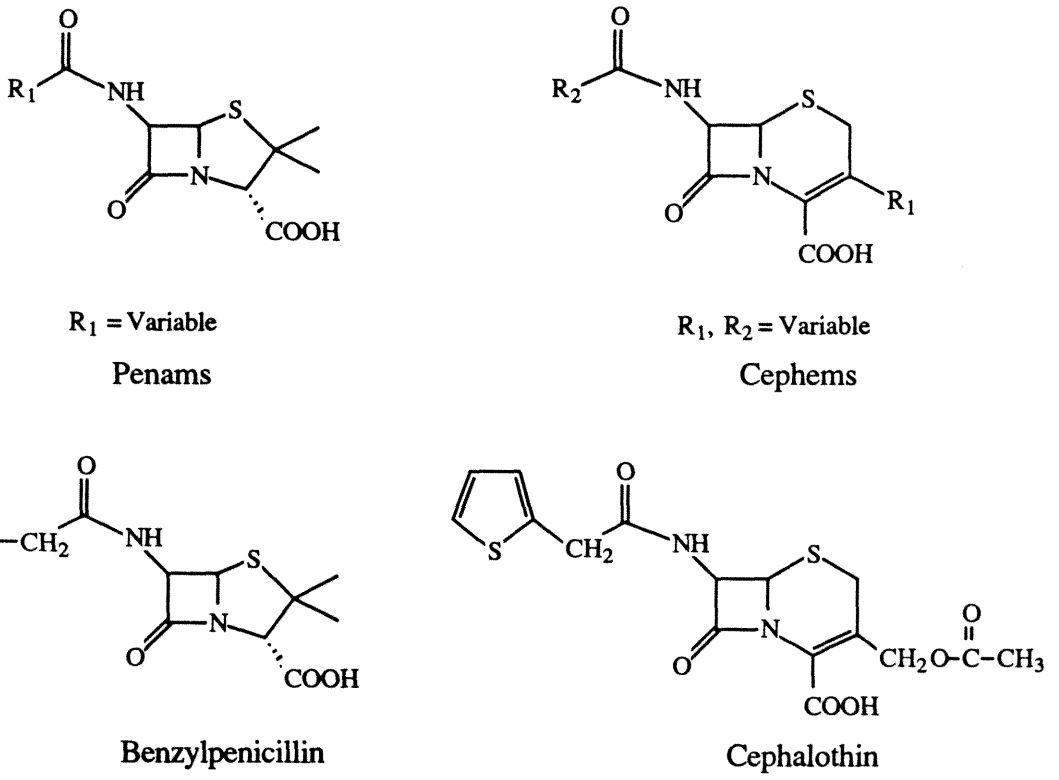

Figure 1. Structure of penam and cephem antibiotics.

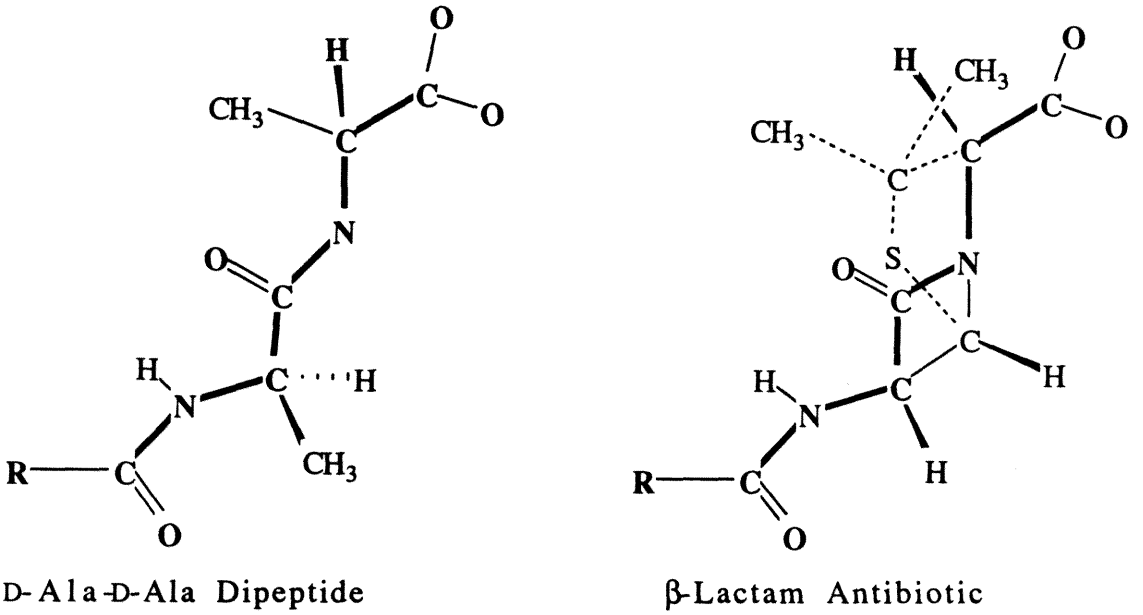

Figure 2. Structural comparison of the β-lactam antibiotics (left) and the D-Ala-D-Ala dipeptide (right), emphasizing the close analogy of the two molecules[3].

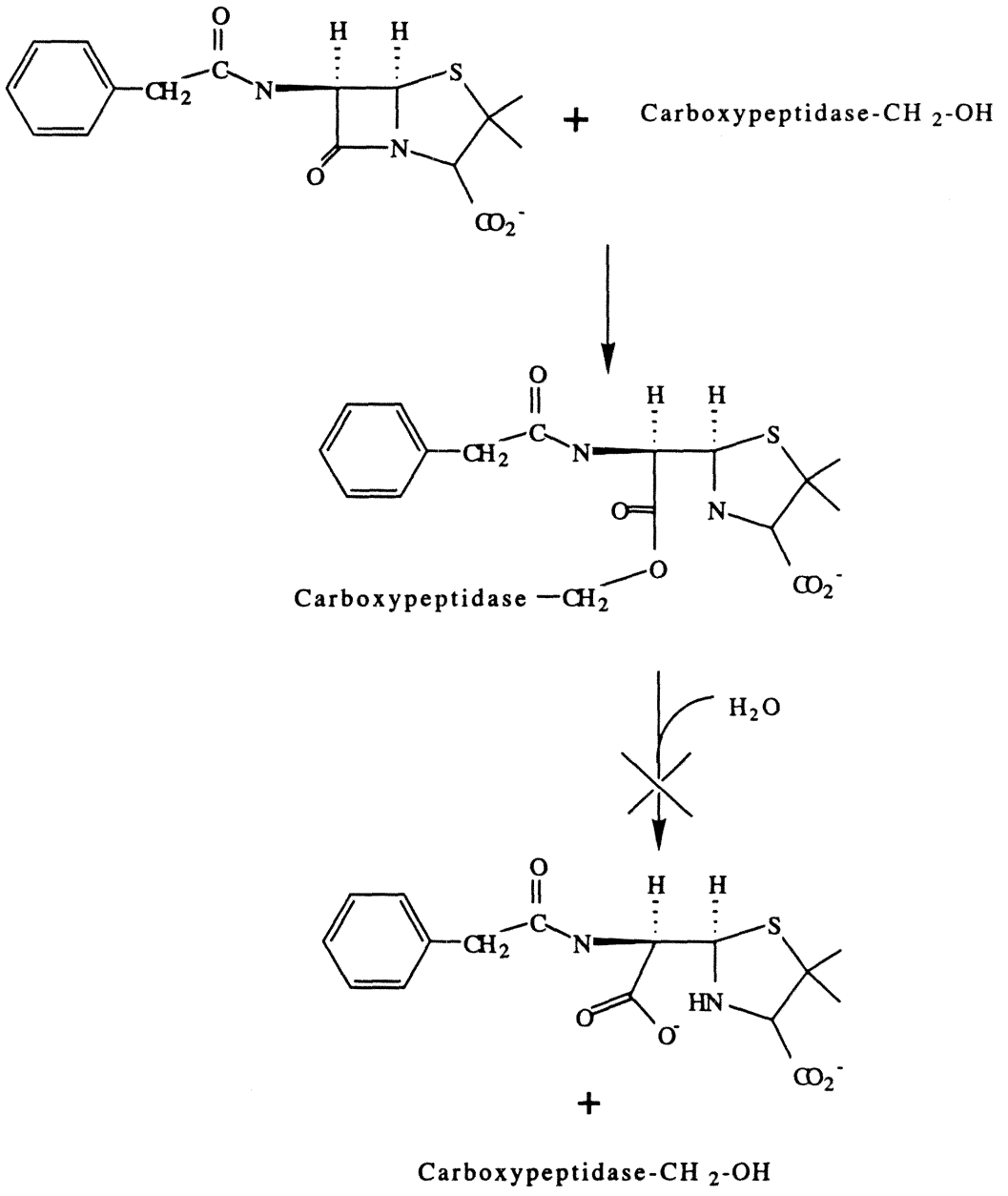

Figure 3. Schematic mechanism of β-lactam antibiotic inhibition of the carboxypeptidases and transpeptidases, by the blocking of the hydrolysis of the acyl-enzyme intermediate.

species occupying the active site, the enzyme is blocked, thus preventing the crosslinking of the cell wall peptidoglycans and resulting in the eventual lysis of the cell. These enzymes are classified as penicillin-binding proteins (PBPs) for their essentially irreversible binding of penicillin.

β-Lactamase

In 1940, Abraham and Chain discovered that extracts from certain bacteria would inactivate the antibiotic effect of penicillin[4]. These extracts were shown to contain a variety of enzymes capable of hydrolyzing the β-lactam ring of penicillin, yet were not inactivated in the process, as were the PBPs. Over 80 of these β-lactamases have since been discovered and have been classified into three groups based on sequence homology, size, substrate specificity, and on their requirement for metal ions to catalyze the lactam hydrolysis.

The class A β-lactamases all have a molecular weight around 30 kilodaltons and catalyze β-lactam cleavage through the use of a nucleophilic serine in the active site. This class includes the RTEM-1 β-lactamases of *E. coli, Staphylococcus aureus* PC1, *Bacillis licheniformis* 749/C, and *Bacillis cereus* 569/HI. The class A enzymes have a substrate preference for the penam antibiotics over the cephems. Sequence homologies between members of this class are considerable, ranging from 26 to 65%[5]. The class B β-lactamase consists of only two enzymes, from *B. cereus* and *Proteus maltophilia,* and differ considerably from the class A enzymes in molecular weight (approximately 23 kD) and in catalytic mechanism. Containing a metalloenzyme complex with either zinc(II) or cobalt(III) at the catalytic center, these enzymes appear to function as general base catalysts, with no indication of an acyl-enzyme intermediate formation during the hydrolysis[6]. The third β-lactamase class, the class C enzymes, consists of the largest enzymes, with molecular weights at ~40 kilodaltons, and include β-lactamases from *Citrobacter freundii* and *Enterobacterium cloacae* P99. The class C enzymes have been demonstrated to function through the use of a nucleophilic serine

in the active site[7]. Both the class B and C β-lactamases preferentially hydrolyze cephems.

Both the class A and class C β-lactamases attack the β-lactam of penam and cephem antibiotics through a nucelophilic serine, in a mechanism similar to that of the PBPs (Figure 4). However, unlike the PBPs, these β-lactamases are further capable of rapid hydrolysis of the acyl-enzyme complex. The similarities in the mechanisms of both penicillin binding, and formation of the enzyme-substrate complex, between the serine β-lactamases and the penicillin binding proteins, suggests a possible evolutionary relationship between these enzyme groups[8]. This relationship is strengthened by the regional sequence homologies and the highly similar gross tertiary structural elements of the three enzyme families (Figure 5). The number and relative positioning of secondary structural elements differ between the three families, yet certain elements remain constant. All three families display a two-domain structural motif, with one domain consisting solely of α-helices, and the second domain having a mixed structure, containing a minimum five-stranded β-sheet sandwiched between two sets of α-helices. The active site is defined by the interface between the two domains, bordered on one side by the β-7 strand and on the other by the α-2 and α-10 helices, with the active-site serine residing at the amino-terminal end of the α-2 helix, in the bottom of the active-site cleft.

A serine β-lactamase of particular interest is the RTEM-1 enzyme, a class A β-lactamase from *E. coli*[9]. This enzyme has been extensively studied, due to its availability as a resistance marker on the ubiquitous plasmid, pBR322, and its clinical relevance with regard to numerous pathogens. RTEM-1 is a soluble protein of 28,000 daltons. It is synthesized as a 286 amino acid preprotein, with a 23 residue leader sequence, which is cleaved off during

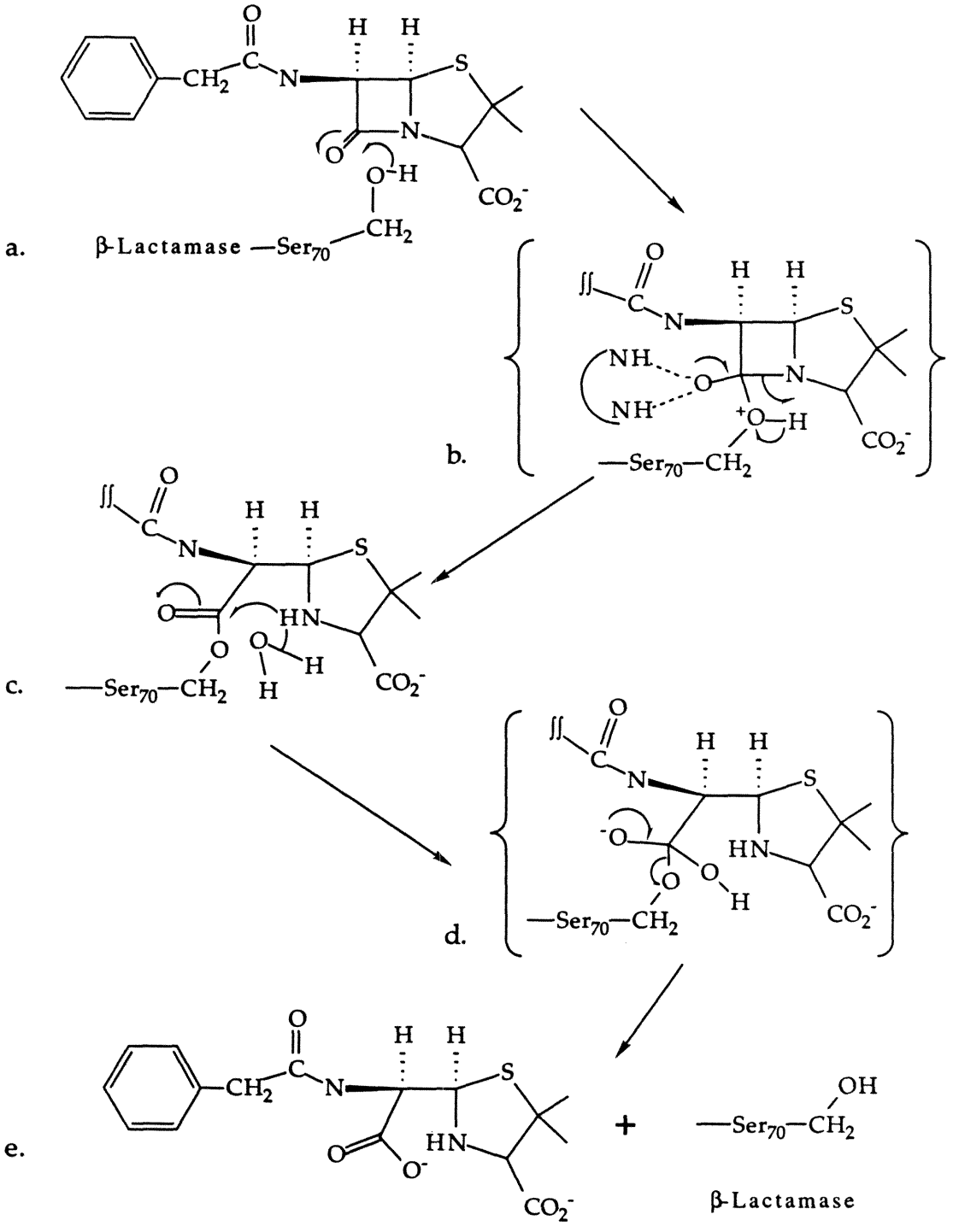

Figure 4. Mechanistic overview of β-lactam antibiotic hydrolysis by β-lactamase. The two tetrahedral transition states are indicated by braces.

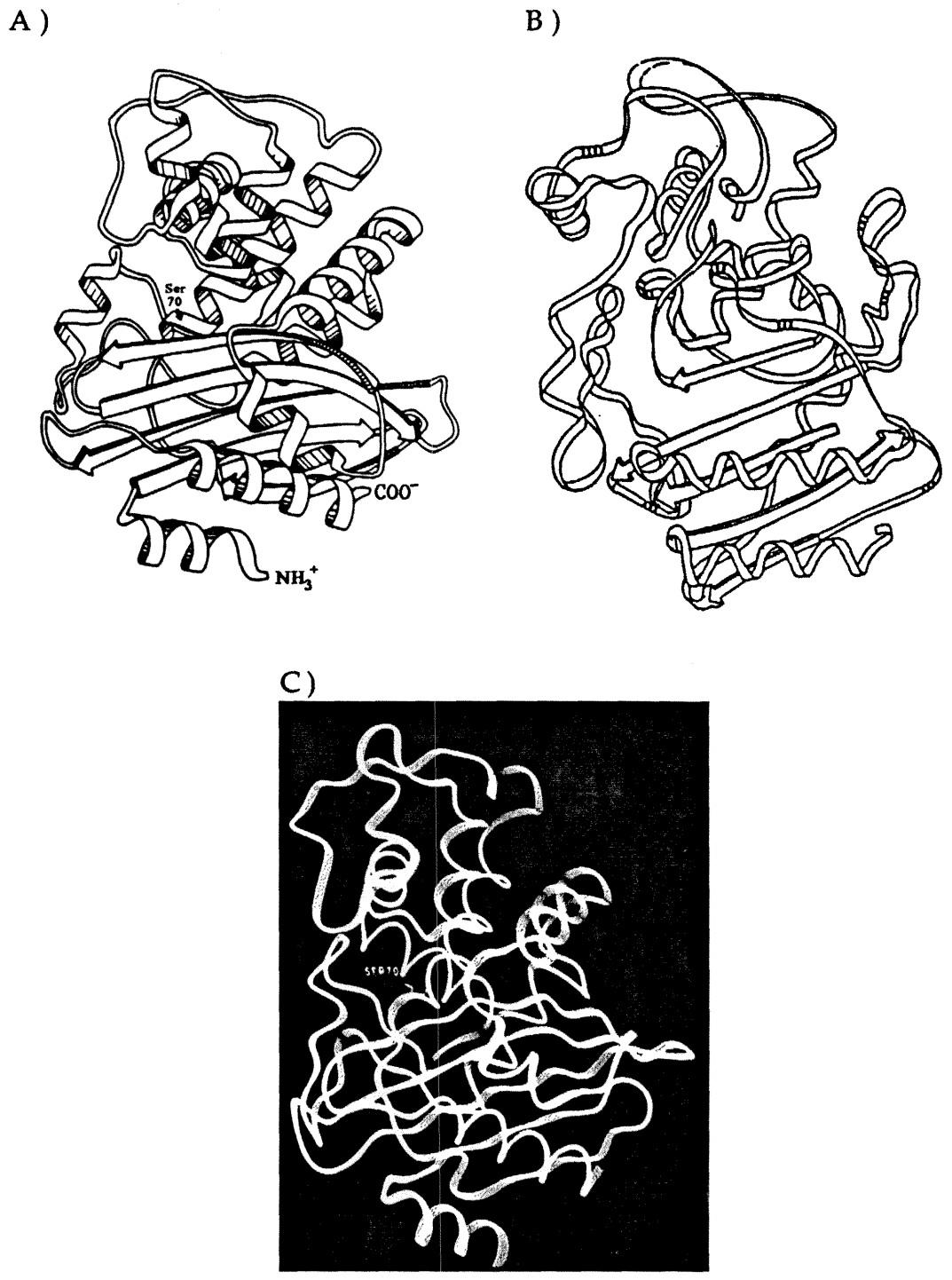

Figure 5. Three-dimensional structures of the class A β-lactamases from *B. licheniformis*[20], *S. aureus* PC1[21], and of carboxypeptidase R61 (Kelly, J. A. *et al.*, 1986, *Science*, 231, 1492). Examination of the structures reveals a strong conservation of secondary structure between the two enzyme classes.

export of the protein into the periplasm of the cell. A number of other RTEM-1 β-lactamases have been isolated and sequenced and differ from RTEM-1 in as little as a single amino acid change (Gln 39 to Lys in RTEM-2). (The numbering of Ambler is used[10].) The RTEM-1 enzyme has been used in the examination of protein secretion[11], expression of fusion proteins[12], and as a selectable marker[13]. The RTEM β-lactamases preferentially hydrolyzes the penam antibiotics at rates approaching evolutionary perfection. The apparent second-order rate constant, k_{cat}/K_M, is on the order of $10^8\ M^{-1}\ s^{-1}$, nearly the rate of a diffusion-controlled mechanism.

As stated earlier, the mechanism of β-lactam substrate hydrolysis by RTEM-1 involves the nucleophilic attack of the active-site serine 70 hydroxyl upon the β-lactam carbonyl group (Figure 4). The attack proceeds through a tetrahedral intermediate. This negatively charged intermediate is stabilized by the oxyanion hole, made up of the main chain amides of residues Ala 237 and the catalytic serine 70, similar to that observed in the serine proteases[14]. The resulting acyl-enzyme complex is then rapidly hydrolyzed by a water molecule, possibly bound in the active site, forming the inactive penicilloic acid.

Though the overall mechanism of β-lactamase activity is understood, the role of many specific residues has not been well characterized. A detailed understanding of the catalytic mechanism of these enzymes would not only further the understanding of the structure/function relationship of enzymatic processes, but would also aid in the rational design of new resistant antibiotics.

Mutagenic Function Studies

As was detailed in Chapter 1, residues conserved among the members of a enzymatic family are highly apt to play crucial roles in the functioning of the enzyme. There are 27 such invariant amino acids in the known class A β-lactamase sequences. Our laboratory has investigated the role of a number of these conserved residues through the use of cassette and site-directed mutagenisis. Research has investigated serine 70 residue through the generation of specific mutants[15], as well as residues at positions Thr 71, Lys 73, Ala 172 and Ala 237, through site-saturation studies[16,17,18,19].

The selection of these residues for study was based almost solely upon their conserved nature within the class A family, and not upon any structural information relating these residues to a catalytic function. In fact, one of the residues studied, alanine 172, proved to have no discernable catalytic function, with the majority of amino acid substitutions at this position producing the wild type activity. The recent publication of X-ray crystal structures for the class A β-lactamases from *B. licheniformis*[20] and from *S. aureus* PC1[21], at 2.0 Å and 2.5 Å resolutions respectively, have greatly aided in the selection of interesting residues for study. The selections of lysine 234, asparagine 132, and glutamate 166 residues as candidates for investigation, were based upon their three-dimensional structural positions, as well as on their invariance within the class A β-lactamases.

Similar analyses have implicated the conserved residue, serine 130, as possibly serving a significant role in the catalytic mechanism of the class A enzymes. In the *B. licheniformis* β-lactamase structure (Figure 6), this residue is shown to be positioned at the top of the substrate binding pocket, and has

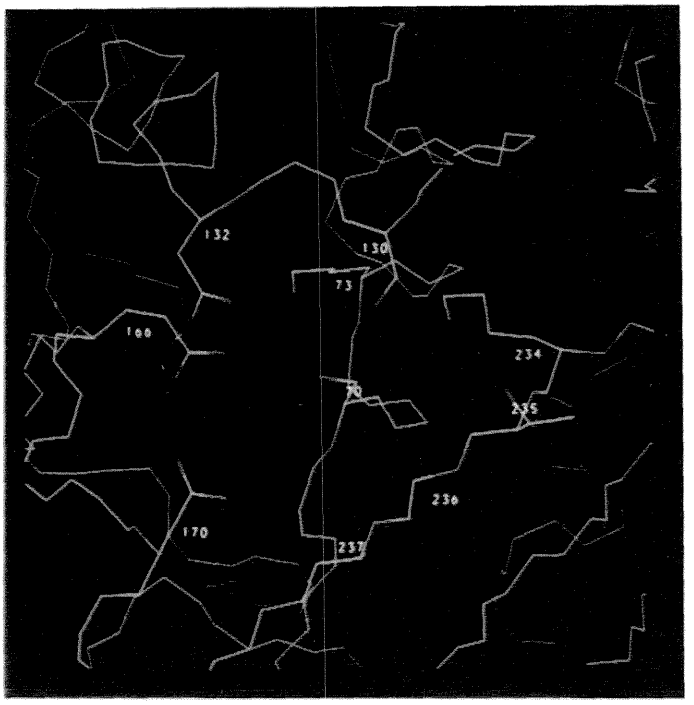

Figure 6. The active site of β-lactamase from *B. licheniformis*. The carbon backbone is portrayed in blue with conserved residues or those believed to be involved in the catalytic mechanism displayed in color. The serine 130 residue is shown at the center-top, pointing into the active site.

been proposed to hydrogen bond to, and orient, the ε-amino group of the conserved lysine 234 residue.

The role of lysine 234 has been investigated in our laboratory, by D. M. Long, through site saturation[22]. The determination of kinetic parameters for the phenotypically active mutants demonstrated the 234 residue to be critical in substrate binding, with all substitutions, aside from Lys 234 Arg, possessing a Michaelis constant, K_M, at least three orders of magnitude greater than that of the wild type. The catalytic rate constant, k_{cat}, exhibited little change for many of the mutants. These results are easily explained by the necessity of the 234 residue in the positioning of a positive charge into the catalytic pocket in order to form a salt bridge with the carboxylate group of the substrate. The serine 130 residue would thus be secondarily involved in the binding of substrate in the active site.

Because of the lack of a good structural model for the RTEM-1 enzyme, the substrate binding site of RTEM-1 had been modeled in our laboratory by using steric energy minimization techniques on the known α-carbon coordinates of the active-site residues from the PC-1 β-lactamase structure along with the residue side chains from the RTEM-1 enzyme[22]. In contrast to the purported role of serine 130 in substrate binding, as suggested by the *B. licheniformis* β-lactamase structure, these analyses predicted that the hydroxyl side chain of serine 130 points away from the active site of the enzyme, placing it too far away from lysine 234 for hydrogen bond formation.

In this chapter, I report on the investigation of the role of the serine 130 residue in β-lactam hydrolysis through the use of site-saturation mutagenesis. The kinetic parameters k_{cat} and K_M were determined for a number of phenotypically active mutants, and these are compared to the values obtained for β-lactamase with mutations at the Lys 234 residue.

Materials and Methods

Enzymes and Chemicals

Acrylamide and bis-acrylamide were purchased pre-mixed in 19:1 and 39.5:0.5 ratios, for protein and sequencing gels respectively, from Boehringer Mannheim Biochemicals (BMB). Nitrocellulose membranes were purchased from Schleicher & Schuell, Inc. NuSieve GTG Low Melting Point (LMP) agarose for DNA fragment isolation was supplied by FMC BioProducts. Molecular biology grade agarose, isopropyl-β-D-thioglactoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-glactoside (X-gal), and 10X ligase buffer were purchased from International Biotechnologies, Inc. Liquified phenol and chloroform were purchased from Fisher Scientific, Inc. Kanamycin sulfate was purchased from BMB; all other antibiotics were obtained from Sigma Chemical Company. Ultra-Pure urea and cesium chloride were purchased from Schwarz/Mann Biotech. Rabbit anti-serum to denatured wild type β-lactamase was previously prepared in our laboratory[23]. Restriction enzymes, T4 DNA ligase, DNase free RNase, calf intestinal alkaline phosphatase (CIAP), and T4 polynucleotide kinase were supplied by BMB. The water used was deionized and glass-distilled.

Bacterial Strains

Plasmid DNA were harbored in *Escherichia coli* strains XL1Blue^{tm} or D1210. Culture medium for plasmid preparations was L-broth (10 g tryptone, 5 g yeast extract, 10 g NaCl in 1 liter). L-agar plates were made with the addition of 15 g bacto-agar per liter.

DNA

Plasmid pJN, containing the IPTG inducible *tac* promoter controlling the RTEM-1 β-lactamase gene and the kanamycin resistance marker, was constructed by J. Nietzel in our laboratory[24]. Plasmid pJN-SAX was a modification of plasmid pJN by T. Richmond which added two restriction sites, Sac I and Sac II (Ksp I), at positions 4787 and 4751 respectively, to allow for the cassette replacement of residues 122 through 134[25].

Ethanol precipitations of DNA were performed by adding a 0.1 volume of 3 M sodium acetate and a 2.5 volume of absolute ethanol to the DNA solution, and precipitating for 15 minutes on dry ice. Larger volumes were precipitated using a 0.8 volume of *i*-propanol in place of absolute ethanol. The samples were centrifuged at 14,000 RPM for 15 minutes at 4°C. Pelleted DNA was then washed twice with 100 µl of 70% ethanol and dried *in vacuo*. The DNA was resuspended in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). All DNA concentrations were estimated from UV absorbance at 260 nm, and purity estimated from the A_{260}/A_{280} ratio.

Synthetic oligonucleotides were synthesized by the Caltech Microchemical Facility using phosphoramidite chemistry[26] on the Applied Biosystems automated DNA synthesizer, model 380A. Sixty nanomoles from a 0.2 µmole synthesis were purified through preparative polyacrylamide gel electrophoresis (20%, 0.1 X 20 X 40 cm gels; 500 V, 12 hours) in TBE buffer (89 mM boric acid, 89 mM Tris base, 0.2 mM EDTA). DNA was visualized through UV shadowing onto a fluorescent indicator TLC plate. Appropriate bands were excised from the gel, and the DNA extracted from the crushed gel into 1 ml of 2 M NaCl in TE overnight at 37°C. Samples were desalted using two sequen-

tial G-25 Sephadex spin columns (2.5 ml resin bed, equilibrated in TE; 2,500 RPM, 10 minutes).

Plasmid Preparations

Plasmid DNA was purified from *E. coli* cultures by alkaline lysis[27]. Mini-preps were performed by growing 2 ml of culture to saturation. The cells were pelleted in a microfuge at room temperature (RT) for five minutes. Cells were resuspended in 100 µl Solution I (50 mM Glucose, 10 mM EDTA, 1 mg/ml lysozyme, 25 mM Tris-HCl pH 8.0) and allowed to react at RT for five minutes. Five units of DNase free RNase was added at this step to remove excess RNA. 200 µl of freshly made Solution II (0.2 M NaOH, 1% SDS) was added, followed by incubation for five minutes at 0°C. The pH was neutralized with 150 µl of cold (4°C) Solution III (1% formic acid, 60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid in 100 ml) and the mixture centrifuged for 10 minutes at 4°C to pellet the cellular debris. The supernatant was removed and extracted twice with 100 µl of a 1:1 ratio of TE saturated phenol and chloroform. The DNA was then precipitated with ethanol. Large-scale preparations were further purified by ultracentrifugation in cesium chloride (1 g/ml) gradients, containing 50 µg/ml ethidium bromide at 45,000 RPM for 20 hours[28].

Transformations and Plating

Cells were made competent for transformation by plasmid DNA through an adapted Hanahan procedure[29]. *E. coli* cells were grown at 37°C to an OD_{550} of ~0.3 in 50 ml SOB media (20 g tryptone, 5 g yeast extract, 0.6 g NaCl, 0.2 g KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ in 1 liter). Two tubes of cell culture (25 ml) were placed on ice for 15 minutes and then centrifuged at 4°C for five minutes at 4000 RPM. The pellets were drained, resuspended in 8 ml transforma-

tion buffer I (12 g RbCl, 9.9 g $MnCl_2 \cdot H_2O$, 1.5 g $CaCl_2 \cdot H_2O$, 150 g glycerol, 30 ml 1 M KOAc pH 7.5 in 1 liter; adjusted to pH 5.8 with 0.2 M acetic acid), and allowed to incubate on ice for 15 minutes. Cell suspensions were centrifuged (4°C, 4000 RPM, five minutes), drained, and resuspended 2 ml in transformation buffer II (1.2 g RbCl 11 g $CaCl_2 \cdot H_2O$, 150 g glycerol, 20 ml 0.5 M MOPS pH 6.8 in one liter). Competent cells were transformed within two hours of preparation or frozen in liquid nitrogen and stored at -70°C. Frozen cells were used after thawing on ice for 10 minutes.

Transformations were performed by gently mixing plasmid DNA (5 to 25 ng) with 300 µl competent cells in a 1.5 ml eppendorf tube. Samples were incubated on ice for 40 minutes, followed by heat shock exposure at 42°C for 90 seconds. Cells were then incubated on ice for an additional five minutes before the addition of 800 µl SOC media (SOB media with an additional 10 mM of glucose) and incubating tubes for one hour at 37°C. Tubes were gently centrifuged (~1000 RPM for 30 seconds), and approximately 900 µl of the supernatant discarded. Pellets were then resuspended in the remaining supernatant and plated onto L-agar containing appropriate antibiotics. Plates were incubated at 37°C overnight.

Kinasing and Annealing Synthetic Oligonucleotides

To 12 pmoles each of the purified complementary oligonucleotides were added 2.5 µl 10X kinase buffer (100 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$), 2.5 µl 100 mM ATP, 1.8 µl 100 mM dithiotheritol (DTT), and 2-5 units of T4 polynucleotide kinase in a total volume of 25 µl. The reactions were incubated for 45 minutes at 37°C. The kinase was denatured by placing the samples in boiling water for five minutes. Typically, two liters of boiling water were used, and

the kinased oligonucleotides were left to equilibrate gradually to RT in the large vessel, allowing the complementary oligonucleotides to anneal.

Restriction Digest and Ligation

Restriction digests were typically performed on 5 µg of plasmid DNA, using 4-12 units of restriction enzyme in a reaction volume of 15 µl, incubated at 37°C for one to two hours.

Terminal phosphates were removed from whole restriction digests by adding 5-20 µl of calf intestine alkaline phosphatase diluted to 0.1 units per ml in CIAP dilution buffer (50 mM Tris-HCl pH 8.0, 0.1 mM EDTA) to DNA samples and incubating at 37°C for one hour. Samples were then incubated for 30 minutes at 75°C, twice phenol/chloroform extracted, followed by precipitation with ethanol. Typically, DNA pellets were redissolved in 25 µl TE and used in ligation reactions directly, without further isolation.

The annealed synthetic cassette (0.8 pmoles) was added to ~0.08 pmoles of the phosphatased pJN-SAX digest, along with 2.5 µl of 10X ligation buffer and 2 units of T4 DNA ligase. The final volume was brought to 25 µl. Control reactions were performed with the absence of the cassette DNA to monitor the reformation of the parent plasmid. The ligation reactions were carried at 16°C for 16 hours, followed by transformation of competent *E. coli* D1210 cells with 0.5 µl of the reaction mixtures. Cells were screened for the insert by plating onto kanamycin (25 mg/l) L-agar plates.

Plasmid Sequencing

Plasmid DNA was sequenced by the dideoxy method[30] for denatured, double-stranded DNA[31]. The sequencing primers were 17-18 bases in length and bound approximately 100 bases upstream of the region to be sequenced. Plasmid DNA (5 µg) was mixed with 25 pmoles of sequencing primer in total

volume of 10 µl of TE. The plasmid was denatured and the primer annealed to the DNA by boiling for five minutes, followed by flash freezing in a dry ice/ethanol bath. Chain-extension reactions were performed with slight variations on the protocol described in the Sequenase^{tm} kit from United States Biochemical[32]. The annealed primer-DNA complex was incubated with 0.5 µl labeling mix, 2 µl 100 mM DTT, 2 µl Sequenase reaction buffer, 1 µl [α-^{35}S]-dATP (1000 Ci/mmole), and 2 µl of a one-eighth dilution of Sequenase enzyme, for three minutes at RT. The reaction mixture was then partitioned (3 µl) into each of the four dideoxynucleotide termination mixes (2.5 µl), mixed, and incubated for 10 minutes at 37°C. Reactions were stopped by the addition of 4 µl of Maxam-Gilbert loading buffer[33], and incubated at 100°C for two to five minutes. Samples were loaded onto 5-8% polyacrylamide/TBE gels, run at 50 mA for three hours, and autoradiographed 12-48 hours using Kodak XAR film. Figure 7 displays a typical sequence.

Protein Gel Electrophoresis and Western Staining

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots were performed to determine presence and purity of β-lactamase in samples. Protein samples (5-20 µl) containing between 0.1 µg and 10 µg of protein were added to an equal volume of loading buffer (10% v/v glycerol, 5% v/v 2-mercaptoethanol, 3% w/v SDS, 62.5 mM Tris-HCl pH 6.8, 0.05% w/v bromophenol blue) and boiled for five minutes. For cell samples; 100 µl of cell cultures were centrifuged, the supernatant removed, and the cells resuspended in 30 µl of loading buffer and boiled for five minutes. Samples were loaded on a 15 cm 12-15% acrylamide gel with a 4% stack. Gels were run at a constant current of 10 mA for ~12 hours in Tris-glycine SDS buffer (12 g Tris base, 57.6 g glycine, 3.2 g SDS in 4 liters). Bands were visualized in the acrylamide gel by

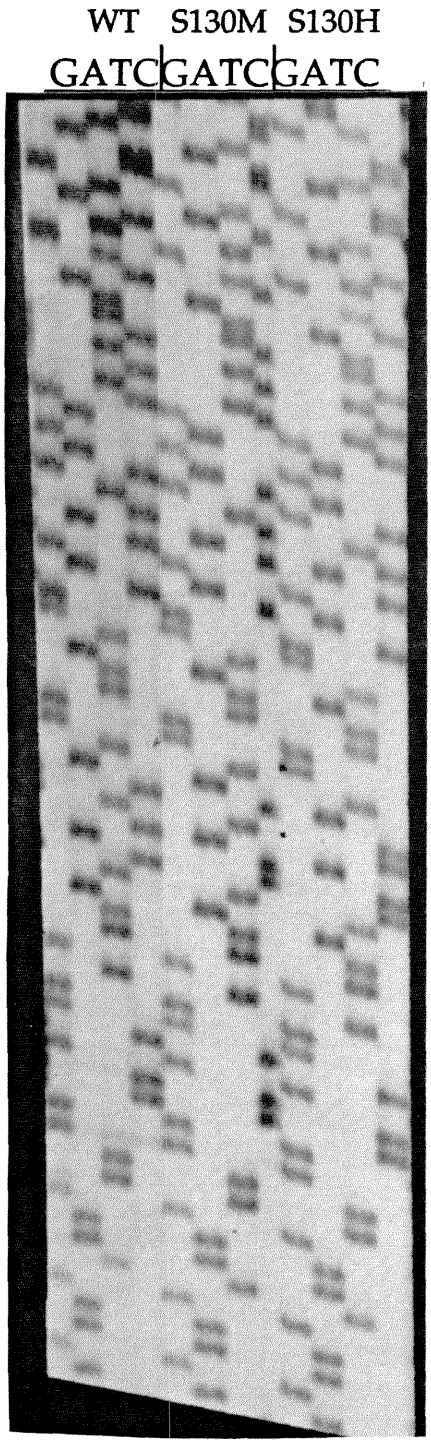

Figure 7. A typical sequencing gel for mutants at position 130. Double-stranded dideoxy sequencing was used, labelling with [α-^{35}S]-dATP.

Brilliant Blue G colloidal staining[34]. For Western blots, protein bands were transferred from the acrylamide gel onto a nitrocellulose membrane, using an electroblotting cell in a modified Tris-glycine buffer (without SDS and containing a 20% v/v of methanol) at a constant current of 100 mA for one to two hours. β-Lactamase was visualized by binding of rabbit anti-β-lactamase antiserum, which is then stained using the Vectastain^{tm} ABC immuno-peroxidase system[35].

Phenotypic Assays

Phenotypic screening of cells harboring mutant β-lactamase were performed in two separate procedures. Cell lines were grown to saturation overnight in LB media. Ten microliters of saturated media was diluted into 1 ml of media and 100 µl of the diluted stock was plated onto L-agar plates containing various concentrations of antibiotic. Plates were incubated overnight at 37° C. The antibiotic resistance conferred by the mutant β-lactamase was determined by the amount of growth observed.

Antibiotic disk susceptibility assays were performed on Mueller-Hinton media agar plates[36]. For each mutant, saturated cells were streaked onto the surface of a plate, using a sterile cotton swab, so as to produce an even lawn of cell growth. Sterile cotton disks (7 mm) containing measured amounts of antibiotic were carefully placed onto the surface of the plate, in a pattern that maximizes the distance between any two disks. Up to seven disks, each with a different antibiotic or antibiotic concentration, could be placed onto a 100-mm agar plate. Antibiotic disks were either purchased from Difco or were prepared by aliquoting fixed amounts of antibiotic solutions onto sterile disk blanks and allowing them to dry within a sterile hood. Plates were incubated

overnight at 37° C. Zones of non-growth around each disk were measured to assess susceptibility of each mutant to different antibiotics.

Protein Expression

β-Lactamases with mutations at residue 130 were expressed from the pJN-SAX plasmid, utilizing the IPTG inducible *tac* promoter. *E. coli* D1210 cells harboring the mutant pJN-SAX vectors were grown in L-broth, containing 50 μg/ml kanamycin, at 37° C until saturated. Typical growth volume was one liter. IPTG was added to a final concentration of 100 mM, and growth allowed to continue for 45 minutes at 37° C.

β-Lactamase, located in the periplasm of the cell, was released using osmotic extrusion. The media was centrifuged at 10,000 RPM for 10 minutes to pellet the cells and the supernatant discarded. The pelleted cells were resuspended in sucrose solution (1.25 M sucrose, 1 mM EDTA, 25 mM Tris-HCl, pH 7.0), 20 ml per 250 ml of original media, and shaken at room temperature for 30 minutes. Samples were centrifuged for 30 minutes at 10,000 RPM, the supernatant discarded, and the pellets resuspended in an equal volume of cold H_2O (4° C) and shaken for 30 minutes at 4° C. For the remainder of the purification, all protein samples were maintained at 4° C. The samples were once again centrifuged at 10,000 RPM for 30 minutes. The supernatant, containing the periplasmic proteins, was retained. Supernatants from the same cell growths were pooled and the sample volumes were reduced to ~5 ml using Diaflow cells with 10,000 molecular weight cut-off (MWCO) membranes, under a nitrogen pressure of 55 psi. Any contaminating cellular debris was then removed from the samples by filtering through Scheicher & Schuell 0.22 μm Uniflow filters.

β-Lactamase was purified from the concentrated periplasmic samples through fast protein liquid chromatography (FPLC) on a MonoQ^{tm} anion exchange column (resin bed of 10 mm diameter x 10 cm) from LKB-Pharmacia. The column was equilibrated in solution A (25 mM triethanolamine (TEA), pH 7.65). All of the crude protein sample was loaded onto the column at a flow rate of 2.5 ml/min, using a 10 ml SuperLoop^{tm} for the injection loop. Proteins were eluted from the column with a salt gradient using 1 M NaCl in 25 mM TEA, pH 7.65 (solution B). The gradient profile used was: five minutes, 0% B; 40 minutes, 0% to 12% B; 15 minutes, 12% to 100% B; 10 minutes, 100% B; 10 minutes, 0% B. Protein elution was monitored through the eluent absorbance at 280 nm; the presence of β-lactamase in individual fractions was confirmed with SDS-PAGE and Western blot analysis. Peak fractions were pooled and dialyzed against 100 mM potassium phosphate, pH 7.0 to a dilution of 1:10^6. Protein concentrations were determined from the OD_{281} using an extinction coefficient[37] of 29,400 M^{-1} cm^{-1}.

Kinetics

Enzymatic reaction rates were determined by monitoring the decrease in substrate concentration through the measurement of the circular dichroism (CD) generated by the chiral ring structure near the β-lactam bond in the substrate[22]. The benzylpenicillin substrate possess a molar ellipticity, [Θ], at 231.8 nm of 393 degree M^{-1} cm^{-1}. The reaction product, penicilloic acid, has no CD signal at this wavelength, providing a means of directly measuring the concentration of the substrate in solution, as well as a maximal $\Delta[\Theta]_{231.8}$ and therefore sensitivity for the reaction. In addition, the ellipticity of the enzyme

at 231.8 nm is small, allowing large amounts of the enzyme to be used in detecting low levels of activity.

Kinetics were measured on a Jasco J600 spectropolarimeter, equipped with a 1-cm water-jacketed cell and a Lauda K-2/R temperature-controlled circulating water bath; the temperature could be maintained to ±0.2°C. Measurement parameters for assaying β-lactamase activity on benzylpenicillin were previously determined by D. M. Long[38]. The spectropolarimeter was configured with a 1500 μm slitwidth, a 0.5 second integration time, and a one-second sampling rate. Reactions were performed at 30°C with all reaction components, except the enzyme, pre-equilibrated to 30°C. A known amount of β-lactamase was added to a 2 ml sample of benzylpenicillin in 100 mM potassium phosphate, pH 7.0, thoroughly mixed, and transferred to the water jacketed-cell, all within a period of 15 seconds. The circular dichroism of the solution was monitored at 231.8 nm for a period of two to 10 minutes. A number of reactions were run for each mutant enzyme, with a range of substrate concentrations selected to span both sides of the K_M value of the mutant. Initial rates were measured from reaction profiles using linear regression analysis. Kinetic parameters were determined from Hanes-plots for each mutant[39].

Enzymatic pH profiles were created using data from initial rate assays in potassium phosphate at pHs of 6.0, 6.5, 7.0, 7.5, and 8.0 at a fixed benzylpenicillin concentration of 100 μM.

Product Inhibition Studies

Penicilloic acid for use in product inhibition studies was produced through the treatment of benzylpenicillin with commercially purchased Penicillinase (Sigma Chemical Company). To 10 ml of a 28 mM solution of

benzylpenicillin was added 10 units (1 unit is defined as the quantity of enzyme that hydrolyzes 1 μmole of substrate per minute) of Penicillinase, and the reaction mixture allowed to incubate for 30 minutes. An additional 10 units of enzyme was added, and the solution incubated for another 30 minutes. The reaction was assayed for the presence of any remaining benzylpenicillin using CD. The Penicillinase was removed from the solution by Ultrafiltration through a 5 kD MWCO filter. The product was collected in the effluent; the enzyme was retained by the filter. The final penicilloic acid (PA) solution was assayed for any remaining Penicillinase activity by monitoring a reaction of 100 mM benzylpenicillin with 10% v/v of the PA solution in the spectropolarimeter for one hour. The rate of penicillin hydrolysis with the PA solution was within 1% of the self-hydrolysis rate of benzylpenicillin.

Product inhibition was assessed through initial rate reactions with the addition of varying concentrations of penicilloic acid. β-Lactamase activity against 100 μM benzylpenicillin was assayed in the presence of 0, 50, 100, or 200 μM concentrations of penicilloic acid under conditions described above.

Results

Construction and Expression of Serine 130 Mutants

The site-saturation of the Ser 130 residue of RTEM-1 was performed through a cassette replacement using the engineered restriction sites Sac I and Ksp I in the plasmid pJN-SAX (Figure 8). Cassette oligonucleotides were constructed with base-pair degeneracies of four-fold, four-fold, and two-fold, respectively, at the three base positions of the Ser 130 codon. This degeneracy results in 32 possible codons, coding for all 20 amino acids and the amber termination codon. The particular mutation expressed from a clonal line is completely random, and in order to obtain all 21 possible codings, with a 95% confidence factor, 120 separate transformants needed to be picked and sequenced[40].

A simple two-fragment ligation was performed using a dephosphorylated, Sac I/Ksp I digested pJN-SAX vector with the kinased synthetic cassette. The removal of the terminal phosphate groups from the digested pJN-SAX vector allowed the ligation to be performed without purification of the digest fragments, by preventing the parent vector from religating. One hundred and twenty kanamycin-resistant transformants were picked onto master plates and sequenced by means of double-stranded, dideoxy sequencing. Eighteen amino acid substitutions, including the wild type and the amber stop codon, were found in the 120 plasmids sequenced. Mutants with isoluecine and phenylalanine at position 130, however, were missing from this set. In addition to the two missing residues, only a single colony carrying the cysteine 130 substitution was found, the sequencing of which displayed a high amount of background, indicative of a mixed colony contamination.

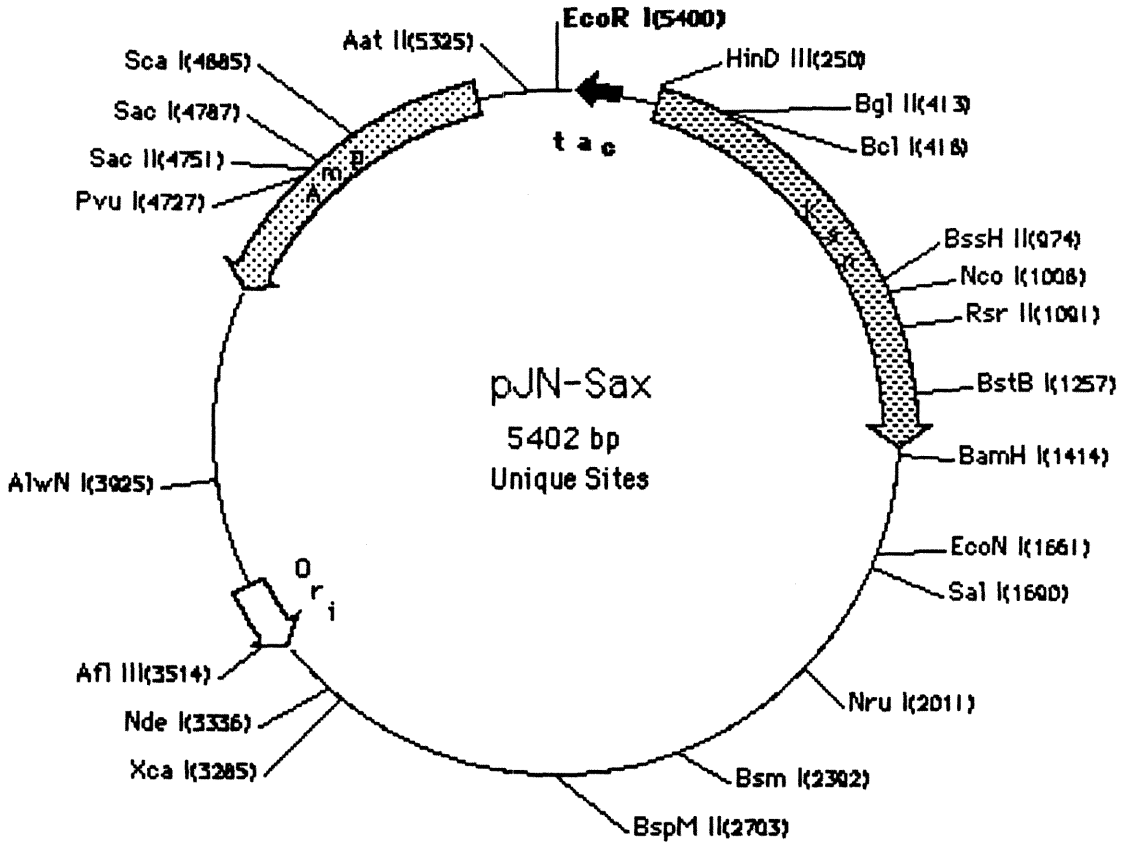

Figure 8. Restriction map of pJN-SAX expression plasmid for RTEM-1 β-lactamase. The two constructed restriction sites, Sac I and Sac II, are shown in the middle of the gene for RTEM-1.

In order to obtain mutants with the three missing or ambiguous substitutions, a second cassette was constructed, containing the degenerate sequence, (T/A),(T/G),T, at codon position 130. This four-fold degeneracy codes for the amino acids Phe, Ile, Cys, and Ser. Kanamycin-resistant transformants were once again sequenced, and the three missing amino acids were isolated from the first 16 colonies picked.

Characterization and Purification of Mutants

Phenotypic analyses were performed on *E. coli* cells harboring each of the 20 different mutant β-lactamase coding vectors, as well as control cell lines both expressing the wild type enzyme, and without the RTEM-1 gene entirely.

Antibiotic disk susceptibility assays were used to measure the resistance of each mutant to five different β-lactam antibiotics: ampicillin, benzylpenicillin, cephalothin, cefoxitin, and cefotaxime. Agar plates were spread with log-phase growths of cells harboring each of the mutant β-lactamase vectors, so as to produce an even lawn. Disks containing fixed amounts of the various antibiotics were set onto the agar, allowing the antibiotics to diffuse into the media. The resistance of the cell lines towards each antibiotic, and thus the activity of the mutant β-lactamase, was assessed by measuring the diameter of the halo of non-growth around each disk. "Resistance" was defined as the inverse of the measured diameter normalized to the resistance of cells harboring the wild type enzyme against ampicillin.

Figure 9 is a bar graph of phenotypic activity for each mutant for four of the antibiotics. (All mutants were equally susceptible to cefotaxime.) Three mutants, glycine, asparagine, and threonine, displayed noticeable resistance to the two penam antibiotics tested. In addition, the Ser 130 Thr mutant displayed a resistance to the cephem antibiotic, cephalothin, equivalent to that of the wild type and substantially higher than either of the other two active mutants. This is of particular note, because the phenotypic activity of the Thr mutant is the lowest of the three active Ser 130 substituted β-lactamases. All mutants were equally ineffective at providing antibiotic resistance against the two later generation cephem antibiotics.

The phenotypic activities of the Ser 130 mutants were further defined by plating cells onto L-agar containing a range of ampicillin or cephalothin concentrations. Table 1 shows the results of the phenotypic plate assay for concentrations of ampicillin from 10 μg/ml to 400 μg/ml. This data shows the same three mutants displaying significant β-lactamase activity, with the glycine mutant conferring ampicillin resistance up to 400 μg/ml and the

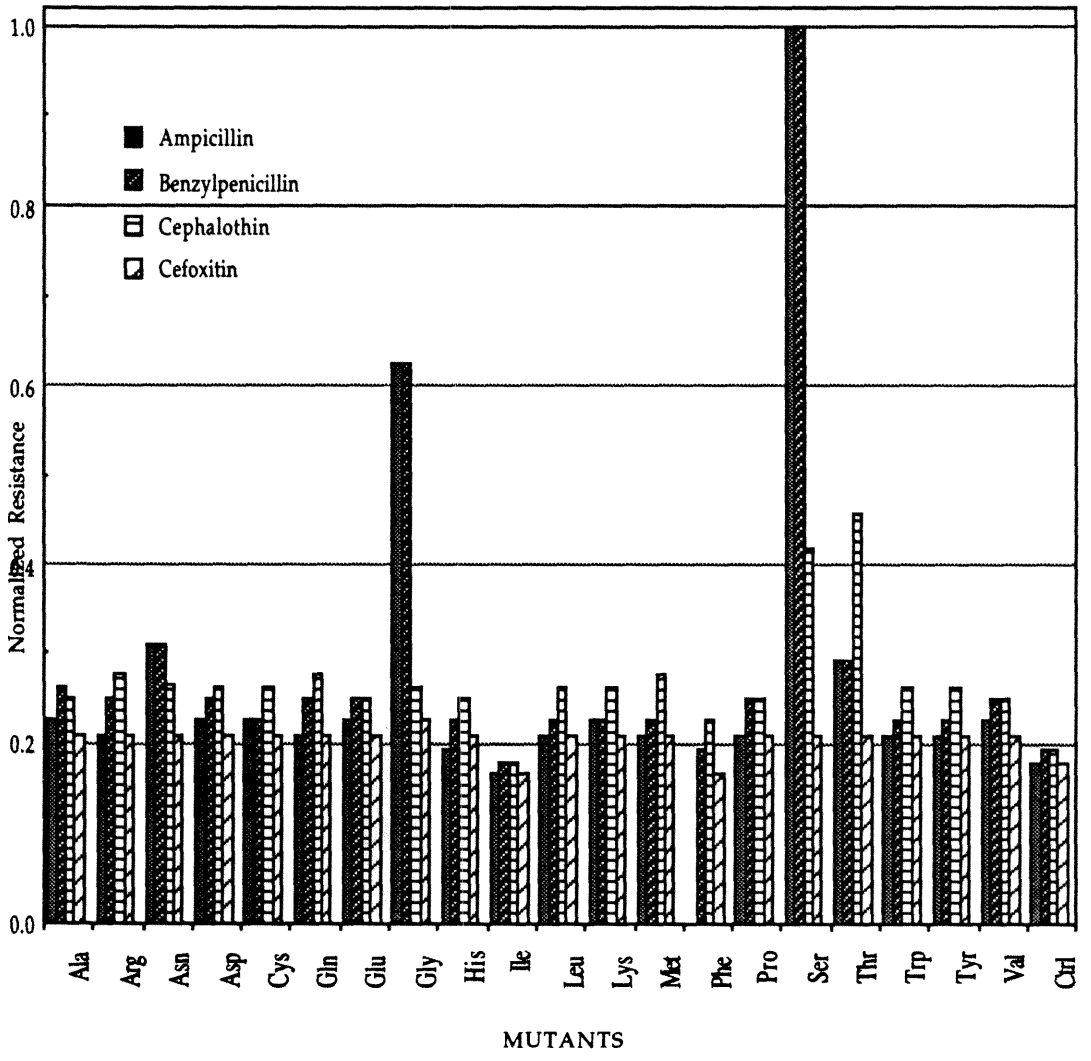

Figure 9. Bar chart of phenotypic resistance of the serine 130 mutants of RTEM-1 β-lactamase on the four antibiotics, ampicillin, benzylpenicillin, cephalothin, and cefoxitin. Resistance was calculated as the inverse of susceptibility measured from the antibiotic disk susceptibility assay, normalized to the susceptibility of the wild type enzyme.

Amino Acid	Benzylpenicillin (mg/l)	Cephalothin (mg/l)
Serine (WT)	>1000	25
Alanine	10	0
Arginine	0	0
Asparagine	75	10
Aspartate	0	0
Cysteine	10	0
Glutamate	0	0
Glutamine	50	0
Glycine	400	10
Histidine	0	0
Isolucine	0	0
Leucine	0	10
Lysine	0	0
Methionine	0	0
Phenylalanine	0	0
Proline	0	0
Threonine	75	50
Tryptophan	10	25
Tyrosine	0	0
Valine	0	0
Control (Minus RTEM-1)	0	0

Table 1. Plating assay maximum resistance of serine 130 mutants to the antibiotics ampicillin and cephalothin.

threonine and asparagine mutants both displaying growth up to 75 μg/ml. The plating assay additionally detected a fourth mutant with noticeable activity. Cells harboring the Ser 130 Gln mutant displayed ampicillin resistance to a concentration of 50 μg/ml. A few of the other mutants displayed scattered small amounts of colony growth, particularly at the lower

antibiotic concentrations. The numbers of colonies were far below that of any of the active mutants, generally less than 10 colonies, and these growths were concluded to be artifacts.

The results of the phenotypic plating assay for cephalothin resistance are also shown in Table 1. A number of the mutants, including two of the mutants demonstrated active on ampicillin, show a small amount of growth on the lowest concentration of 10 μg/ml cephalothin, but not on any higher concentrations. Since these growths were only a small number of colonies, and tended to be clumped together on the plate, they once again were considered to be artifacts. However, two other mutants, Ser 130 Val and Ser 130 Thr, displayed noticeable cephalothin resistance. Of particular note, the threonine mutant conferred resistance to cephalothin concentrations as high as those of the wild type RTEM-1 enzyme (25 μg/ml), and possibly as high as 50 μg/ml. (With only 10 colonies on the 50 μg/ml cephalothin plate, the possibility of this growth being artifactual was not ruled out.)

The proper folding and thermal stability of mutant β-lactamases were assayed through SDS-PAGE and Western blot analysis. Figure 10 shows Western blots, using anti-β-lactamase antisera, for the 20 Ser 130 substitutions, along with controls of wild type β-lactamase and *E. coli* D1210 cells. All but four mutants (Lys, Leu, Pro, and Tyr) were shown to be stable at 37°C, and produced in amounts on the order of that of the wild type. Cells harboring all the mutant vectors were regrown at 30°C, and Western blots again performed, with the same results (not shown).

The four active mutants (Gly, Thr, Asn, and Gln) were selected for kinetic study. These mutants, along with wild type β-lactamase, were grown up in 500 ml of L-broth media, and isolated as described in the Materials and Methods section. The final purification was performed by FPLC, using condi

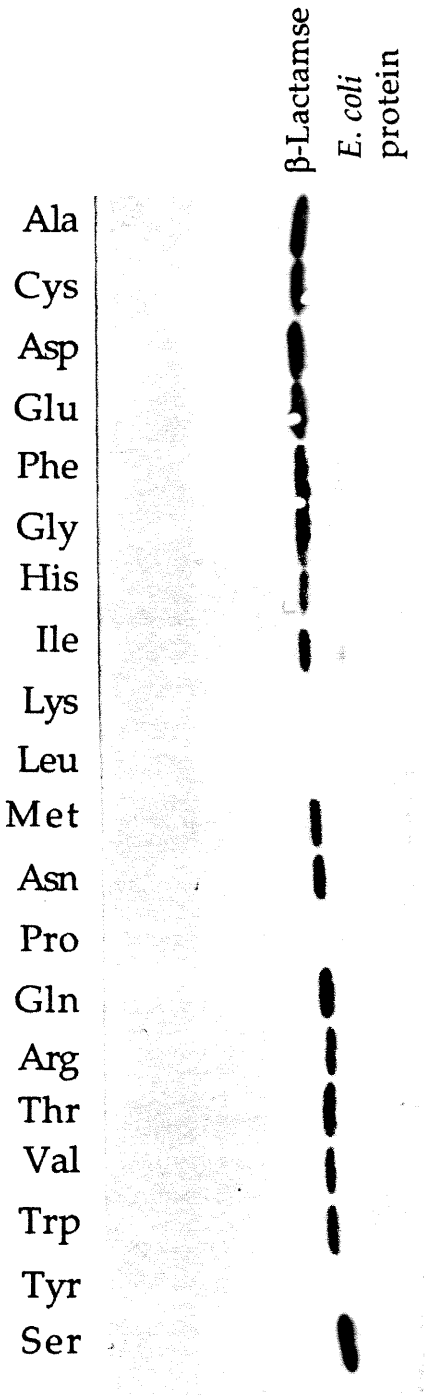

Figure 10. Western bolt analyses, using anti-β-lactamase antisera, of serine 130 mutants, displaying expression of enzyme and stability of mutants to proteolytic digestion in the periplasm of the cell. Four mutants (Lys, Leu, Pro, and Tyr) were unstable. A few of the other mutants have reduced stability with respect to the wild type enzyme (bottom lane). The circular artifacts on three of the bands are the result of air bubbles trapped between the gel and the membrane during electro-blotting.

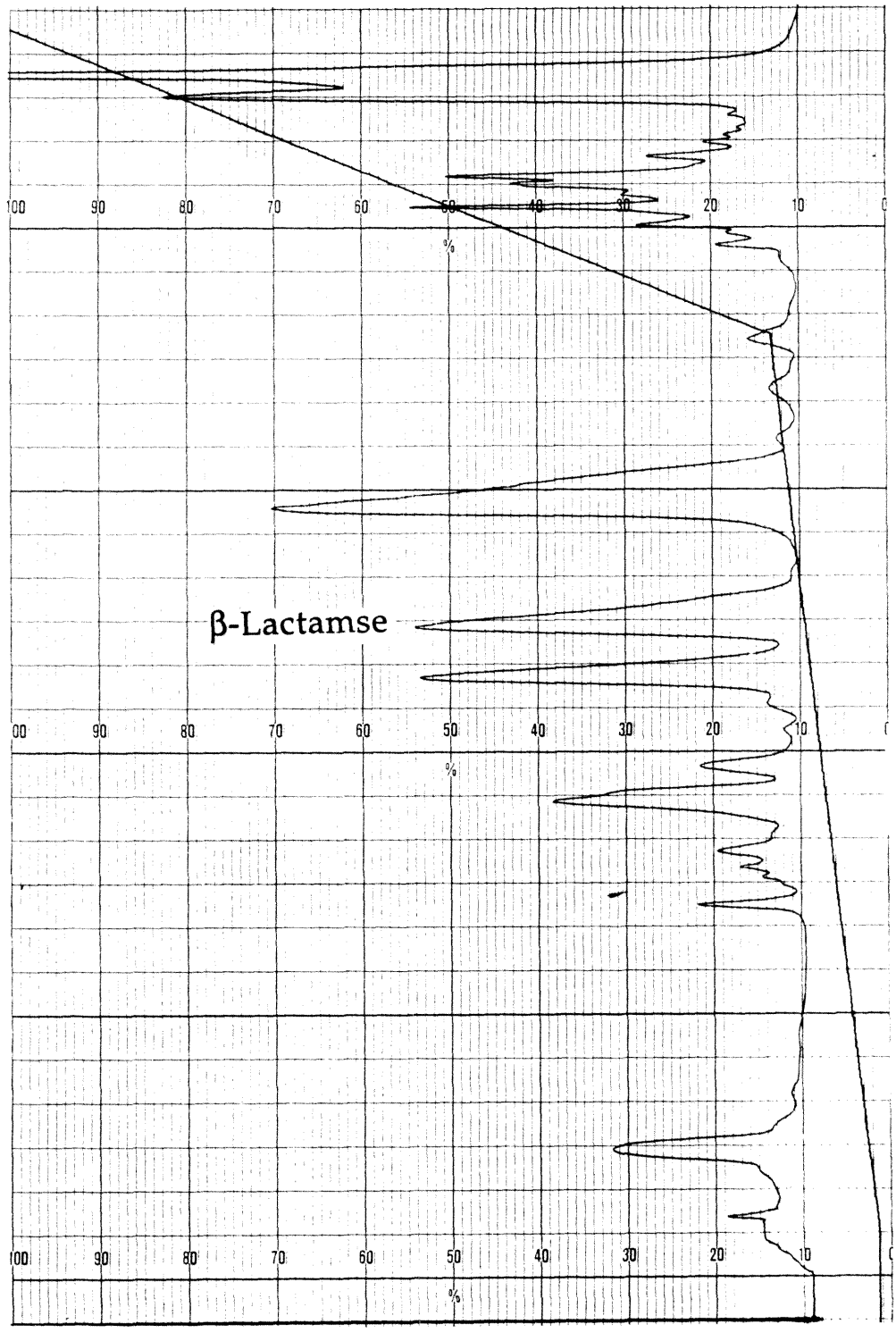

Figure 11. Typical FPLC chromatogram of periplasmic extruded, crude β-lactamase. The peak containing purified β-lactamase was identified by activity assay on benzylpenicillin and by SDS-PAGE. Time scale is two minutes per division. Fractions were collected on 0.5 minutes intervals.

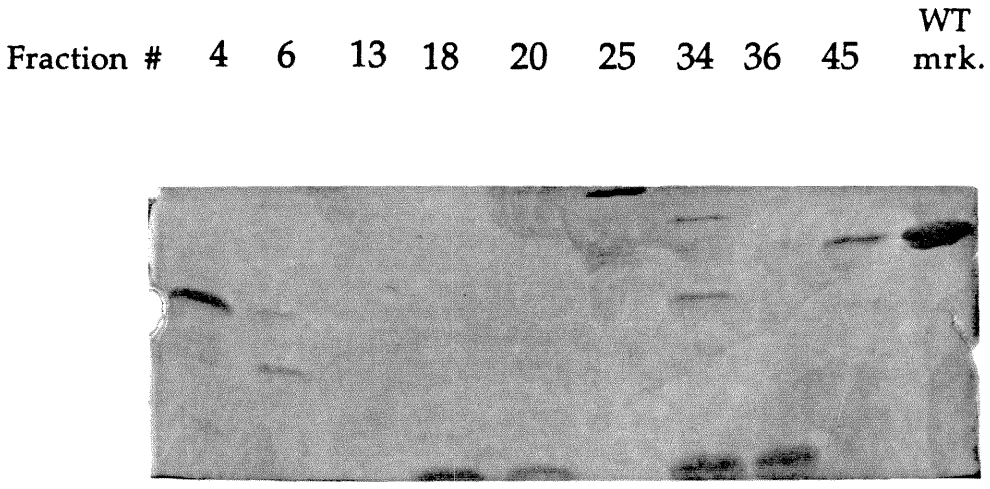

Figure 12. Coomassie-stained SDS-PAGE gel of fractions from an FPLC purification of crude β-lactamase, showing the purity of collected fractions. Lane #45 depicts fraction containing purified β-lactamase. The far right lane contains the wild type marker.

tions developed in our laboratory[38]. Figure 11 shows a typical FPLC elution trace. The purity of pooled peaks was determined by Coomassie-stained SDS-PAGE (Figure 12).

Kinetics

Michaelis-Menten kinetic parameters (k_{cat} and K_M) were determined for the wild type and the four mutant β-lactamases (Gly, Thr, Asn, and Gln) on the β-lactam substrate benzylpenicillin. Initial rate measurements were made for each of the β-lactamases on substrate concentrations ranging from one-half to four fold the K_M value of each enzyme. The kinetic parameters were determined from Hanes replots of the initial rate data[39]. A typical kinetic run is shown in Figure 13.

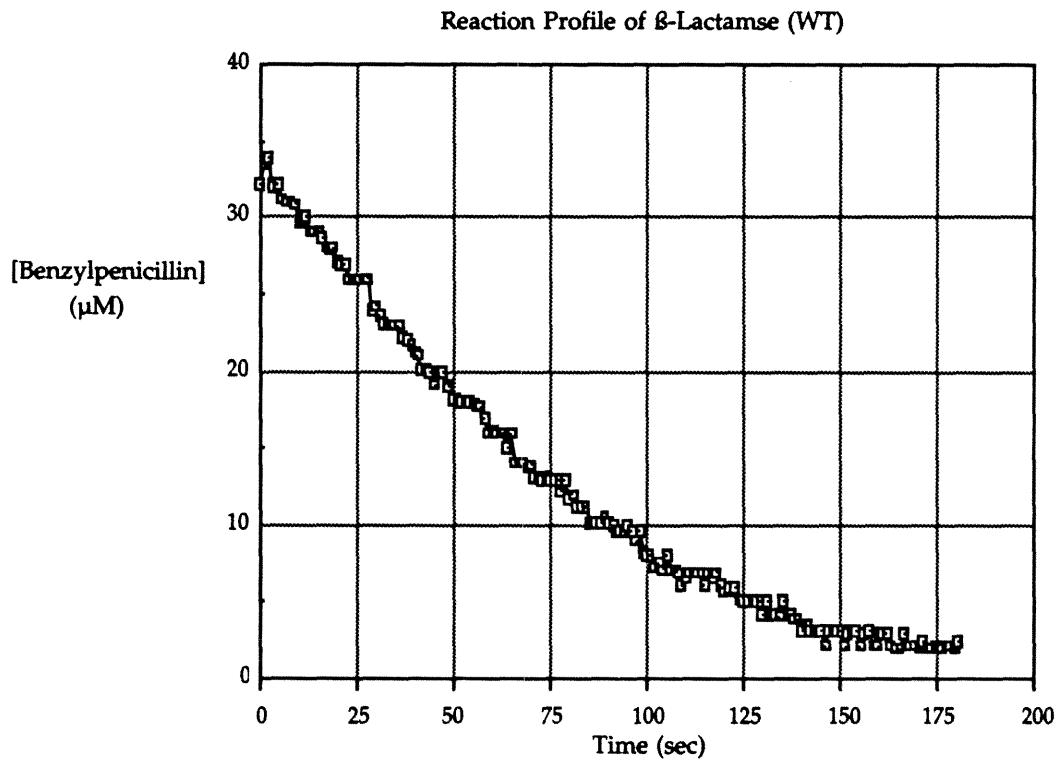

Figure 13. Typical reaction trace of wild type β-lactamase on benzylpenicillin, calculated from measured values of ellipticity versus time. Conditions were 100 mM potassium phosphate, pH 7.0, 30° C. The sharp "rise" in concentration from time 0 to 2-3 seconds is an artifact of the integration mechanism of the spectropolarimeter, and initial substrate concentrations are determined by extrapolating the initial rate back to time zero.

Table 2 displays the kinetic parameters for the four serine 130 mutants and compares them to the values obtained for the wild type enzyme. All four mutants possess reduced activity (k_{cat}/K_M) on the benzylpenicillin substrate, ranging from 4% to 0.002% of the wild type activity. The Michaelis constants of the mutant enzymes vary little with respect to the wild type; none of the K_M values measured are more than an order of magnitude different than the wild type K_M. The majority of the decreased catalytic activity of the Serine 130 mutants is distinctly attributed to decreases in the rate constant, k_{cat}.

Mutant	K_M (μM)	k_{cat} (sec^{-1})	k_{cat}/K_M ($M^{-1}sec^{-1}$)	Relative Activity
Serine (WT)	15.0 ±0.02	981 ±22	6.55×10^7	1.0
Glycine	95.7 ±0.16	246 ±11	2.58×10^6	3.9×10^{-2}
Threonine	21.6 ±0.09	2.42 ±0.05	1.12×10^5	1.7×10^{-3}
Asparagine	143.5 ±0.09	7.93 ±0.26	5.52×10^3	8.3×10^{-4}
Glutamine	46.8 ±0.37	0.062 ±0.016	1.33×10^3	2.0×10^{-5}

Table 2. Michaelis-Menten parameters for wild type and four serine 130 mutants of RTEM-1 β-lactamase on benzylpenicillin. Parameters were calculated from Hanes replots of initial reaction rates of the enzymes on various concentrations of substrate. Error values reported are the standard deviation of the data.

The extent of product inhibition was determined through competitive inhibition studies. Kinetic initial rate assays were performed on the Gly, Thr, and Asn mutants, as well as on the wild type enzyme, with varying concentration of the reaction product, penicilloic acid (PA), from 0 to 200 μM. Initial rates for reactions with and without PA were within 5% of each other for all the mutants assayed. There was in fact no detectable trend associated with the concentration of penicilloic acid, demonstrating a clear lack of

product inhibition at the concentrations assayed, both for the wild type and the Ser 130 mutants.

The pH dependency of the catalytic rates of the glycine, theronine, and asparagine mutants was measured and the results plotted in Figure 14. The pH profiles of the three mutants exhibited no significant changes from the profile obtained for the wild type enzyme.

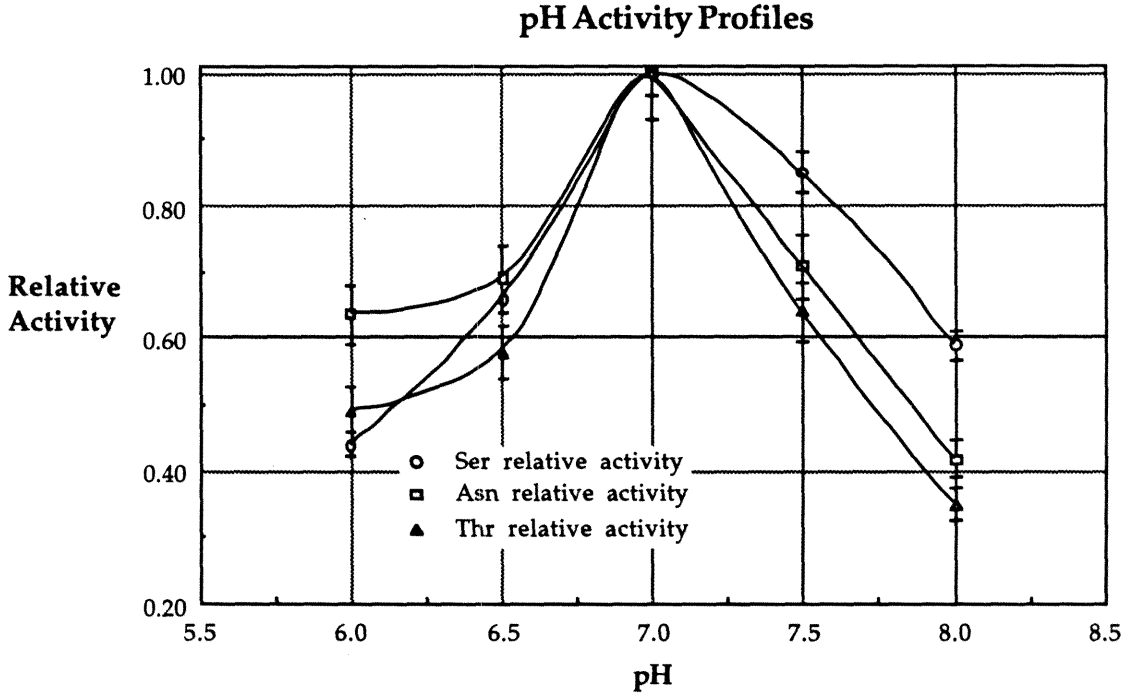

Figure 14. Plot of relative enzymatic activity for two serine 130 mutants versus pH.

Discussion

Mutagenesis

The selection of residue Ser 130 as a subject for mutagenic studies was based both upon X-ray crystallographic information localizing the residue in the active site of the enzyme and on the residues conserved within the class A β-lactamases. The serine 130 residue is particularly interesting to study since, though it appears to be a critical component of the enzyme, its function in the β-lactamase mechanism is not known.

Site-saturation of Ser 130 took advantage of two engineered restriction sites previously created in the RTEM-1 expression vector pJN-SAX by T. Richmond in our laboratory. One hundred and twenty colonies from the initial site-saturation mutagenesis were sequenced, yielding 17 of the 20 possible mutants. Sequences of the cysteine mutant contained a high amount of background, and were therefore suspected of being from mixed cells lines. The absence of amino acids Ile and Phe at position 130, coupled with the lower-than-expected number of colonies expressing the methionine or valine substitutions, would suggest that the percentage of thymine in the second codon position was lower than the optimal distribution of 25%. Though the construction of degenerate oligonucleotides attempts to evenly distribute base incorporation, it is not uncommon to see the distribution deviate noticeably from an equal apportionment[18].

The two elusive mutants, along with an unambiguous cysteine mutant, were readily obtained from a second cassette mutagenesis, using degenerate oligonucleotides (T/A, T/G, T) coding only for the four amino acids Phe, Ile,

Cys, and Ser at the 130 position. The coding for serine in the cassette was a result of the base degeneracy chosen for the mutagenisis.

Phenotypic Screening

With all 20 possible serine 130 substitutions, including the amber stop codon mutation, realized and identified, phenotypic studies were performed to determine if any of the β-lactamase mutants conferred resistance to any of the tested antibiotics, and to what extent. Antibiotic disk susceptibility and phenotypic plating assays identified three mutants at position 130 with marked β-lactamase activity: asparagine, glycine, and theronine. Though these mutants displayed substantially reduced activity compared to the wild type enzyme, any β-lactamase activity is noteworthy given the conserved nature of the Ser 130 residue. All other mutants conferred an antibiotic resistance to cells on the order of the control cell line, which did not harbor the RTEM-1 expression plasmid.

To ascertain if any of the mutants were inactive due to a deficiency of enzyme expression, Western blot analyses were performed on whole cell lysates, assessing the amount of β-lactamase protein present for each of the mutants. Though a number of factors, including protein processing, secretion, and stability, might result in a lack of detection of protein from cells expressing mutant enzymes, G. Dalbadie-McFarland *et al.* has shown in two instances, that single-site mutations of the RTEM-1 enzyme resulted in no significant difference in processing and secretion of mutant enzyme with respect to the wild type enzyme[41]. A lack of detection of β-lactamase mutants in whole cell lysates is thus probably a result of altered folding properties or conformations that effect the proteolytic stability of the enzyme to the cellular proteases. Fifteen of the 19 amino acid substitution mutants displayed

amounts of Western staining material on the order of that of the wild type β-lactamase. Four mutants (Lys, Leu, Pro, and Tyr), however, displayed no detectable levels of β-lactamase in the cells. These four residues do not possess an obvious set of similar characteristics that might explain how their inclusion at this position would render the enzyme susceptible to proteolysis. One could speculate that the positive charge of a Ser 130 Lys substitution would interact unfavorably with the side chain of Lys 234, decreasing the stability of the enzyme. However, this does not explain why the Ser 130 Arg mutant is not destabilized to an equivalent extent. Of particular note is the tyrosine mutant, which might have been expected to function similarly to that of serine had its inclusion produced a stable enzyme.

Kinetics and the Catalytic Mechanism

A major goal of enzymology is to dissect the detailed workings of enzymatic mechanisms, to understand the steps that make up a particular enzymatic reaction, and to assign a role to each particular "player" in the mechanistic "drama." However, as was described in Chapter 1, the function of a particular residue or structural moiety is often more complex than a single simple mechanistic action. Such may well be the case for the serine 130 residue in the RTEM-1 β-lactamase.

The X-ray crystal structure of β-lactamase from *B. licheniformis* shows the hydroxyl group of serine 130 to be within hydrogen-bonding distance of the ε-amino group of lysine 234. Frere *et al.* have predicted that this hydrogen bond is essential in orienting the lysine 234 residue and the β-3 strand in which it resides.

Analyses of both the *B. licheniformis* and the *S. aureus* PC1 β-lactamase structures have led to substrate binding models in which lysine 234 is

assigned a critical role in binding the carboxylate group adjacent to the β-lactam bond, found in virtually all of the β-lactam antibiotics. This requirement for substrate binding has been borne out in site-saturation experiments on Lys 234 performed in our laboratory[22]. These results demonstrate that mutants of residue 234 that do not possess a positively charged group appropriately positioned in the active site exhibit substantial increases in K_M, with little or no change in the rate constant, k_{cat}. From the hypothesized role of Ser 130, and the known function of Lys 234 as a substrate binding moiety, it would be expected that mutations at the serine residue would cause effects in substrate binding similar to mutations at 234, and as such be evinced as increases in K_M.

However, kinetic studies of serine 130 mutants have yielded contrasting results. As previously shown in Table 2, substitutions at 130 have little effect on the value of K_M, yet produce substantial decreases in the observed turnover rate, k_{cat}. Though this does not yield information concerning the existence or importance of the hydrogen-bond interaction between residues Lys 234 and Ser 130, it does implicate serine 130 as a more active participant in the catalytic mechanism.

As a potential explanation of these conflicting data, I hypothesized that the effect of the Ser/Lys interaction might not be an enhancement of the affinity of the enzyme for the substrate, but a reduction of product binding. Possibly by constraining the position of the lysine amino group, the serine residue would decrease the binding energy of the penicilloic acid carboxylate to the Lys 234 ammonium in the enzyme-product complex. In such a scenario, changes in the Ser/Lys hydrogen bond could decrease the rate of release of the hydrolyzed product, thus decreasing the observed value for k_{cat}.

Product inhibition studies were performed on three of the Ser 130 mutants (Gly, Thr, and Asn) and on the wild type enzyme. Enzymatic rates of hydrolysis of benzylpenicillin were measured in the presence of various concentrations of the product penicilloic acid. The results from these experiments clearly demonstrated there is no difference in the effect of penicilloic acid concentration on the reaction rates for the four enzymes. In fact, no product inhibition is seen for any of the enzymes, including the wild type enzyme, at product concentration levels up to 400 μM. The kinetic derivation of the complete equilibrium mechanism (Figure 15) shows that k_{cat} is a function of k_3, the dissociation rate of product from the enzyme, but not of k_{-3}, the association rate of product and enzyme. Product inhibition studies, like other competitive inhibitor analyses, measure the product dissociation constant K_p, which is the ratio of the individual "on" and "off" rates. Specifically, if mutations at Ser 130 cause product dissociation to become the rate-limiting step, we would expect to see a difference in K_p between the mutants and wild type enzyme. However, such mutations might effect product "on" and "off" rates equally, resulting in the value of K_p remaining unchanged for the mutants, yet still generating the observed decrease in k_{cat}. Nevertheless, these results likely suggest that slow product dissociation is not the cause of the decreased turnover rate in the Ser 130 mutants, yet verification necessitates determination of the individual rate constants for the mutant enzymes. Further studies using a stop-flow apparatus to determine the individual rate constants would settle this uncertainty.

In the recent report of the X-ray crystal structure for the class C β-lactamase from *Citrobacter freundii* to 2.0 Å, the authors have postulated a different mechanistic role for the 130 hydroxyl[42]. In this class C β-lactamase, the structural position of the conserved Ser 130 residue in the class A enzymes is

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P$$

$$k_{cat} = \frac{k_2 k_3}{k_{-2} + k_2 + k_3} \qquad K_M = \frac{k_{-1}k_{-2} + k_{-1}k_3 + k_2 k_3}{k_1(k_{-2} + k_2 + k_3)} \qquad K_P = \frac{k_3}{k_{-3}}$$

Figure 15. Kinetic analysis of the equilibrium model of the β-lactamase catalyzed hydrolysis reaction. The model predicts that k_{cat} for the forward reaction is a function of the product dissociation rate.

replaced with a tyrosine, numbered 150. This structure also suggests the existence of a hydrogen bond between the Tyr hydroxyl group and the equivalent of Lys 234 in the C. *freundii* enzyme. However, the authors have further predicted, based upon structure superposition studies with trypsin, that the Tyr 150 oxygen functions as the general base during β-lactam hydrolysis, storing the serine proton in a manner similar to the function of His 57 in the trypsin mechanism. This requires the pK_a of the tyrosine to be shifted 3-4 units down towards neutral. Though a three-unit shift in the pK_a of a functional group within the controlled environment of the active site is not unknown[43], applying the same mechanistic role to the Ser 130 hydroxyl of the class A enzymes requires a pK_a shift of over nine units, a substantially more difficult supposition to justify.

Both the *B. licheniformis* and the *S. aureus* PC1 β-lactamase structures have predicted that enzyme acylation proceeds without the proton relay mechanism associated with the serine proteases; instead the proton from Ser 70 is transferred directly to the nitrogen of the β-lactam during formation of the acyl-enzyme intermediate. It is speculated that Lys 73 is intimately involved in this process by orienting the Ser 70 proton towards the β-lactam bond and providing an electrostatic gradient to drive the proton from the ser-

ine oxygen to the lactam nitrogen. Mutagenic studies have demonstrated that the positive charge of the Lys residue is a critical component of catalysis[17].

The direct transfer of the serine 70 proton to the β-lactam nitrogen during acylation precludes the deacylation hydrolysis from proceeding through the reverse of this reaction mechanism, as in the serine proteases. It has been hypothesized that a water molecule, bound in the active site, is responsible for the nucleophilic hydrolysis of the acyl-enzyme bond. In this model, Glu 166 is predicted to act as a general base, with Asn 132 possibly involved in binding the attacking water. The role of Glu 166 in deacylation has been corroborated by experiments demonstrating that mutations at Glu 166 result in the accumulation of the acyl-enzyme complex[44,45]. Though there is currently no direct evidence supporting a role in deacylation for Ser 130, the positioning of the residue in the active site, potentially hydrogen-bonded to Asn 132 or a water molecule bound by Glu 166, along with its clear involvement in the catalytic mechanism, suggests this serine may also influence deacylation, possibly through the proper orientation of the attacking water molecule.

Lastly, it is generally believed that the serine 130 residue is involved in the hydrogen-bonding network within the active site of the enzyme. Analysis of the mutations of Ser 130 provides indirect evidence of a hydrogen-bonding role for the residue. All three residue substitutions at the 130 position that resulted in active enzyme possessed functionality capable of maintaining the hypothesized hydrogen-bonding requirements of this position. This is illustrated in similar mutagenesis experiments performed on T4 lysozyme[46]. In this study, Thr 157 was shown to play an important role in the thermal stability of the protein, by hydrogen bonding to two other moieties in the enzyme. Though all mutations at this position reduced the stability of the enzyme,

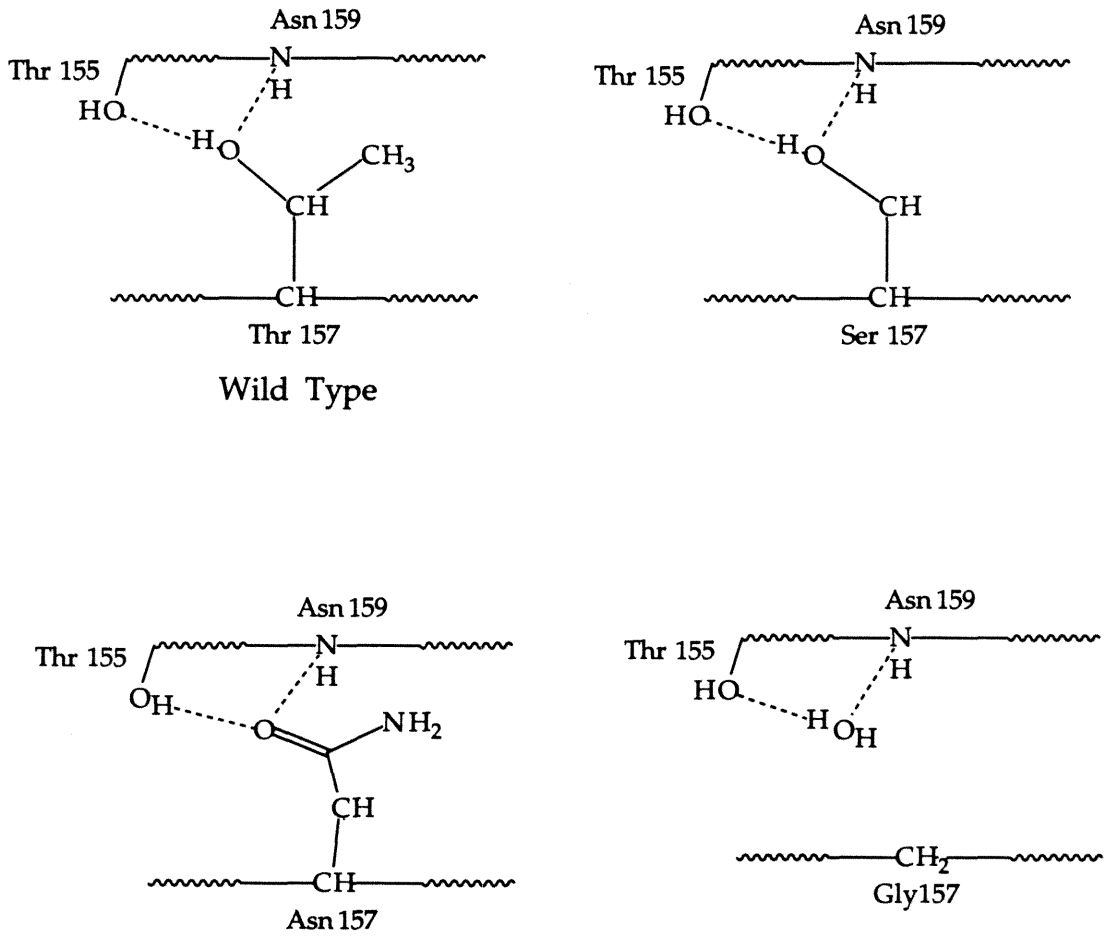

Figure 16. Schematic diagram of hydrogen-bonding interactions in three mutants of T4 lysozyme[46]. The three mutants of theronine 157 are able to maintain most of the hydrogen-bonding contacts that exist in the wild type structure, even though in some cases the polarity of the bond is switched. These interactions are a potential model for the serine 130 mutants of RTEM-1 β-lactamase.

three residues (Ser, Asn, and Gly) were substantially less destabilizing than the other 10 mutations examined. The X-ray crystal structures of these mutants were determined. Figure 16 schematically depicts the hydrogen-bonding network of the three less destabilizing mutants (Ser, Asn, and Gly) of Thr 157 in lysozyme. Though the function of Thr 157 in lysozyme is quite different than that of Ser 130 in β-lactamase, the ability of the serine, threonine, glycine, and asparagine residues to all participate in similar hydrogen-bonding networks, and the observation that these are the acceptable substitutions

at Ser 130 in RTEM-1, strongly implicates the serine 130 residue as a hydrogen-bonding moiety in the active site. Additionally, the fact that these Ser 130 mutants are able to maintain the hydrogen-bonding functionality of the residue might explain why these mutants did not exhibit a marked increase in K_M, as was predicted.

In summary, kinetic analyses of Ser 130 mutants have demonstrated that the residue plays a vital role in the mechanism of β-lactam hydrolysis in RTEM-1. This is a substantial augmentation to the hypothesized role of Ser 130 as a secondary substrate binding moiety proposed by Moews *et al.* in their report of the structure of β-lactamase from *B. licheniformis.* Additionally, the ability of the three active mutant residues to maintain hydrogen-bonding interactions within the active site strongly implicates the Ser 130 hydroxyl in a hydrogen-bond donor or acceptor role. Though the exact functional role of serine 130 has not been discerned, its position in the enzyme suggests it might be involved in the deacylation step of β-lactam hydrolysis.

Conclusion

I performed mutagenesis studies on the conserved residue serine 130 in the RTEM-1 class A β-lactamase. The residue position was substituted with all 20 possible amino acids, and transformed *E. coli* cells were screened for resistance to penam and cephem antibiotics. Three mutants (Gly, Thr, and Asn) were discovered to confer resistance to β-lactam antibiotics at levels substantially higher than the background controls. These three mutants, along with the Ser 130 Gln substitution and the wild type enzyme, were purified, and the kinetic parameters K_M and k_{cat} were determined for all five enzymes. The kinetic analyses demonstrated less than an order of magnitude change in K_M between the wild type enzyme and any of the mutants. However, the mutant enzymes displayed a decrease in the enzyme turnover rate, k_{cat}, of up to four orders of magnitude, relative to the wild type. These results clearly demonstrate the involvement of the Ser 130 residue in the catalytic mechanism of β-lactam hydrolysis. Discerning its role in either the acylation or deacylation mechanisms will require further kinetic analysis.

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