Specificity Determinants of the Class A ß-lactamase RTEM-1

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One must learn by doing the thing;
    for though you think you know it
You have no certainty until you try.

-Sophocles

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Abstract

Factors determining the substrate specificity of RTEM-1 ß-lactamase have been studied with the use of cassette mutagenesis. Three studies were performed to elucidate these factors. First, two chimeric proteins were created, substituting the active site helix of RTEM-1, residues 71-82, with the corresponding sequence from PBP5 of *E. coli.* In Chimera2, the potential disulfide bond between Cys77 and Cys123 was preserved, whereas it was removed in Chimera1 by a C77S mutation. The expression of either chimera produced no observable phenotypic effects. Both chimeras were present in *E. coli* only at reduced temperatures (25°C). Chimera2 was more stable to periplasmic proteases than Chimera1, suggesting that the disulfide bond was formed, increasing the stability. The formation of the disulfide was verified by an SDS-PAGE mobility shift assay. The behavior of Chimera2 and RTEM-1 on ion-exchange and chromatofocusing columns and the circular dichroism (CD) spectra of the two enzymes suggested a significant structural difference. Chimera2 was much more sensitive to thermal denaturation than RTEM-1, as monitored by CD. Chimera2 displayed no detectable activity as a ß-lactamase against benzylpenicillin or as a depsipeptidase against diacetyl-Lys-D-Ala-D-lactate in assays with detection limits of 3×10^{-3} sec^{-1} and 3×10^{-3} sec^{-1}, respectively. Radiolabelled acyl-enzyme could not be detected following incubation of Chimera2 with [^{14}C]-benzylpenicillin.

In the second study, residues 233, 234, 235 and 237 of RTEM-1 ß-lactamase were substituted with residues believed to be important in the differing substrate specificities of the penicillin-recognizing enzymes. Mutants were generated to yield all possible combinations of the substitutions D233H, K234H, K234N, K234Q, S235T and A237N, resulting in thirty-two sequences, including the wild-type sequence. Results of phenotypic screening were as follows: 1) Mutants with a substitution at Lys234 were phenotypically

inactive against all antibiotics tested. 2) The substitution D233H destabilized the enzyme as seen by differences in the conferred resistance at 30°C vs. 37°C. 3) The substitution S235T led to increased resistance to cephalothin compared to RTEM-1. Molecular modeling studies suggest that the instability imparted by the D233H substitution may be due to the accommodation of the five-membered ring of histidine and the loss of a favorable contact between Asp233 and Arg222. These studies also suggest that the incorporation of the methyl group in the S235T mutants into the packed space within the structure alters the orientation of the hydroxyl group. This hydroxyl group is hydrogen-bonded to the ammonium group of the conserved Lys234, which has been shown previously to be necessary in the binding of the substrate.

In the third study of RTEM-1 substrate specificity, residues 237, 238 and 240, were simultaneously randomized, and mutants were screened for the ability to confer resistance to the antibiotic cefotaxime. The three residues were chosen for study based on the sequences of the naturally occurring extended-spectrum ß-lactamases, which are active against the third generation cephems. Sixty-eight different mutant enzymes were found to possess extended activity. In general, small amino acids were found at position 237 (Ser, Gly, Ala, Thr), a range of residues were found at position 238 (Ala, Asn, Arg, Ser, Thr, Asp, Gly), and a positively charged residue (Lys, Arg, His) was commonly found at position 240. Glycine was found at position 238 if and only if proline was present in position 240. All of the mutants conferred a reduced level of resistance to ampicillin and benzylpenicillin compared to the wild-type RTEM-1. The majority of the mutants conferred increased resistance to cephalothin and cloxacillin. The values of k_{cat} and K_M, were determined for three of the mutants, ASK, ASE and AAK. The values of k_{cat} on the substrate benzylpenicillin were only slightly reduced from the values obtained for the wild-type RTEM-1 enzyme. The values of K_M for both benzylpenicillin and cefotaxime were reduced substantially, with the value of K_M on cefotaxime for the most active mutant, ASK, reduced to 120μM, compared with K_i of > 3 mM for the wild-type RTEM-1. With a

consideration of the structural differences between benzylpenicillin and cefotaxime and the results of molecular modeling studies, the results suggest that the effect of these mutations on the substrate specificity of the enzyme is not a simple enzyme-substrate contact as was previously proposed but instead suggest that the substitutions allow a conformational change not accessible to the wild-type enzyme.

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Abbreviations and Nomenclature

The three-letter amino acid code followed by a number indicates the amino acid at the given position; e.g., Ser235 refers to the serine residue at position 235. Substitution mutations are designated by the one-letter code for the native amino acid, the residue number and the one-letter code for the substituting residue; e.g., S235T refers to the single site mutation where the serine at position 235 has been replaced with threonine. Mutants with substitutions at two sites are designated by the notation for the individual mutations separated with a slash (/); e.g., the notation S235T/A237N refers to a mutant where the serine at position 235 has been replaced with threonine and the alanine at position 237 has been replaced with arginine.

Chapter 1

Introduction

The complexity Nature has created using only a small number of chemical structures is exemplified by the many roles that proteins play in biological systems. Built from the same twenty amino acids, proteins act as structural scaffolding, molecule transporters, signal transducers, motors and finely tuned catalysts. The fundamental questions are what determines how a certain sequence of amino acids will fold and how does the sequence determine the specific function it will perform.

Enzymes catalyze reactions that would not otherwise occur *in vivo* or would occur at only slow rates. However, as important as the reactions that enzymes catalyze, are the reactions they do not catalyze. Often an enzyme must discriminate between similar structures and catalyze the reaction of only one. The importance of this selectivity is demonstrated by the family of serine proteases. The enzymes of this family carry out the hydrolysis of peptide bonds. Some of these enzymes will hydrolyze any peptide bond, while others, such as the enzymes of the complement or blood clotting cascades and leader processing enzymes, will recognize very specific sequences and will hydrolyze only these. The specificity of each enzyme is key to its function in the biological system.

Catalytic proteins (enzymes) have been studied extensively by physical methods such as NMR and x-ray crystallography to determine the three dimensional structures of proteins. Kinetic studies and the use of designed inhibitors have provided insights into the possible mechanisms of enzyme action and the roles of specific residues. Primary sequence comparisons of related enzymes and generated mutants can implicate certain residues as necessary for activity. Chemical derivitizations have also been used but have largely given way to the techniques of site-specific mutagenesis. With these techniques, any residue of a protein can be changed to any other and the functional consequences studied. In spite of this wealth of experimental methods, the rules determining how the linear sequence of a protein determines the conformation it adopts and how the folded protein acts as a catalyst are not yet understood. The enzymologist or medicinal chemist is not yet able to design an enzyme inhibitor with any degree of certainty.

With a greater understanding of the factors that affect substrate binding, enzyme inhibitors and ligands that specifically bind known receptors could be designed. Further, this understanding could be used to design new enzymes, creating catalysts with desired functions. More generally, studies of the features of an enzyme that determine its specificity will contribute to the general understanding of the fine interplay between the structure and the function of proteins.

In this thesis, I describe studies of the enzyme RTEM-1 ß-lactamase and the factors determining its substrate specificity. This enzyme is a member of a large family of enzymes that hydrolyze the ring amide bond of the ß-lactam antibiotics. These antibiotics inhibit the enzymes necessary for the growth and maintenance of the bacterial cell wall by mimicking the structure of their substrate. The targeted enzymes are referred to as the penicillin-binding proteins (PBPs) though some are more sensitive to other types of ß-lactams. The PBPs function as D-alanyl-D-alanyl carboxypeptidases and transpeptidases that crosslink the peptidoglycan strands that constitute the cell wall. (The PBPs and their functions will be discussed further in Chapter 2.) Bacteria producing a ß-lactamase will be resistant to the antibiotics hydrolyzed by that enzyme.

Since benzylpenicillin was first used clinically forty years ago, many semisynthetic and synthetic ß-lactam antibiotics have been developed and are currently in use. These antibiotics consist of an essential nucleus of a ß-lactam ring that may be fused to a saturated or unsaturated five- or six-membered ring that may contain sulfur or oxygen (Figure 1). These core ring structures are further modified by various side chains. In virtually all of these compounds there is a carbonyl-containing group adjacent to the lactam nitrogen which is occasionally derivatized to form a prodrug with improved pharmacological properties. The most common core structures are the penams and cephems often represented by their earliest known members, benzylpenicillin and cephalosporin C.

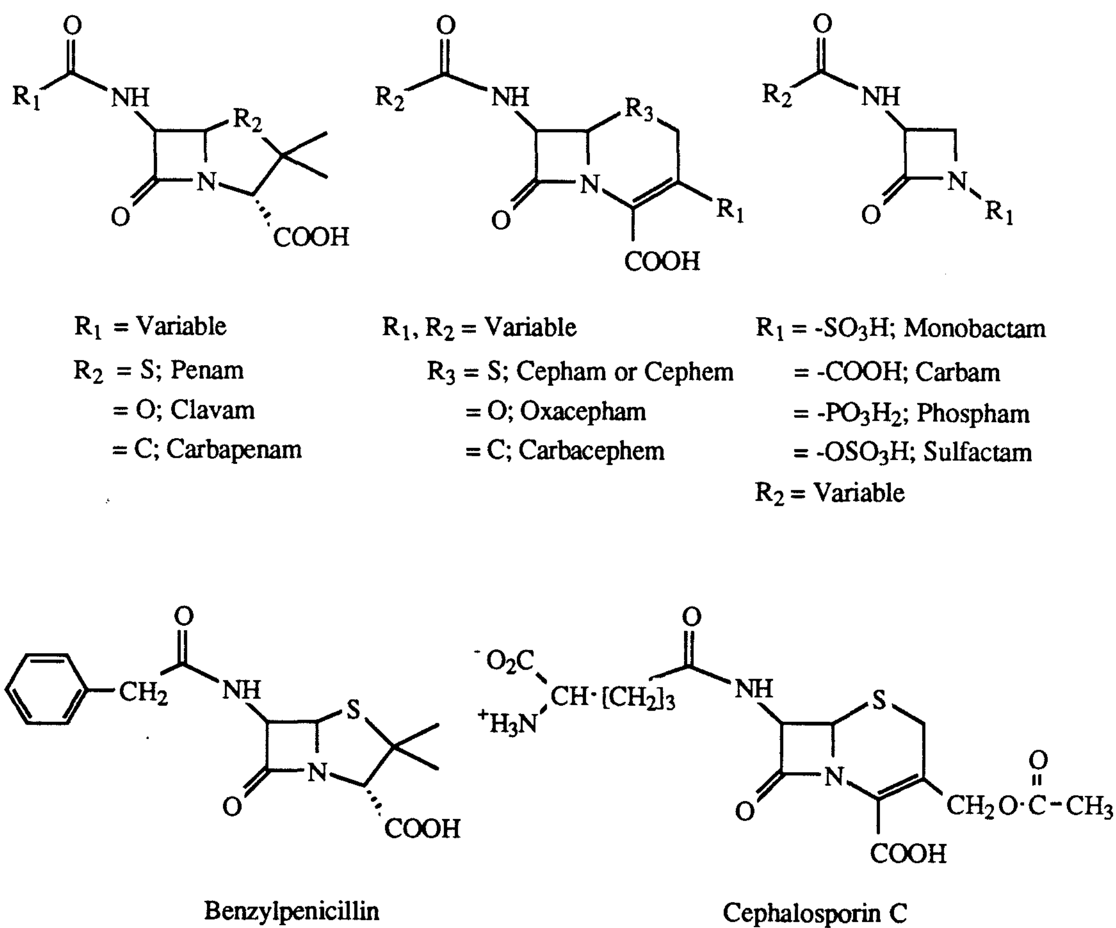

Figure 1. Core structures of ß-lactam antibiotics, the penam benzylpenicillin and the cephem cephalosporin C.

Several schemes have been developed for the classification of the many types of ß-lactamases. The early schemes were based primarily on substrate profiles, with some distinctions made based on inhibition profiles or reaction with antisera. The first scheme in general use was that of Richmond and Sykes, which divided the ß-lactamases into five classes[1]. With the development of an activity assay performed directly from isoelectric focussing gels, isoelectric points could be easily compared and were a major criterion in the classification scheme of Mathews and Sykes[2]. The more commonly used scheme of Ambler divides the ß-lactamases into three classes based primarily on molecular weight, sequence data and substrate specificity profiles. The class A ß-lactamases are the smallest (molecular weight 29 kDa), are usually plasmid-encoded and are more active in the hydrolysis of penams than of cephems. The class C ß-lactamases are larger (molecular weight 40 kDa), are usually chromosomally encoded, and are more active in the hydrolysis of cephems than penams. The class B enzymes are metalloenzymes, containing an active site zinc, with a mechanism of hydrolysis different from the other classes which all have an active site serine[3]. A fourth class, class D, has been added to include the oxacillin-hydrolyzing ß-lactamases[4]. In the classification scheme of Bush[5-7], class 1 represents chromosomally encoded cephalosporinases, class 2 represents the penicillinases and broad-spectrum ß-lactamases, which may be either plasmid or chromosomally encoded, class 3 represents the metalloenzymes and class 4 represents the penicillinases not inhibited by clavulanic acid. The numerous enzymes of class 2 are further subdivided into six subclasses based on substrate and inhibition profiles. The RTEM-type enzymes studied in this work are type IIIa in the scheme of Richmond and Sykes, class A in the scheme of Ambler, and group 2b in the scheme of Bush.

The serine ß-lactamases and PBPs form a homologous superfamily of penicillin-recognizing enzymes (Figure 2). While the ß-lactamase sequences differ substantially from the PBPs, several conserved regions have been identified[8]. Among these is the region containing the active site serine, where the sequence is S-X-X-K in all known serine

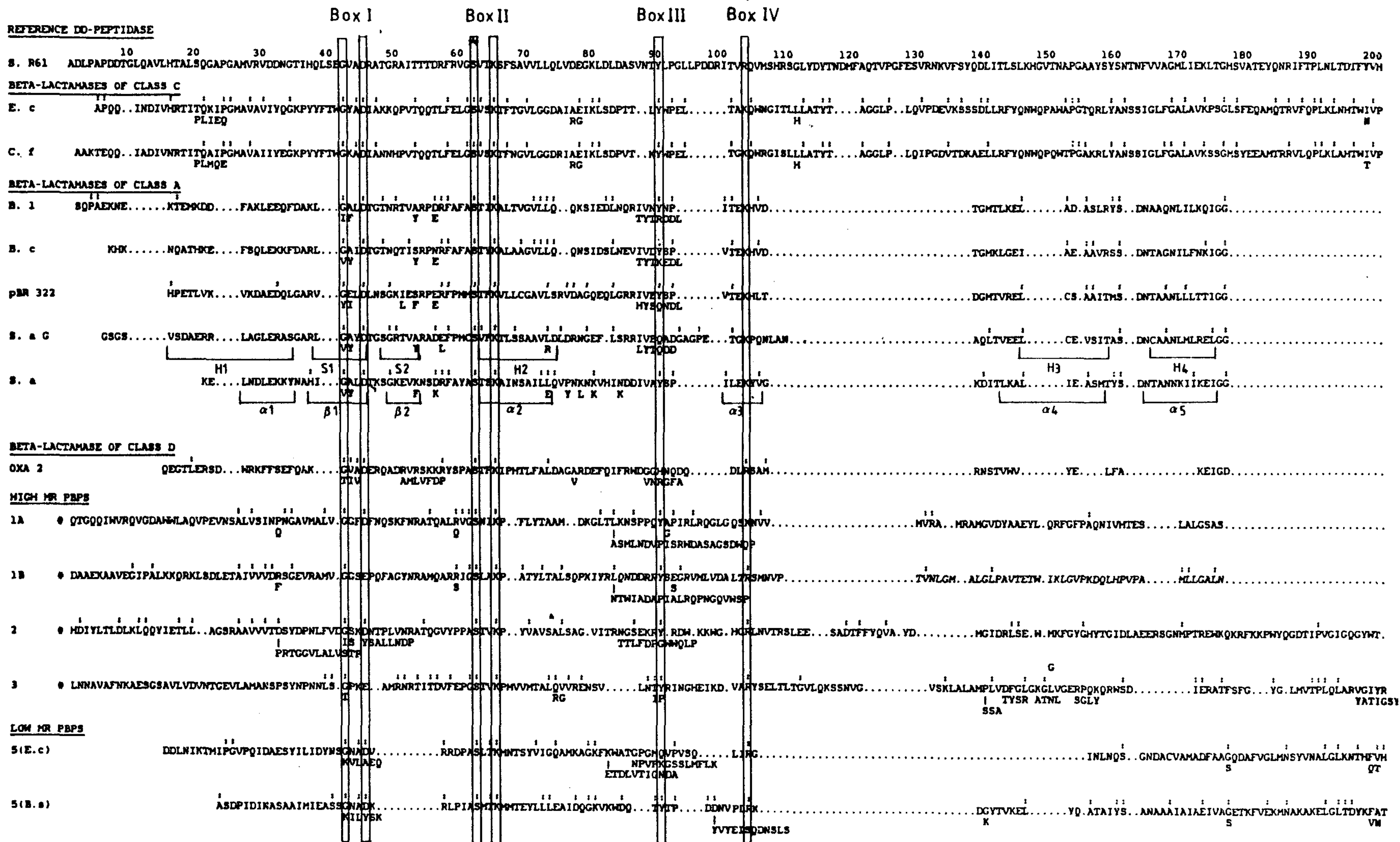

Figure 2. Amino acid sequences of penicillin recognizing enzymes and domains. Boxes indicate conserved segments. Abbreviations of sources are: S. R61, *Streptomyces* R61; E. c., *Escherichia coli* K12; C. f, *Citrobacter freundii* OS60; B. 1, *Bacillus licheniformis* 749/c; *Bacillus cereus* ß-lactamase I; S. aG, *Streptomyces albus* G; S. a, *Staphylococcus aureus*, Oxa-2, *Salmonella typhimurium*; High MW PBP's are all of *E. coli*; B. s, *Bacillus subtilus*. (Ref. 8)

```
                                  Box V                                         Box VI                    Box VII
REFERENCE DD PEPTIDASE
                 210        220        230        240        250        260        270        280        290        300        310        320        330        340
S. R61   PDTVIPGTHANGYLTPDEAGGALVDBTEQTVSWAQTGAAVISSTQDLDTFFSALMSGQLMSAAQLAQMQQWTTVNSTQGYGLGLRRRDLSCGISVYGHTGTVQGYYTYAFASKDGQAHVTALANTSNNVNVLNTMARTLESAFCGKPTT

BETA-LACTAMASES OF CLASS C
E. c     PAE..EKNYAWGYR..EGKPGAL.D.AE........AYGVKSTIEDMARWVQSNLKPTLQQGIQLAQSRYWQTGDMYQGLGWEMLDWPVNPDASWVHKTGATGGFGSYAFIPEKELGIVM.LANKNYPNPARVDAAWQILNALQ
                        AVHVS                                LDINEX                                    |                  V
                                                                                                     SIINGSDNKIALAARPVKAITPPTPAVR

C. f     QSE..QKNYAWGYL..EGKPGQL.D.AE........AYGVKSSVIDMARWVQANMDATLQQGIELAQSRYWRIGDMYQGLGWEMLNWPLKADASWVHKTGSTGGFGSYAFVPEKNLGIVM.LANKSYPNPARVEAAWRILEKLQ
                        PVHVS                                SHVQEX                                    |                  V
                                                                                                     SIINGSDSKVALAALPAVEVNPPAPAVK

BETA-LACTAMASES OF CLASS A
B. l     PESLKKELRKIG....DEVTPERFEP.ELNEVNP..GETQDTSTA..RALVTSLRAFALLPSEKRELLIDWMKRNTTG..DALIRA.GVPDGWEVADKTGAASGTRNDIAIWPPKGDPVVLAVLSSRDKKDAKYDDKLIAEATKVVMKALNMNGK
                        N                                      EDK                                              Y      I

B. c     PKGYEKALRHMG....DRITSNRFET.ELNEAIP..GDIRDTSTA..KAIATNLKAFTVLPAEKRKILTEWMLGNATG..DKLIRA.GIPTDWVVGDKSGAGSGTRNDIAVWPPNRAPIIIAILSSKDEKEAIYDNQLIAEATKVIVKALR
                        M                                      GNA                                              Y      V

pBR 322  PKELTAFLHNMG....DHVTLDRWEP.ELNEAIP..NDERDTTMP..AAMATTLRKLLTLTLASRQQLIDWMEADKVAGP..LLRS.ALPAGWFIADKSGAGEGSRGIIAAGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW
                        R                                      GEL                                              R      L *

S. a G   PAAVTRFVRSLG....DRVTLDRWEP.ELNSAEP..GRVTDTTSP..RAITRTBBRLVLGNPRDRRLLTSWLLANTTSG..DRFRA.GLPDDWTLGDKTGAGRGTNNDAGVWPPGRAPIVLTVLTAKTEQDAARDDGLVADAARVLAETLG
         |_________|    R                       |_____________|DAL|_________|      |_______|     |____|   |Y|     T  |     |_________|    |____________|
             H5                                       H6              H7               H8             S3     S4           S5              H9

S. a     IKKVKQRLKELG....DKVTPVRYEI.ELNYYSP..KSKKDTSTP..AAFGKTLNBLIALSKENKKFLLDLMLNNKSG..DTLIKD.GVPKDYKVADKSGQATASRNDVAFYPGQSEPIVLVIFTNKDNKSDKPNDKLISETAKSVMKEF
         |_________|    N     |__|                |__________|NGN|_________|          |______|      |_____| |Y|  V K  |______|       |__________|
             α6                α7                      α8             α9                 α10           β3   I   β4          β5              α11

BETA-LACTAMASE OF CLASS D
OXA2     DKA.RRYLKKIDYGNADPSTSNG.DYWE........GSLAISAQEQIA.FLRKLYRNELFRVEHQRLVKDLMIVEA............GRNWILRAKTGWEGGWWVG.WVWPTGSVFFALNIDTPNRMDDLFKREAIVRAILRSI.EALPPNPAVNSDAAR
                                 I                          P                                             RM     E

HIGH MR PBPS
1A       ............FTPMANGGFLVDKIENDQGGVIFEAKPKVACPECDIPV                  GEPGWDGTGWRAG..RDLQRRDI.....GGKTGTNSSKDAWFSGYGPGVTSVWIGFDDHRRNLGTTASGAIKDQISGYEGGAKSAQPA   *
                        |           PNFIS                  |****************|                                          V            H
                        QVARGYAVM                          619              693

1B       ............LTPIESGGAE.DGKVLYQSFPQAERAVP.....................AQAAYLTIWTMQQVVQR.GTG.RQLGAKYPLHLAQKTGTTNNNVDTWFAGIDGSTTITWVGRDNNQPTKLYGASGAMSIYQRYLANQTPTPLNLV   *
                        |  NRAPLSALRSVI                                                     N                  V
                        VAQAFQTIA

2        ............ATPIQNDGAE.DGKQVPHVQPHEPVGD......................IHSGWELAKDGMY.GVANRHKYFASPYKIAAKSGTAQVFETYNAHKIAERLRDHKLMTAFAPYNNPQVAVAMILENGGAGPAVGTLM   *
                        |  IVKVPHLLMST    P                                      PNGTA A         GLKAN
                        MSKALMILI

3        PLSI...TKVDPPV.PGER..VFPESIV............................RTVVHMMESVALPGGG.....GVKAAIKG....YRIAIKTGTAKKVYI.AYT.AGVAPASQ...PRFALVVVIND..PQAGKYYGGAVSAPVFGAI   *
                                                                                                              GPDGRYINK

LOW MR PBPS
5(E. c)  GLDADGQYS....SARDMAGQALIDVPN...........EYSIYKEKE.FTFNGIRQLNRNG.....LIWDNS.LN..............VDGIKTGHTDKAGY.NLV...ASATEGQM...RLISAVMGGR.TFK..GREAESKKLLTWGFRFFETVNPLKVGK   *
                        LI   R

5(B. s)  GLENKDLHGHQPESAKDMAADHLIDVPE........ILETSSIAKTK..FREGTDDEMDMP.......NWNFM.LK...GLVSEYKKAT...VDGIKTGSTDSAGS.CFT...GTAERNGM...RVITVVLNAK.GNLHTGRFDETKKMFDYAFDNFSMKEIYAE   *
                     |   VL   T
                     GTSVNEESEV
```

Figure 2. Continued

ß-lactamases and PBPs. Another conserved region is referred to as the HTG box (residues 234-236 in the numbering scheme of the class A ß-lactamases[3]). This sequence has been shown by mutagenesis to be important in the functioning of the class A ß-lactamases as well as PBP3 of *E. coli*[9] and will be discussed in greater detail in Chapter 3. Within the ß-lactamases, the sequence homology is greater particularly among members of the same class. For example, the class A ß-lactamases RTEM-1 and SHV-1 have 68% homology[10]. The amino acid sequences of several of the commonly studied class A ß-lactamases are given in Figure 3.

Both the PBPs and the serine ß-lactamases function through the formation of an acyl-enzyme intermediate with the active site serine. In the case of the ß-lactamases, the penicilloyl-enzyme intermediate is readily hydrolyzed and the innocuous product is released. In the case of the PBPs, the peptidyl-enzyme intermediate is attacked by water in the case of the carboxypeptidase, or by a peptide of the peptidoglycan in the case of the transpeptidases. When the PBPs react with a ß-lactam, the resulting acyl-enzyme intermediate is stable and the enzyme is covalently inhibited.

The organization of the secondary structural features of the ß-lactamases and PBPs have been found to be conserved. The structures of the class A ß-lactamases from *S. albus* G, *S. aureus* PC1 and *B. licheniformis* have been determined by X-ray crystallography to 3.0, 2.5 and 2.0 Å resolution, respectively[11-13]. These enzymes, shown in Figure 4, have similar overall structures despite their limited homology. The crystal structure of the water soluble carboxypeptidase-transpeptidase from *Streptomyces* R61 has also been determined[14] and compared to the low-resolution structures of the ß-lactamases from *B. cereus* and *B. licheniformis*[15,16] (Figure 5). These comparisons reveal a high degree of structural homology, again in spite of a limited sequence homology. The construction of water soluble derivatives of PBP2, PBP3 and PBP5 of *E. coli* has been reported, and crystallization studies are under way[17-19]. These structural and functional homologies

```
TEM-3   MSIQHFRVALIPFFAAFCLPVFAHPETLV  K VKDAEDQLGARVGYIELDLNSGKILESFRPEERFPMMSTFKVLLCGAVLSRV  DAGQEQLGRRIHYSQNDLVK  YSPVTEKH  LTDGMTVRELCSAAITM
TEM-1   MSIQHFRVALIPFFAAFCLPVFAHPETLV  K VKDAEDQLGARVGYIELDLNSGKILESFRPEERFPMMSTFKVLLCGAVLSRV  DAGQEQLGRRIHYSQNDLVE  YSPVTEKH  LTDGMTVRELCSAAITM
SHV-2   MRYIRLCIISLLATLPLAVHASPQPLE  Q IKLSESQLSGRVGMIEMDLASGRTLTAWRADERFPMMSTFKVVLCGAVLARV  DAGDEQLERKIHYRQQDLVD  YSPVSEKH  LADGMTVGELCAAAITM
SHV-1   MRYIRLCIISLLATLPLAVHASPQPLE  Q IKLSESQLSGRVGMIEMDLASGRTLTAWRADERFPMMSTFKVVLCGAVLARV  DAGDEQLERKIHYRQQDLVD  YSPVSEKH  LADAMTVGELCAAAITM
OHIO-1  MRYFRLCIISLLATLPLAVHAGPQPLE  Q IKLSESQLSGSVGMIEMDLAPPGTLTAWRADERFPMMSTFKVVLCGAGLARV  DAGDEQLERKIHYRRQDLVD  YSPVSEKH  LADGMTVGEECAAAITM
LEN-1   MRYVRLCVISLLATLPLVVYAGPQPLE  Q IKQSESQLSGRVGMVEMDLANGRTLAAWRADERFPMVSTFKVLLCGAVLARV  DAGLEQLDRRIHYRQQDLVD  YSPVSEKH  LVDGMTIGELCAAAITL

PSE-4   MKFL LAFSLLIPSVVFASSSKFQQVE  QDVKAIEVSLSARIGVSVLDTQNGEYWD YNGNQRFPLTSTFKTIACAKLLYDA  EQGKVNPNSTVEIKKADLVT  YSPVIEKQ  VGQAITLDDACFATMTT
CARB-3  MKFL LAFSLLIPSVVFASSSKFQQVE  QDVKAIEVSLSARIGVSVLDTQNGEYWD YNGNQRFPLTSTFKTIACAKLLYDA  EQGKVNPNSTVEIKKADLVT  YSPVIEKQ  VGQAITLDDACFATMTT
CARB-4  MKLL LVFSLLIPSMVFANSSKFQQVE  QDAKVIEASLSAHIGISVLDTQTGEYWD YNGNQRFPLTSTFKTIACAKLLYDA  EQGEINPKSTIEIKKADLVT  YSPVIEKQ  VGQAITLDDACFATMTT

BCE5H   MILKNK RMLKIGICVGILGLSITSLEAFTGESLQVEAKEKTGQVKHKNQA  THKEFSQLEKKFDARLGVYAIDTGTNQTIS YRPNERFAFASTYKALAAGVLLQQ  NSIDSLNEVITYTKEDLVD  YSPVTEKH  VDTGMKLGEIAEAAVRS
BCE5B   MKNK KMLKIGMCVGILGLSITSLVTFTGGALQVEAKEKTGQVKHKNQA  THKEFSQLEKKFDARLGVYAIDTGTNQTIA YRPNERFAFASTYKALAAGVLLQQ  NSTKKLDEVITYTKEDLVD  YSPVTEKH  VDTGMTLGEIAEAAVRY
BLIP    MKLWFSTLKLKKAAAVLLFSCVALAGCANNQTNASQPAEKNEKTEMKDD  FAKLEEQFDAKLGIFALDTGTNRTVA YRPDERFAFASTIKALTVGVLLQQ  KSIEDLNQRITYTRDDLVN  YNPITEKH  VDTGMTLKELADASLRY
BCEZ    MFVLNKFFTNSHYKKIVPVVLLSCATLIGCSNSNTQSESNKQTNQTNQVKQENKRNHAFAKLEKEYNAKLGIYALDTSTNQTVA YHADERFAFASTSKSLAVGALLRQ  NSIEALDERITYTRKDLSN  YNPITEKH  VDTGMTLKELADASVRY
ROB-1   MLNKLKIGTLLLLTLTACSPNSVHSVTSNPQPASAPVQQSATQATFQQTLANLEQQYQARIGVYVWDTETGHSLS YRADERFAYASTFKALLAGAVLQS  LPEKDLNRTISYSQKDLVS  YSPETQKY  VGKGMTIAQLCEAAVRF
CACAOI  VACGQASGSESGQQPGLGGCGTSAHGSADAHEK  EFPALEKKFDAHPGVYAIDTRDGQEIT HRADEPFAYGSTFKALQAGAILAQVLRDGREVRRGAEADGMDKVVHYGQDAILP  NSPVTEKH  VADGMSLRELCDAVVAY
PC1     MKKLIFLIVIALVLSACNSNSSHAK  ELNDLEKKYNAHIGVYALDTKSGKEVK FNSDKRFAYASTSKAINSAILLEQ  VPYNKLNKKVHINKDDIVA  YSPILEKY  VGKDITLKALIEASMTY
SABLA   VRLTQAPPSRRTLMTLGAGATMAA LLPAGGAAYASTSTAKAPAAEGISGRLRALEKQYAAPLGVFALDTGTGADRS YRAGERFPMCSVFKALAAAAVLRDV  GARREFLTKRIHYTEKFVKDA GYIPVTGKPENIAGG MTGAELCAAAVSE
ALBUS   VHPSTSRPSRRTLLTATAGAALAAATLVPGTAHASSGGRGHGSGSVSDAERRLAGLERASGARLGVYAYDTGSGRTVA YRADELFPMCSVFKTLSSAAVLRDL  DRNGEFLSRRILYTQDDVEQADGAGPETGKPQNLANAQLTVEELCEVSITA
                                                        α1       β1       β2          α2                                              α3        α4

TEM-3   SDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTMPAAMATTLRKL LTG  ELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAS ERGSRGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW*
TEM-1   SDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTMPAAMATTLRKL LTG  ELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAG ERGSRGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW*
SHV-2   SDNSAANLLLATVGGPAGLTAFLRQIGDNVTRLDRWETELNEALPGDARDTTTPASMAATLRKL LTS  QRLSARSQRQLLQWMVDDRVAGPLIRSVLPAGWFIADKTGAS ERGARGIVALLGPNNKAERIVVIYLRDTPASMAERNQQIAGIGAALIEHWQR*
SHV-1   SDNSAANLLLATVGGPAGLTAFLRQIGDNVTRLDRWETELNEALPGDARDTTTPASMAATLRKL LTS  QRLSARSQRQLLQWMVDDRVAGPLIRSVLPAGWFIADKTGAG ERGARGIVALLGPNNKAERIVVIYLRDTPASMAERNQQIAGIGKALYEHWQR*
OHIO-1  SDNSAANLLLPAVGGPAGLTAFLRQIGDNVTRLDRFETELNEALPGDARDTTTARSMAATLRKL LTS  HGLSARSQRQLLQWMVDDRVAGRSIRSVLPAGWFIADKTGAG ERGARGIVALLGPNNKAERIVVIYLRDTPASMAERNQQIAGIA  QR*
LEN-1   SDNSAGNLLLATVGGPAGLTAFLRQIGDNVTRLDRWETALNEALPGDARDTTTPASMAATLRKL LTA  QHLSARSQQQLLQWMVDDRVAGPLIRAVLPPGWFIADKTGAG ERGARGIVALLGPDGKPERIVVIYLRDTPASMAERNQHIAGIG  QR*

PSE-4   SDNTAANIILSAVGGPKGVTDFLRQIGDKETRLDRIEPDLNEGKLGDLRDTTTPKAIASTLNKF LFG  SALSEMNQKKLESWMVNNQVTGNLLRSVLPAGWNIADRSGAG GFGARSITAVVWSEHQAPIIVSIYLAQTQASMEERNDAIVKIGHSIFDVYTSQSR*
CARB-3  SDNTAANIILSAVGGPKGVTDFLRQIGDKETRLDPIEPDLNEGKLGDLRDTTTPKAIASTLNKL LFG  SALSEMNQKKLESWMVNNQVTGNLLRSVLPAGWNIADRSGAG GFGARSITAVVWSEHQAPIIVSIYLAQTQASMAERNDAIVKIGHSIFDVYTSQSR*
CARB-4  SDNAAANIILNALGGPESVTDFLRQIGDKETRLDRIEPELNEGKLGDLRDTTTPNAIVNTLNEL LFG  STLSQDGQKKLEYWMVNNQVTGNLLRSVLPEGWNIADRSGAG GFGARSITAVVWSEAQSPIIVSIYLAQTEASIADRNDAIVKIGRSIFEVYSSQSR*

BCE5H   SDNTAGNILFNKIGGPKGYEKALRHMGDRITMSNRFETELNEAIPGDIRDTSTAKAIATNLKAFTVGN  ALPAEKRKILTEWMKGNATGDKLIRAGIPTDWVVGDKSGAG SYGTRNDIAVVWP PNSAPIIV LISSKDEKEAIYNDQLIAEATKVIVKGS*
BCE5B   SDNTAGNILFHKIGGPKGYEKALRKMGDRVTMSDRFETELNEAIPGDIRDTSTAKAIARNLKDFTVGN  ALPHQKRNILTEWMKGNATGDKLIRAGVPTDWVDADKSGAG SYGTRNDIAIVWP PNRSPIIIAILSSKDEKEATYDNQLIKEAAEVVIDAIK*
BLIP    SDNAAQNLILKQIGGPESLKKELRKIGDEVTNPERFEPELNEVNPGETQDTSTARALVTSLRAFALED  KLPSEKRELLIDWMKRNTTGDALIRAGVPDGWEVADKTGAA SYGTRNDIAIIWP PKGDPVVLAVLSSRDKKDAKYDDKLIAEATKVVMKALNMNGK*
BCEZ    SDSTAHNLILKKLGGPSAFEKILREMGDTVTNSERFEPELNEVNPGETHDTSTPKAIAKTLQSFTLGT  VLPSEKRELLVDWMKRNTTGDKLIRAGVPKGWEVADKTGAG SYGTRNDIAIIWP PNKKPIVLSILSNHDKEDAEYDDTLIADATKIVLETLKVTNK*
ROB-1   SDNSATNLLLKELGGVEQYQRILRQLGDNVTHTNRLEPDLNQAKPNDIRDTSTPKQMAMNLNAYLLGN  TLTESQKTILWNWLDNNATGNPLIRAATPTSWKVYDKSGAG KYGVRNDIAVVRI PNRKPIVMAIMSTQFTEEAKFNNKLVEDAAKQVFHTLQLN*
CACAOI  SDNTAANLLFDQLGGRRGSTRVLAQLGDHTTSMDRYEQELGSAVPGDPRDTSTPRAFAEDLRAFAVEDGEKAALAPNDREQLNDWMSGSRTGDALIRAGVPKDWKVEDKSGQV KYGTRNDIAVVRP PGRAPIVVSVMSHGDTQDAEPHDELVAEAGLVVADGLK*
PC1     SDNTANNKIIKEIGGIKKVKQRLKELGDKVTNPVRYEIELNYYSPKSKKDTSTPAAFGKTLNKLIANG  KLSKENKKFLLDLMLNNKSGDTLIKDGVPKDYKVADKSGQAITYASRNDVAFVYPKGQSEPIVLVIFTNKDNKSDKPNDKLISETAKSVMKEF*
SABLA   SDNGAGNLLLRELDGPTGITRFCRSLGDTTTRLDRWEPALNSAEPDRVTDTTSPGAIGRTFGRLIVGS  ALPAGDRKRLTGWLVANTTNRPTFRAGLPDDWVLADKTGGGEQYGVANDVGVVQP PGRAPLVLSVLSTKFDPKGPTDNPLVAKAAALVAGELT*
ALBUS   SDNCAANLMLRELGGPAAVTRFVRSLGDRVTRLDRWEPELNSAEPGRVTDTTSPRAITRTYGRLVLGD  ALNPRDRRLLTSWLLANTTSGDRFRAGLPDDWTLGDKTGAG RYGTNNDAGVTWP PGRAPIVLTVLTAKTEQDAARDDGLVADAARVLAETLG*
           α5          α6                α7             α8             α9          α10     β3      β4         β5                  α11
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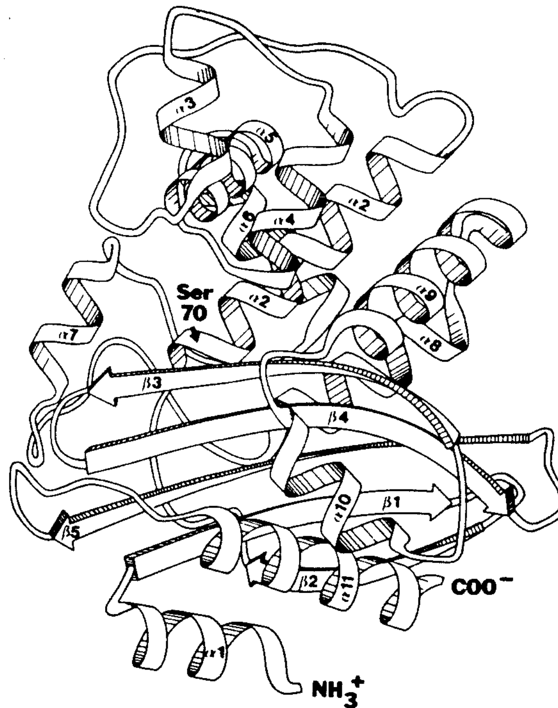
Figure 3. Amino acid sequences of class A ß-lactamases. (Ref. 34) ß-lactamases are from the following sources: ALBUS, *Streptomycese albus* G; SABLA, *Streptomyces aureofaciens*; PC1, *Staphylococcus aureus* PC1; CACAOI, *Streptomyces cacaoi* KCC-SO352 (chromosomal); ROB-1, *Haemophilus influenzae* type b strain F990; BCEZ, *Bacillus cereus* 569/H lactamase III; BLIP, *Bacillus licheniformis* 749/c; BCE5B, *Bacillus cereus* 5/B lactamase I; BCE5H, *Bacillus cereus* 569/H lactamase I; CARB-4, *Pseudomonas aeruginosa* P83 372; CARB-3, *Pseudomonas aeruginosa* Cilote; PSE-4, *Pseudomonas aeruginosa* Dalgleish; LEN-1, *Klebsiella pneumoniae* LEN-1; OHIO-1, *Enterobacter cloacae* 075; SHV-1, *Klebsiella pneumoniae* R974; SHV-2, *Klebsiella ozaenae* 2180; TEM-1, *Salmonella paratyphi* B; TEM-3, *Klebsiella pneumoniae* CF104. Asterisks indicate residues conserved in all sequences.

A)



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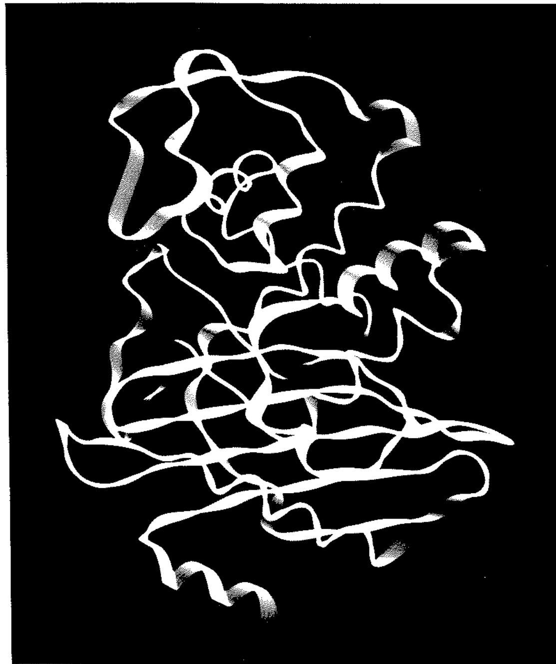
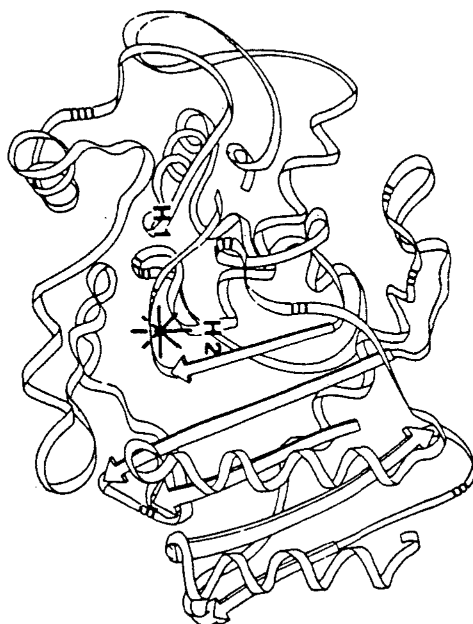


Figure 4. Structures of two class A ß-lactamases as determined by X-ray crystallography.
A) ß-lactamase of *Staphylococcus aureus* PC1 at 2.5Å resolution (Ref. 12).
B) ß-lactamase of *Bacillus licheniformis* 749/C at 2Å resolution (Ref. 13).

A)



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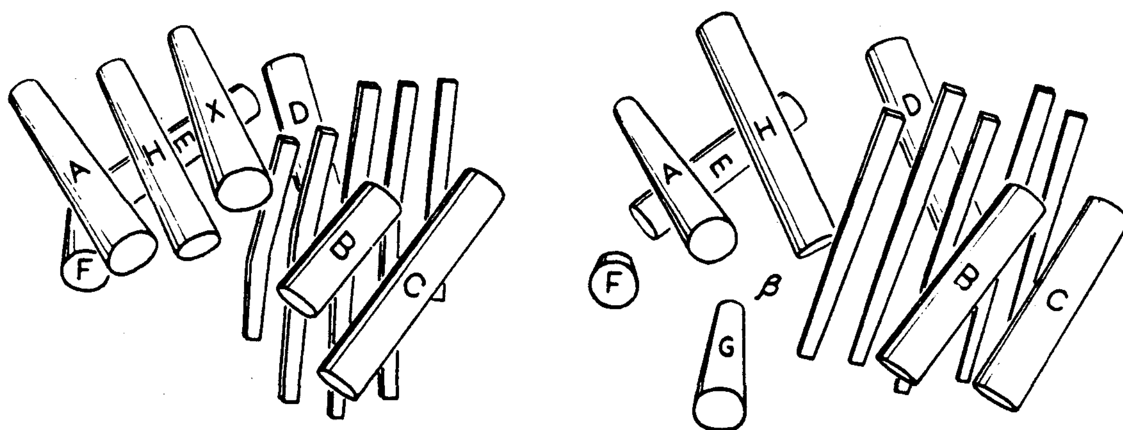

Figure 5. Structure of a D-alanyl carboxypeptidase of *Streptomyces* R61 and its comparison with the structure of a class A ß-lactamase. A) Main chain folding scheme of a D-alanyl carboxypeptidase of *Streptomyces* R61 at 2.8Å resolution (Ref. 14). B) Secondary structure elements of the ß-lactamase of *B. licheniformis* (left) and structure shown in A presented in an analogous manner (Ref. 16).

among the PBPs and ß-lactamases suggest an evolutionary relationship between these enzymes.

The roles of several residues of the class A ß-lactamases have been studied by a variety of means, but the details of the catalytic mechanism are not yet known. Since no substrate analogs are available, the structures determined are those of unbound forms of the enzymes. The location of the substrate binding site has been predicted by computer modeling based on the penicillin and cephalothin complexes of the homologous carboxypeptidase of *Streptomyces* R61[20]. The role of Ser70 as the nucleophile forming the acyl-enzyme intermediate was the first to be elucidated[21,22], and the requirement for activity of a primary nucleophile at this site has been shown[23-25]. (Numbering is according to Ambler[3].) Site-saturation studies of Thr71 revealed this residue to be important in structural stability but to have no catalytic function[26]. Lys234 has been shown to be important in substrate binding and is suggested to form a salt bridge with the carbonyl group adjacent to the lactam nitrogen present in virtually all ß-lactamase substrates[27,28]. Studies using a modified substrate revealed the presence of an oxyanion hole analogous to that found in the serine proteases[29]. X-ray crystallographic studies have suggested the oxyanion hole is formed by the backbone amides of residues 237 and 70, and by the partial positive charge of the active site helix dipole, residues 70-82[13,14]. Residue 237 has also been implicated in substrate specificity as mutations in this residue created by chemical mutagenesis[30] and cassette mutagenesis[31] were found to have elevated levels of activity on cephalothin. The cysteine at residue 77 forms a disulfide with Cys123 in RTEM and contributes to the stability of the wild-type enzyme as well as mutants with substitutions at other sites[32]. Several other conserved residues, including K73, E166, S130 and N132, have been subjected to mutagenesis, but their roles remain unclear. The catalytic mechanism of the hydrolysis remains elusive. The structure of the binding site is discussed in greater detail in Chapter 3.

The class A ß-lactamases are well suited to studies of the factors that determine enzyme specificity. The clinical significance of these enzymes has led to the identification and characterization of a large number of members of this family whose sequences and kinetic behavior may be compared. The wealth of PBP inhibitors produced by antibiotics research provides large numbers of diverse compounds which may be studied as substrates and potential substrates for the ß-lactamases. While crystallization has been difficult, structural data are now available at . In addition, RTEM-1 ß-lactamase is suitable experimentally; it is a monomeric, stable enzyme that is readily purified in high yield and is easily assayed directly using spectrophotometric techniques. The gene is available in a convenient vector for mutagenesis studies, and the ease of phenotypic screening and selection provides rapid identification of active or inactive mutants. In short, the ß-lactamases are well characterized and experimentally suitable enzymes.

In addition to contributing to a general understanding of enzymes, studies of RTEM-1 ß-lactamase may provide useful information for the design and development of ß-lactam antibiotics. While resistance to the ß-lactam antibiotics may arise through alterations of the target PBPs, the most common form of resistance is ß-lactamase production. As ß-lactamase-mediated resistance spread, newer antibiotics were developed that were stable to the ß-lactamases. The clinical use of the new antibiotics creates an environmental pressure that selects for naturally occurring ß-lactamase mutants that are able to hydrolyze the antibiotics and confer resistance to them. This has been called the "ß-lactamase cycle" by Sykes and Bush[33]. If the substrate-specificity determinants of the ß-lactamase are understood, it may be possible to slow the cycle by the informed design of ß-lactam antibiotics and to design specific inhibitors of the ß-lactamases.

I have taken three approaches to the study of the substrate-specificity determinants of RTEM-1 ß-lactamase. In Chapter 2, I describe the construction and analysis of two PBP/RTEM chimeras designed to study the possibility of reducing the amidase specificity of RTEM-1 and allowing the acceptance of a D-Ala-D-Ala substrate. The work described in

Chapter 3 consists of the construction and analysis of mutant RTEM-1 ß-lactamases that incorporated sequence differences among the penicillin-recognizing enzyme family. The effect of the mutations on the penam vs. cephem preferences was studied. In Chapter 4, the roles of three residues found substituted in the naturally occurring extended-spectrum ß-lactamases were studied by simultaneous randomization. The study takes advantage of the variety of substrates and potential substrates of the ß-lactamases and the phenotypic selection that the enzyme provides.

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Chapter 2

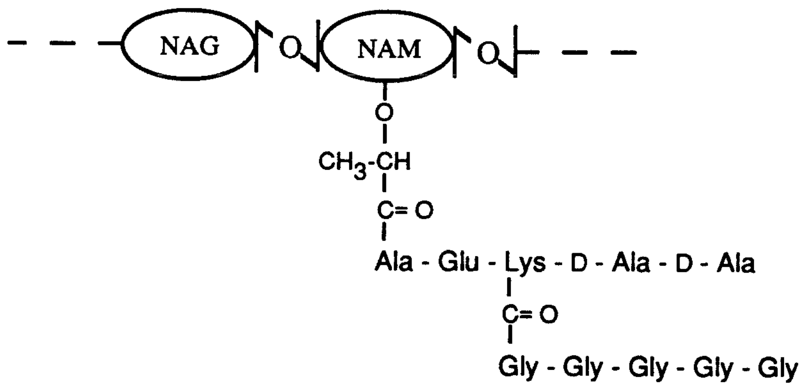
Exchange of a Secondary Structural Element Between Two Closely Related Proteins: Chimeras of RTEM-1 and PBP5 of *E. coli*

Introduction

Bacteria are contained within a peptidoglycan sacculus that provides structural support to the cell. Without it, the bacteria will readily lyse in response to osmotic pressure. The peptidoglycan consists of alternating N-acetylglucosamine (NAG) and N-acetylmuramate (NAM) sugars crosslinked by peptides bound to the NAM sugars (Figure 1). The construction of the structure begins with the growth of the pentapeptide chain on an activated NAM sugar. A NAG is then added to the NAM, followed by the addition of glycines to the ε-amino group of the pentapeptide to form the pentaglycine chain. The disaccharide is transferred to the growing polymer. Finally, the peptidoglycan is crosslinked by the joining of the pentapeptide of one sugar with the pentaglycine of another via a transpeptidase reaction. In this final transpeptidase reaction, the terminal D-Ala of the pentapeptide is removed. A group of enzymes acting as D-Ala-D-Ala carboxypeptidases are also found, which remove the terminal D-Ala without crosslinking. While the physiological role of this activity is not known, it has been suggested as a mechanism for the regulation of the degree of crosslinking of the cell wall[1].

Since the cell wall is unique and necessary to bacteria, reactions in its construction are ideal targets for antimicrobial therapies. Examples of these are D-cycloserine inhibition of alanine racemase and D-Ala-D-Ala ligase to block completion of the pentapeptide, phosphonomycin inhibition of the activation of the NAM, vancomycin inhibition of transglycosylation and release of a carrier lipid, and bacitracin inhibition of the regeneration of the lipid[2]. The final step in cell wall construction, the transpeptidase and carboxypeptidase reactions, is blocked by the ß-lactam antibiotics. As described in Chapter 1, these antibiotics consist of an essential nucleus of a four-membered ß-lactam ring, which may be fused to a five- or six-membered ring. The most common ß-lactam

A.



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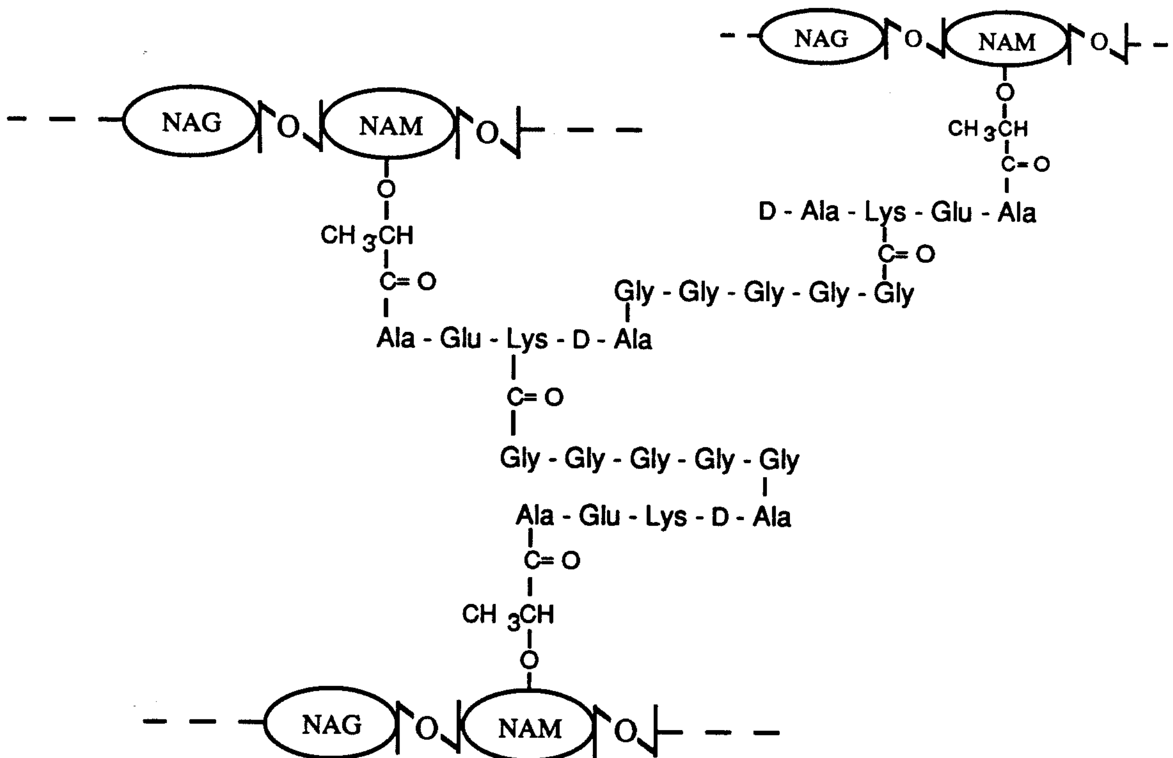

Figure 1. The structure of the peptidoglycan of *S. aureus*. Small modifications are found in other species. A) Before crosslinking B) Crosslinked peptidoglycan.

antibiotics are based on the penam or cephem ring structures and are often represented by the earliest known members of these groups, benzylpenicillin and cephalosporin C.

The enzyme targets of the ß-lactam antibiotics are identified by incubation of bacteria with radiolabelled benzylpenicillin followed by polyacrylamide gel electrophoresis of whole cell extracts. Proteins that covalent bind to the antibiotic are identified as bands by autoradiography. These proteins are termed penicillin-binding proteins (PBPs) and are numbered by decreasing molecular weight. This system of labelling the PBPs leads to the same name being given to nonanalogous proteins from different bacteria. For example, PBP2 of *N. gonorrhoeae* is not necessarily analogous to PBP2 of *E. coli.*

The first step in the transpeptidase or carboxypeptidase reactions is the nucleophilic attack of a serine of the PBP on the nitrogen of the terminal -D-Ala-D-Ala peptide to form an acyl-enzyme intermediate, resulting in the release of the terminal D-Ala. The acyl-enzyme is then subjected to nucleophilic attack either by the glycine of the peptidoglycan in the case of the transpeptidase reaction or by water in the case of the carboxypeptidase reaction. Because of a structural analogy with the -D-Ala-D-Ala peptide, the ß-lactams can form a similar acyl-enzyme intermediate with the PBPs. In this case, however, the acyl-enzyme is stable and the PBP is inhibited (Figure 2).

The PBPs have been divided into two groups. The high molecular weight PBPs (60-100 kDa) are bifunctional, acting as both penicillin-insensitive transglycosylases and penicillin-sensitive transpeptidases. The two functions are performed by two separate domains of the enzymes[3-5]. The low molecular weight PBPs (35-50 kDa) function as carboxypeptidases. Some of the low molecular weight PBPs carry out transpeptidase reactions under certain conditions *in vitro*, but it is not known if this reflects an *in vivo* role. The roles of the PBPs in the cell wall biosynthesis may be elucidated by comparison of the sensitivity of the PBPs to various antibiotics *in vitro* with morphological changes in bacteria grown in the presence of the antibiotics. In general, it is the inhibition of the transpeptidase activity of the high molecular weight PBPs that is lethal, while inhibition of

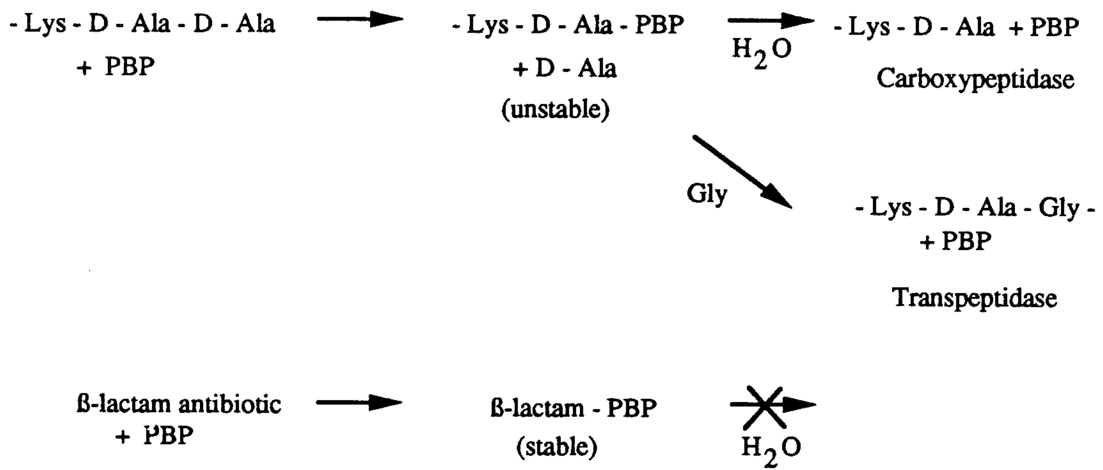

Figure 2. Mechanism of the carboxypeptidase and transpeptidase reactions of the penicillin-binding proteins and their inhibition with the ß-lactam antibiotics.

the carboxypeptidase activity of the low molecular weight PBPs is well tolerated. Often more than one PBP must be inhibited for lysis to occur, accounting for the synergism between some combinations of ß-lactam antibiotics in clinical therapy[3]. Eight PBPs have been identified in *E. coli*. A summary of these enzymes is given in Table 1.

Table 1. The PBPs of *E. coli* [a]

Protein	MW	Activity	Function (Effect of inhibition)	Sensitivity to ß-lactams
1a&1b	91	Bifunctional	Elongation (rapid lysis if both 1a & 1b are inactivated)	cephs. >pens.
2	66	Bifunctional (with rod A)	Cell Shape (large round cells)	pens. >cephs.
3	60	Bifunctional	Cell Division (filamentation)	
4	49	tpase/cpase	Cpase 1A (no defect)	
5	42	cpase	Cpase 1B	pens. >cephs.
6	40	cpase	Cpase 1B (no defect)	pens. >cephs.
7	30	unknown	Unknown (lysis of non-growing cells)	pens. >cephs.

[a] Ref. 3, 6, 7, 8

Bacterial strains resistant to ß-lactam antibiotics may arise by mutations in the target PBP, which reduce the affinity for the ß-lactam or by increased expression of a compensatory PBP. Both mechanisms of resistance have been identified in methicillin-resistant *S. aureus* and non-ß-lactamase producing *N. gonorrhoeae* as well as in several other pathogenic bacterial species. More commonly, however, resistance is due to the production of a ß-lactamase that hydrolyzes the ß-lactam bond, destroying the antibiotic.

As discussed in Chapter 1, there are analogous structural and functional features among the serine ß-lactamases and the PBPs.

In this work, two chimeras were constructed between the ß-lactamase RTEM-1 and PBP5 of *E. coli* to address two general questions. The first is the question of substrate specificity. When compared to the PBPs, the ß-lactamases must restrict the accepted substrates; i.e., the PBP will react with both the ß-lactams and the D-Ala-D-Ala peptide, while the ß-lactamases will not accept the D-Ala-D-Ala peptide. The lack of carboxypeptidase activity in the ß-lactamase is necessary to cell survival as demonstrated by the morphological changes seen in *E. coli* expressing slightly increased levels of the carboxypeptidase PBP5[9]. What features of the enzyme's structure account for the increased specificity of the ß-lactamases? The second question addressed is one of protein stability. In this work, the twelve amino acids comprising the active site helix of RTEM-1 ß-lactamase are replaced with the analogous sequence of PBP5 of *E. coli*. While the sequence taken from PBP5 is expected to form an α-helix in the PBP5 structure, the contacts between the helix residues and the surrounding structure in the RTEM-1 will clearly be different from the contacts made by the helix residues in PBP5. What effect will this have on the stability of the chimera, and will the twelve residues adopt a helical structure?

In addition to the considerations discussed in Chapter 1, the use of the penicillin-recognizing enzyme family to address these questions is appropriate because of the likely evolutionary link between these enzymes. This link suggests that it may be possible to alter the properties of the ß-lactamase with limited sequence changes in what is essentially the reverse of evolution.

Prior to initiation of this study, an RTEM-1/PBP5 chimera was constructed that replaced the nineteen nonconserved residues between residues 56 and 77 in the RTEM-1 sequence with the corresponding sequence from PBP5 with an unintentional insertion of a glutamate residue between residues 59 and 60 (Y.H.C., Figure 3). This chimera was

```
            50                  60                  *                   80
RTEM-1      D L N S G K I L E - S F R P E E R F P M M S T F K V L L C G A V L S R V
PBP5        D Y N S G K V L A - E Q N A D V R R D P A S L T K M M T S Y V I G Q A M
Y.H.C.      D L N S G K V L A E E Q N A D V R R D P A S L T K M M T S G A V L S R V
M.R.L.      D L N S G K V L A - E Q N A D V R R D P A S L T K M M T S G A V L S R V
Chimera1    D L N S G K I L E - S F R P E E R F P M M S L T K M M T S Y V I G Q R V
Chimera2    D L N S G K I L E - S F R P E E R F P M M S L T K M M T C Y V I G Q R V
```

Figure 3. Sequences of RTEM-1 ß-lactamase, PBP5 of *E.coli* and constructed chimeras around the active site serine. The active site serine is labelled with an asterisk (*). Numbering is according to Ambler (Ref. 10) for the ß-lactamases. The active site serine is residue 44 in the numbering of the PBP5 (Ref. 11). Residues changed in the chimeras are underlined. Y.H.C.- Chimera constructed and studied by Y.H. Chang (Ref. 12). M.R.L. - Chimera constructed and studied by M.R. Labgold (Ref. 13). Chimera1 and Chimera2 are described in this work.

designed based upon the sequence homology between PBP5 and RTEM-1, as the project was performed before high-resolution structural data for the serine ß-lactamases became available. The chimera was active, with turnover numbers of 3.5×10^{-3} min^{-1} on the carboxypeptidase substrate ^{3}H-diacetyl-Lys-D-Ala-D-Ala (1.3% of the activity of the wild-type PBP5), and 1.3 sec^{-1} on the ß-lactamase substrate benzylpenicillin.[12] When the crystal structure of *S. aureus* PC1 ß-lactamase was solved[14] the sequence replaced in the chimera was shown to comprise a strand of ß-sheet well removed from the putative substrate binding site, a loop of random coil along the edge of the binding site and the first seven residues of the active site helix.

In this work, chimeric proteins were constructed in which residues 71-82 of RTEM-1 ß-lactamase were replaced by the analogous sequence, residues 45-56, of *E. coli* PBP5. In both the RTEM-1 and PBP5 structures, these sequences are expected to comprise the active site helices, based on the structural data available for the ß-lactamase of *S. aureus* PC1 and the carboxypeptidase of *Streptomyces* R61 (Figure 4). The sequence of PBP5 was chosen because PBP5 is expected to be more closely related to RTEM-1 than the other PBPs. This expectation is based on the greater sequence homology PBP5 has with the class A ß-lactamases over any of the other PBPs (25% around the active site serine[11,16]) and the weak ß-lactamase activity PBP5 possesses. The half-life of the PBP5-penicilloyl acyl-enzyme is twelve minutes at 25°C or five minutes at 30°C, as compared with half-lives greater than sixty minutes for most other PBP-penicilloyl acyl-enzymes[6,17]. The chimeras were designed to leave Arg83 unchanged, as this charged residue may stabilize the α-helix by interacting with the helix dipole. The sequences of RTEM-1 and PBP5 in this region are shown in Figure 3. In the sequence of Chimera1, a disulfide between Cys77 and Cys123 present in RTEM-1 ß-lactamase is removed by the Cys77Ser mutation. The disulfide is not conserved among the class A ß-lactamases[18] but has been shown to be important in the thermal stabilization of the wild-type RTEM-1 ß-lactamase as well as RTEM-1 mutants with substitutions at other sites[19]. Chimera2 consists of the same

replacement as Chimera1 with the exception of Cys77, leaving the potential disulfide intact. The sequences of these two chimeras are shown in Figure 3.

A)

B)

Figure 4. Ribbon representations of the structures of two penicillin-recognizing enzymes as determined by X-ray crystallography. A) The ß-lactamase of *S. aureus* PC1, 2.5Å resolution (Ref. 14). Residues changed in this study are shaded. B) The water soluble carboxypeptidase/transpeptidase of *Streptomyces* R61 at 2.8Å resolution (Ref. 15).

Materials and Methods

Enzymes and Chemicals

Restriction enzymes EagI, NdeI and AatII were purchased from New England Biolabs. All other restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim Biochemicals. All antibiotics and diacetyl-Lys-D-Ala-D-lactate were purchased from Sigma. [^{14}C]-Benzylpenicillin (59mCi/mmole) and Amplify fluorographic reagent were purchased from Amersham. En^{3}Hance fluorographic reagent was purchased from DuPont. All bacterial culture media were purchased from Difco Laboratories.

DNA

Oligonucleotides were synthesized by the Caltech Microchemical Facility using an Applied Biosystems automated DNA synthesizer. One third of a 0.2µM scale synthesis product was resuspended in 20µl loading buffer (80% formamide, 50mM Tris-borate, 1mM EDTA, 0.1% (w/v) each xylene cyanol and bromophenol blue), and purified by polyacrylamide gel electrophoresis (15% 19:1 acrylamide : N,N'-methylenebis-acrylamide, 40% (w/v) urea), in TBE buffer (89mM Tris, 89mM boric acid, 0.2mM EDTA). DNA was visualized by UV shadowing using a fluorescent TLC indicator plate, and the bands were excised, crushed, and soaked overnight in 200mM NaCl, 10mM Tris-HCl pH 7.6, 1mM EDTA at 37°C. Oligonucleotides used in the cassette mutagenesis construction of Chimera1 were subsequently loaded on a NACS PrePac™ column (Bethesda Research Laboratories), washed and eluted in 600µl high-salt buffer (2M NaCl, 10mM Tris-HCl pH 7.2, 1mM EDTA) as per manufacturer's instructions. Desalting was performed using a Sephadex G-25 spin column (Pharmacia, 3mls gel volume in 10mM Tris-HCl pH. 7.5, 1mM EDTA). For oligonucleotides used as primers in DNA sequencing or in site-directed mutagenesis, supernatants from soaked gel slices were

purified using a Sephadex G-25 spin column only, without the NACS PrePac™ column purification. Plasmid pBR322 was purchased from International Biotechnologies Inc. (IBI) A modified plasmid pBR322 containing a silent mutation that introduces an AvaI restriction site at position 3972 was previously constructed by S. Schultz[20]. Plasmid pJN was prepared from D1210 *E. coli* using standard alkaline lysis techniques followed by CsCl gradient purification[21]. Bacteriophage M13mp19 (replicative form) was purchased from Bethesda Research Laboratories. Single-stranded DNA for sequencing was prepared according to Amersham recommendations[22]. Absorbance at 260nm was used to estimate DNA concentrations.

Restriction digests were performed according to manufacturers' recommendations with one exception. The enzyme EagI was found to cleave incompletely at the recommended temperature of 37°C, and digests using this enzyme were performed at room temperature overnight. Restriction fragments were isolated by 1.2% agarose gel electrophoresis (IBI) in TAE buffer (4.84g/L Tris base, 1.14ml/L acetic acid, 1mM EDTA) and isolated using the IBI UEA electroeluter according to the manufacturer's instructions.

DNA Sequencing

DNA sequencing was carried out using the Sequenase™ sequencing system from United States Biochemical Co. as recommended[23], with the following modifications: Double-stranded DNA was prepared by standard mini-prep alkaline lysis of 2ml overnight cultures[21]. After removal of cellular debris, the supernatant was extracted 3-5 times with TE-saturated phenol (TE: 10mM Tris-HCl pH 7.5, 1mM EDTA) and once with 1:1 phenol/chloroform. The DNA was precipitated with ethanol and washed twice with cold 70% ethanol. The NaOH denaturation of double-stranded DNA was performed by the addition of 20μl of 2M NaOH to the dry DNA pellet, incubation at room temperature for five minutes followed by precipitation with ethanol. In later work on Chimera2, denaturation and primer annealing were achieved in one step. The template DNA (1-3μg)

was mixed with the primer (100ng) in 10μl buffer, placed in a boiling water bath for four minutes, and frozen rapidly in a dry ice/ethanol bath. The remainder of the protocol follows the manufacturer's recommendations[23]. Oligonucleotide primers were 17-18 bases in length. Gel electrophoresis was performed using 8% polyacrylamide gels (19:1 acrylamide : N,N'-methylenebisacrylamide, 7M urea) run with TBE buffer (89mM Tris, 89mM boric acid, 0.2mM EDTA) or 5% polyacrylamide gels run with a modified TBE buffer (133mM Tris, 45mM boric acid, 2.5mM EDTA).

Construction of Chimera1 by Cassette Mutagenesis

Chimera1 was constructed through the insertion of a synthetic cassette between a HincII and a constructed AvaI site in the RTEM-1 ß-lactamase gene of pBR322 (Figure 5). The presence of other AvaI and HincII restriction sites in the pBR322 plasmid necessitated the use of a third enzyme and a three piece ligation. The enzyme EagI was used for this purpose.

The oligonucleotides used were 64 and 68 bases in length, with the following sequences (written in the 5' to 3' direction):

CCGAGGAACGTTTTCCAATGATGTCGTTAACGAAAATGATGACGTCATATGTTATCGGTCAAC-
GTGTT

and

AACACGTTGACCGATAACATATGACGTCATCATTTTCGTTAACGACATCATTGGAAAACGTTAA.

The oligonucleotides were mixed (0.2 pmol/μl each in 10mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 50mM NaCl, 1mM dithioerythritol), heated to 95°C in a water bath, and allowed to cool slowly to room temperature to anneal the complimentary strands. The ligation was performed stepwise. The restriction fragments were first ligated at a concentration of 20μg/ml DNA in 50mM Tris-HCl pH 7.6, 10mM $MgCl_2$, 1mM spermidine, 10mM dithiothreitol, 0.4mM ATP and 5U T4 DNA ligase in a total volume of 100μl at 15°C overnight. DNA was precipitated with ethanol, dried, resuspended (10mM Tris-HCl

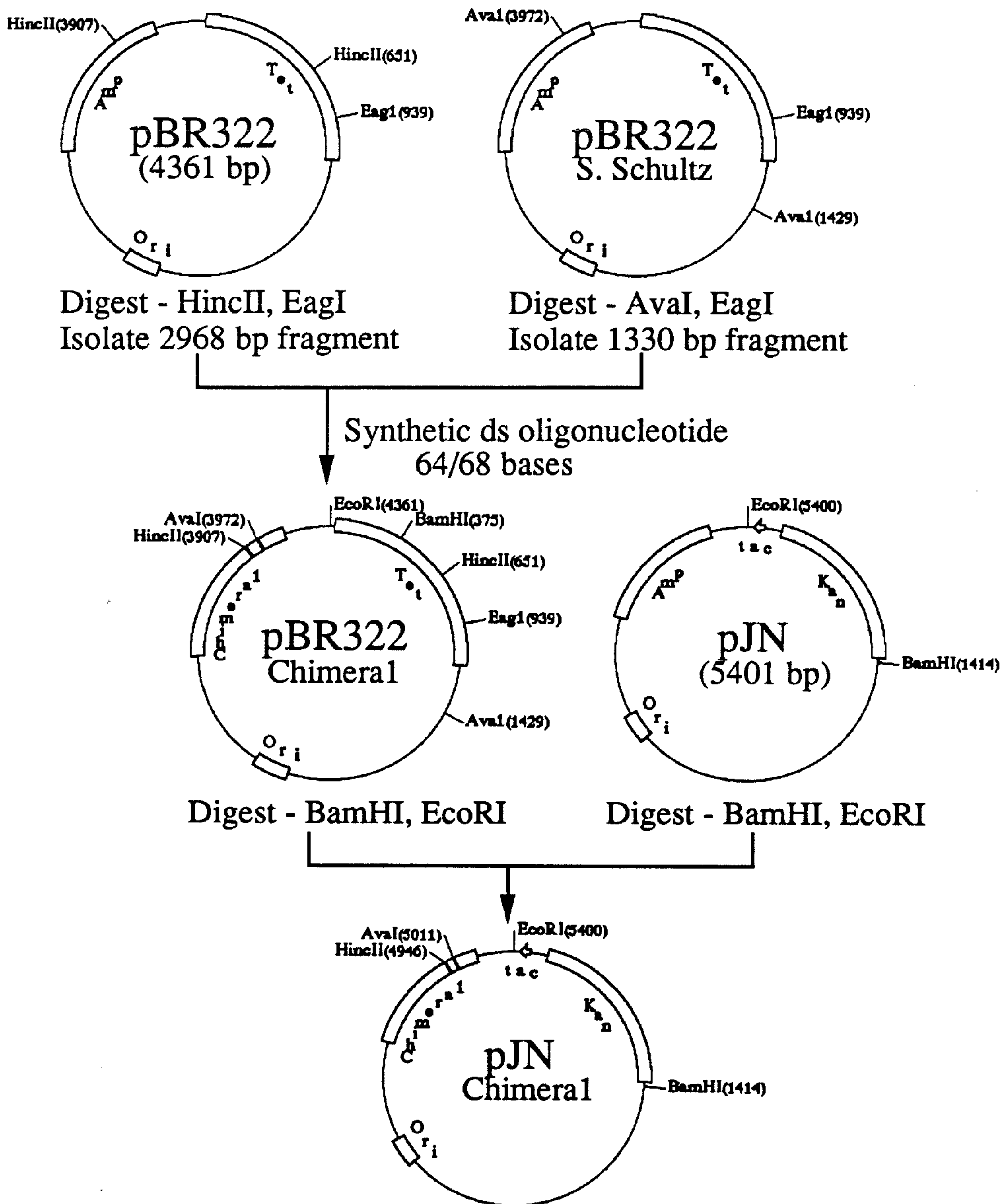

Figure 5. Construction of Chimera1 by mutagenesis and transfer of the Chimera1 gene to the pJN vector for expression.

pH 7.5, 1mM EDTA) and used in ligation reactions with the annealed oligonucleotides (1:10 molar ratio of vector to synthetic insert) performed under the same conditions. Ligation mixtures were used to transform LS-1 *E. coli* to tetracycline resistance by the method of Hanahan.[24] Presence of the insert was verified by DNA sequencing. The Chimera1 gene was then transferred to the pJN expression vector.[25] Both the pJN vector and the pBR322-Chimera1 vector were digested to completion with EcoRI and BamHI and ligated under conditions given above without isolation of restriction fragments. D1210 *E. coli* were transformed and selected for kanamycin resistance. Colonies carrying the pJN plasmid with the chimeric ß-lactamase were identified by an inability to grow on ampicillin-containing plates. The sequence of the Chimera1 gene was verified by DNA sequencing.

Construction of Chimera2 by Oligonucleotide-Directed Mutagenesis

Chimera2 was constructed by oligonucleotide-directed mutagenesis of Chimera1 to reverse the Cys77Ser mutation to the wild-type Cys77 (Figure 6). The Chimera1 gene was inserted into the multiple cloning site of M13mp19 between the EcoRI and SalI restriction sites. Plasmid pBR322-Chimera1 and phage DNA were digested together, extracted with phenol, precipitated with ethanol, resuspended and ligated under the following conditions: 20µg/ml DNA, ligase buffer (International Biotechnologies Inc., 25mM Tris-HCl pH 7.8, 10mM $MgCl_2$, 4mM ß-mercaptoethanol, 0.4mM ATP), 15°C overnight. TG-1 *E. coli* were transformed with the ligation mixtures and plated as recommended by Amersham[22]. Recombinant phage were identified by the formation of clear plaques and the presence of the Chimera1 insert was verified by DNA sequencing. Oligonucleotide-directed mutagenesis was carried out with the Eckstein selection method[26] using the M13 mutagenesis kit of Amersham as recommended[22]. DNA sequencing verified the presence of Chimera2. The Chimera2 gene was placed under the control of the *tac* promoter in the pJN expression vector in the following manner: M13mp19 RF

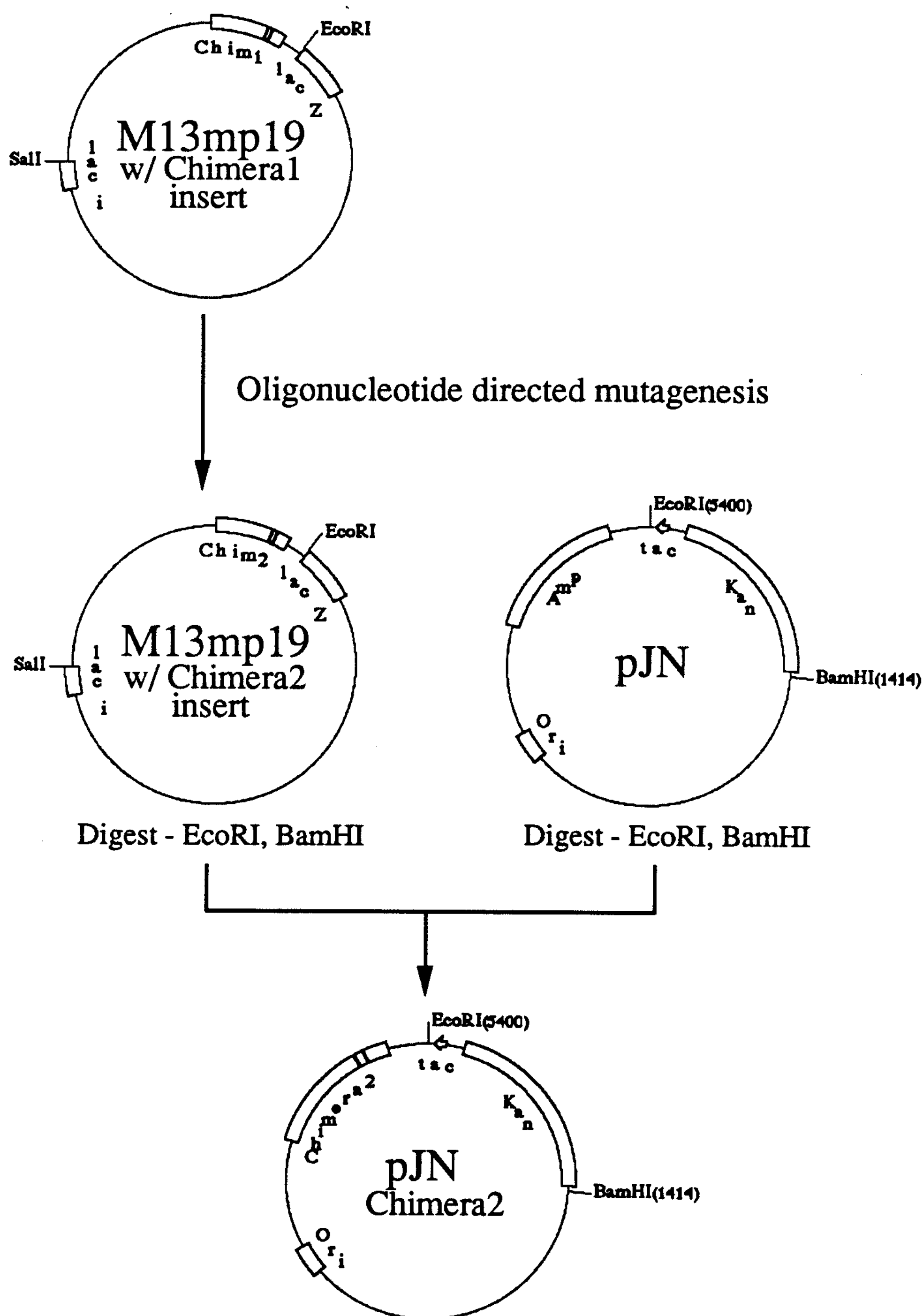

Figure 6. Construction of Chimera2 by mutagenesis and transfer of the Chimera1 gene to the pJN vector for expression.

containing the Chimera2 gene was isolated by standard alkaline lysis methods and digested with EcoRI and SalI. The pJN vector was cleaved with EcoRI, SalI and AvaI. The digestion mixtures were each extracted with phenol followed by chloroform, and the DNA was precipitated with ethanol. Following the isolation of restriction fragments, ligation was carried out under the same conditions used in the transfer the Chimera1 gene to the M13mp19 multiple cloning site. The lac i^Q *E. coli* strain D1210 was transformed to kanamycin resistance with the ligation mixtures by the method of Hanahan[24]. The complete DNA sequence of the Chimera2 gene was verified.

Western Blot Analysis

Western blot analysis of whole-cell lysates was performed to assay the structural stability of the chimeras as reflected by their resistance to cellular proteases. *E. coli* were grown in L broth (10g/L tryptone, 5g/L yeast extract, 5g/L NaCl) containing kanamycin sulfate (50mg/L) to late log phase (ODU_{600nm} = 1) at the indicated temperature. 1.5ml of each cell culture were centrifuged to pellet the cells, which were then resuspended in a volume of loading buffer proportional to the measured absorbance (200µl buffer/ODU, 10% (v/v) glycerol, 3% (w/v) sodium dodecylsulfate (SDS), 31.2mM Tris-HCl pH 6.8, 5% (v/v) ß-mercaptoethanol and 0.05% (w/v) bromophenol blue). Samples were heated to 95°C for five minutes and vortexed briefly. Samples were loaded (30µl each) on a 12% polyacrylamide gel (12% 37.5:1 acrylamide : N,N'-methylenebisacrylamide, 375mM Tris-HCl pH 8.8, 0.1% SDS) with a 4% polyacrylamide stacking gel (4% 37.5:1 acrylamide : N,N'-methylenebisacrylamide, 125mM Tris-HCl pH 6.8, 0.1% SDS) and electrophoresed overnight. (Running buffer is 25mM Tris, 192mM glycine pH 8.3, 0.1% SDS.) Proteins were transferred to nitrocellulose (Schleicher and Scheull BA85, 0.45 micron pore size) using a Bio-Rad TransBlot apparatus with platinum plate electrodes in the following manner: The gel was soaked in the blotting buffer for 30 minutes (25mM Tris, 192mM glycine pH 8.3, 20% (v/v) methanol). The gel was then sandwiched between

nitrocellulose and blotting paper, placed in the TransBlot chamber containing blot buffer and electrophoresed at 150 mA for two and one half hours. The nitrocellulose was washed, incubated in a solution containing normal goat serum to block nonspecific interactions and then incubated in a 1:1000 dilution of rabbit anti-ß-lactamase raised against denatured ß-lactamase as described[25]. The bound antibody was stained using the VectastainABC goat anti-rabbit immunoperoxidase kit (Vector Laboratories) as recommended by the manufacturer. Though the ß-lactamase genes were behind the *tac* promoter in the expression vector pJN in these experiments, the addition of isopropylthiogalactoside (IPTG) was not necessary because the pBR322 ß-lactamase promoter is intact and ß-lactamase is constitutively expressed from this vector.

The reduced and nonreduced forms of ß-lactamase can be easily resolved by SDS-PAGE[27]. To determine if the disulfide between Cys77 and Cys123 is formed in Chimera2, whole cells were resuspended in loading buffer with and without ß-mercaptoethanol, electrophoresed and stained as described above.

Effect of Expression of Chimeras on Cell Morphology

The effect of expression of Chimera1 or Chimera2 on cell morphology was studied by light microscopy. *E. coli* D1210 harboring the pJN plasmid with the chimeric ß-lactamases were grown in L-media (10g/L tryptone, 5g/L yeast extract, 5g/L NaCl) in the presence of kanamycin sulfate (50mg/L). Cells were grown to mid-log phase (ODU ~ 0.5) at 30°C and IPTG was added to a final concentration of 0.1mM. Cells were observed by light microscopy after 1 hour, 3 hours and overnight growth.

Expression and Purification of Chimera2

The gene encoding Chimera2 was placed under the control of the *tac* promoter in the expression vector pJN[25,28]. Protein expression was induced by IPTG and protein isolated by osmotic extrusion as described[25,29] with some modification. Cells were grown to near saturation at 30°C in XB media (25g/L tryptone, 7.5g/L yeast extract, 20mM $MgSO_4$,

50mM Tris-HCl pH 7.5) containing kanamycin sulfate (50mg/L). IPTG was added to a concentration of 0.1mM (24mg/L). Cultures were immediately placed on ice and transferred to 4°C, where they were shaken for 15-20 minutes. The remainder of the procedure was carried out at 4°C. Cells were pelleted (GSA, 5K rpm, 10 min. or J6, 4.2K rpm, 20 min.), resuspended in cold sucrose solution (20ml/g wet cells, 450g/L sucrose, 0.5g/L EDTA, 25mM Tris-HCl pH 7.0) and shaken for 30 minutes. Cells were pelleted again (GSA, 10K rpm, 30 min.), resuspended in ice-cold water (20mls/g wet cells), and shaken vigorously for 30 minutes. Cells were pelleted again (GSA, 10K rpm, 20min.) and the supernatant was removed and filtered through a 0.2micron sterile filter. The volume of the protein solution was reduced by ultrafiltration (Amicon, YM10 membrane). The protein solution was then loaded onto a Q-Sepharose FF (Pharmacia) column (5cm x 5cm) pre-equilibrated with 25mM triethanolamine (TEA) pH 7.7. The column was washed with 25mM TEA pH 7.7. The protein was eluted with 300mM NaCl, 25mM TEA pH 7.65. The eluate was dialyzed overnight against 25mM Tris-HCl pH 7.5, 1mM EDTA. The sample volume was again reduced by ultrafiltration. Protein was purified by FPLC ion-exchange chromatography (Pharmacia) using the protocol developed by D.M. Long. The sample was loaded onto a MonoQ 10/10 column (Pharmacia) with 25mM TEA pH 7.7 and eluted by gradient formation using 1M NaCl, 25mM TEA pH 7.65 at a flow rate of 2.5 ml/min. (t=0 min,0% B; t=64, 19%B; t=77, 100%B). Chimera2 eluted with approximately 120mM NaCl. Fractions containing Chimera2 were determined by SDS-PAGE stained with Coomassie blue in the following manner: 800µl aliquots of each fraction were desalted by Sephadex G-10 spin column (Pharmacia, 3mls gel volume in 10mM Tris-HCl pH 7.5, 1mM EDTA) and lyophilized. Pellets were resuspended in 15µl loading buffer (10% (v/v) glycerol, 3% (w/v) SDS, 31.2mM Tris-HCl pH 6.8, 5% (v/v) ß-mercaptoethanol and 0.05% (w/v) bromophenol blue) and subjected to SDS-PAGE (12% polyacrylamide gel). Gels were stained in the following manner: Fix: Soak gel in 50% (v/v) methanol, 12% (v/v) acetic acid for one hour. Stain: 0.1% (w/v) Coomassie brilliant

blue in 25% (v/v) ethanol, 8% (v/v) acetic acid, 60°C, 10-15 min. Destain: Soak gel in several changes of 25% (v/v) ethanol, 8% (v/v) acetic acid. Appropriate fractions were dialyzed against 100mM potassium phosphate pH 7.0. Wild-type ß-lactamase was expressed and purified by the same protocol with the omission of the Q-Sepharose FF column.

Protein concentration was estimated by UV absorbance at 280nm. The extinction coefficient of wild-type ß-lactamase at this wavelength has been reported as 29,400 $M^{-1}cm^{-1}$.[29] The chimeric enzymes contain one additional chromogenic residue, a tyrosine at position 78. Based on the reported extinction coefficients of tyrosine as 1300 $M^{-1}cm^{-1}$ [30], and that of the wild-type ß-lactamase, the extinction coefficients of the chimeric enzymes were approximated as 30,700 $M^{-1}cm^{-1}$.

During the development of the purification scheme, chromatofocusing of Chimera2 and wild-type RTEM-1 was performed using a MonoP 5/20 column (Pharmacia). The column was pre-equilibrated with 25mM Tris-HCl pH 7.1. 10% (v/v) Polybuffer 74-HCl pH 4.0 (Pharmacia) was used to create the pH gradient and to elute the proteins at a flow rate of 0.8 ml/min. The pH's of 0.8ml fractions collected during a blank run were measured and plotted vs. fraction number to verify creation of a linear pH gradient.

Circular Dichroism

Circular dichroism (CD) was used to study differences in secondary structure between wild-type RTEM-1 and Chimera2, and to study the thermal denaturation of Chimera2. CD scans were taken using a Jasco J600 spectropolarimeter under the following conditions: Protein concentrations were 1-5μM in 100mM potassium phosphate pH 7.0, using a 1mm path length cell, time constant = 2 sec, band width = 1nm, scan rate = 20nm/min. The program SSEAX (Jasco) was used to fit a set of standard reference spectra of five proteins of known structure to the measured spectra of Chimera2 and RTEM-1 to estimate the secondary structural features.

Thermal denaturation was studied by changes in ellipticity at 222nm with increasing temperature[31]. Conditions used were: pathlength = 10mm, slit width = 2µm, 100mM potassium phosphate pH 7.0. The temperature of the recirculator was increased stepwise and allowed to equilibrate 5-10 minutes before the temperature and the ellipticity were recorded.

Activity Assays

Samples were assayed for ß-lactamase activity on the substrate benzylpenicillin by the method of D. M. Long (personal communication). A known amount of enzyme is added to 2ml benzylpenicillin in 100mM potassium phosphate pH 7.0. The sample is mixed, transferred to a water-jacketed cell, and the ellipticity at 231.8nm is measured over time (Slit width = 1500µm, time constant = 0.5 sec.). The ellipticity of the hydrolyzed product is zero at this wavelength allowing the concentration of the substrate to be obtained directly from the CD signal. (Molar ellipticity of benzylpenicillin is 393deg cm^{-1} M^{-1}.) Initial rates were determined by a linear regression fit of the time-course data. Kinetic constants were determined using the Hanes-Woolf replot method[32]. Because of the small signal from the protein at this wavelength, large concentrations of protein may be used to detect low levels of activity. In contrast, earlier assay methods using UV are limited by the high absorbance of both protein and substrate and high concentrations of either are not experimentally accessible.

An assay for carboxypeptidase activity was developed. The products and reactants were separated by HPLC and quantified using UV absorbance at 214nm. The activity of the enzyme on the depsipeptide diacetyl-Lys-D-Ala-D-lactate was measured because the more reactive ester function allows a greater sensitivity to low levels of activity when acylation is the rate limiting step[33]. Protein was incubated with substrate under various conditions, and 100µl aliquots of reaction mixtures were injected onto a C18 reverse phase HPLC column (SynChropak RP-P100, 25cm x 5mm, SynChrom Inc.). The substrate and

product were eluted with the following gradient: Buffer A: 0.1% trifluoroacetic acid (TFA, Pierce Chemicals), buffer B: 80% acetonitrile, 0.1% TFA, t=0, 0% B; t=3, 0% B; t=26, 15%B; t=35, 100%B, flow rate = 1 ml/min. The base-catalysed hydrolysis of the ester (5% triethylamine, pH 11.6, 2 hours, 37°C) provided diacetyl-Lys-D-Ala used in the method development. The substrate and product were completely resolved with retention times of 12 minutes and 15 minutes for the product and substrate, respectively. Quantification was performed by integration of the peaks measured at 214nm.

Radiolabelling of Proteins with [^{14}C]-Benzylpenicillin

Protein samples were incubated with radiolabelled benzylpenicillin, subjected to SDS-PAGE and autoradiographed to detect the formation of an acyl-enzyme. Crude protein preparations, prepared by osmotic extrusion and estimated to be 0.3 mg/ml Chimera2 based on yields of purified protein obtained previously, were incubated with labelled benzylpenicillin at concentrations ranging from 18-90 µg/ml Chimera2 at room temperature for 30 minutes in a final volume of 55µl. 10µl of loading buffer were added and samples were heated immediately to 95°C for 3 minutes (10% (w/v) SDS, 33% glycerol, 100mM Tris-HCl pH 6.8, 0.15% (w/v) bromophenol blue, 166µl/ml ß-mercaptoethanol). Samples were separated by SDS-PAGE (12% polyacrylamide gel, 4% polyacrylamide stacking gel). Detection of radioactivity was increased using the fluorographic reagent En^{3}Hance (DuPont) according to manufacturer's recommendations. The gel was then dried and autoradiographed. The experiment was repeated using purified protein samples at concentrations of 0.1-0.3 mg/ml, benzylpenicillin concentrations of 0.015-1.3 mg/ml, incubation times of 10-30 minutes and temperatures of 16°C or room temperature in 30µl reaction mixtures. 5µl loading buffer was added and the total sample analyzed by SDS-PAGE and autoradiographed as above. ß-lactamase mutants E166C and E166Y[34] were used as controls. For these experiments, Amplify (Amersham) was used for fluorographic enhancement.

Results and Discussion

Chimeric receptors, chimeric antibodies and protein/antibody chimeras have been studied to elucidate roles of various domains as well as to develop potential therapeutic agents[35-38]. In enzymology, the roles of domains and secondary structural elements in enzyme catalysis have been studied by the construction of chimeras of related proteins. Some examples of these are the domain interchange between human and yeast phosphoglycerate kinase[39], the redesign of the recognition helix of 434 repressor to contain the sequence of the P22 repressor[40], and the 28 amino acid substitution in a P450 hydroxylase[41].

In this work, the sequence of the putative active site helix of RTEM-1 is replaced by the corresponding sequence from PBP5 of *E. coli.* The active site helix is directed away from the binding site, and residues 74-82 are not expected to contact the substrate directly (Figure 4). The question addressed by the production of these chimeras is this: Can a change in the amino acid sequence in this segment reduce the specificity of the enzyme? The orientation of an α-helix formed by the replaced segment may be altered and may affect the orientation of the residues in the binding pocket as well as the helix dipole. A change in the sequence may lead to a more flexible enzyme to allow acceptance of a wider range of substrates as was seen for mutations in α-lytic protease[42]. Evidence of the conformational flexibility of the class A ß-lactamases in the presence of differing substrates[43-45] suggests that it may be possible, by mutation, to allow the enzyme access to conformations, and hence to activities, not accessible to the wild-type enzyme. These possibilities also suggest that mutations leading to a less selective enzyme may also lead to an enzyme less stable to thermal denaturation or proteolytic degradation. While this work addresses the question of the stability of the chimeric enzymes, the goal was to create chimeric enzymes that might

have reduced specificity, and the potential destabilizing effects of the substitutions were not a criterion in the design.

Another question addressed by the construction of these two chimeras is the effect of the disulfide bond on the stability of a mutant with many changes in the amino acid sequence. The incorporation of nonnative disulfide bonds to increase the stability of a protein has enjoyed some success, and stabilized forms of subtilisin[46,47], T4 lysozyme[48,49], λ-repressor[50], dihydrofolate reductase[51], and ribonuclease T1[52] have been created. The criteria important in the design of disulfide bonds to stabilize a protein structure are not completely understood, and some incorporated disulfides have led to decreased protein stability. This may be due to the removal of favorable interactions when the wild-type residue is changed to a cysteine, or to the incorporation of strain when the geometry of the Cys-Cys interaction is not ideal for disulfide formation[48]. Incorporated disulfide bonds have also been used to control enzyme activity. The formation of a crosslink in the active site cleft of T4 lysozyme allowed the enzyme to be switched on and off by the redox state of the enzyme[53], and the formation of a crosslink between two α-helices of troponin C that prevented a change in their relative positions during a key conformational change abolished the regulatory activity of the enzyme[54].

In the case of Chimera1 and Chimera2, the problem is reversed, namely, how does the removal of a disulfide with a stabilizing effect on the wild-type enzyme and mutants with single substitutions[19] affect the stability of a chimera with substituted residues in the vicinity of the disulfide? If the altered sequence adopts a structure similar to the wild-type enzyme, the disulfide can be expected to stabilize the structure. However, if the altered sequence does not adopt a structure similar to the wild-type enzyme in the vicinity of Cys77, no prediction can be made on the stabilizing or destabilizing effects of the disulfide.

The phenotypic effects of expression of Chimera2 were studied. Cells harboring plasmid carrying the Chimera2 gene were not able to grow on media containing low levels of ampicillin, suggesting that ß-lactamase activity, if present, was low. If the chimera

possessed sufficient carboxypeptidase activity, expression of the enzyme would result in spherical cells as was seen when the expression of the carboxypeptidase PBP5 was increased. Overexpression of PBP5 is expected to be lethal as suggested by an inability to grow cells harboring a high copy-number plasmid encoding PBP5.[9] No morphological changes were seen in cells expressing Chimera2 under conditions expected to maximize the level of the enzyme present, i.e., lower temperatures and the presence of IPTG. In addition, the presence of the Chimera2 gene does not inhibit the growth of *E. coli*, as the doubling time of the *E. coli* is alike in cells expressing the chimera or the wild-type ß-lactamase.

Western blot analysis of whole-cell lysates was performed to determine the susceptibility of the chimeras to cellular proteases at various temperatures (Figure 7). The chimeric proteins were found to be present in much lower quantities than the wild-type RTEM-1, with the observed levels of the chimeras increasing at lower temperatures. The reduced staining might be explained by a reduction in the antigenicity of the chimeric ß-;lactamases compared to the wild-type enzyme against which antibodies were raised. However, the shapes of the stained protein bands indicated significantly reduced amounts of chimeric protein present; the RTEM-1 bands were broader than the chimera bands, as would be expected if higher amounts of RTEM-1 were loaded on the gel. Western blots showed the Chimera2 to be more stable than Chimera1, suggesting that the disulfide is formed in Chimera2 and increasing the stability of the protein. The formation of the disulfide bond was verified by Western blot analysis of whole cell lysates in the presence and absence of ß-mercaptoethanol. A mobility difference can be easily seen between these two conditions, and two distinct bands were seen when the reduced and unreduced samples of RTEM-1 or Chimera2 were loaded together in the same lane (data not shown). For the disulfide bond to form between Cys77 and Cys123, the two sulfhydryls must be near one another and in the appropriate geometry. Formation of the disulfide suggests that the

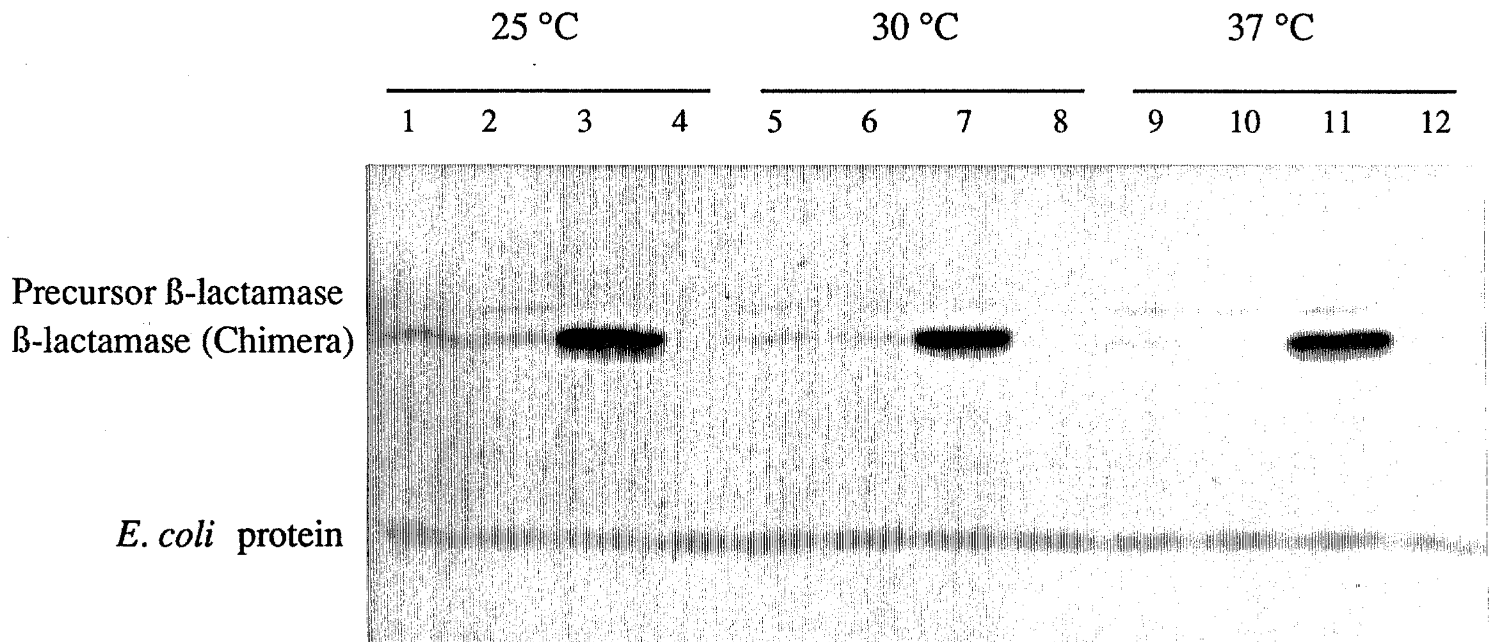

Figure 7. Western blot analysis of wild-type RTEM-1, Chimera1 and Chimera2. Lanes 1, 5 and 9, Chimera2; lanes 2, 6 and 10, Chimera 1; lanes 3, 7 and 11, wild-type RTEM-1; lanes 4, 8 and 12, cells only without plasmid.

α-helix structure of the wild-type enzyme is adopted by the chimera in the vicinity of Cys77.

Because of the instability of Chimera1 demonstrated by Western blot analysis, purification of this enzyme was not attempted. With care, Chimera2 could be prepared with yields of 300μg pure Chimera2 per liter of cell culture. Typical yields of wild-type RTEM-1 were 2-3mg enzyme per liter of cell culture. The standard methods of ß-lactamase preparation by osmotic extrusion were all performed at 4°C. An ion-exchange step was incorporated prior to FPLC purification to obtain better resolution and a higher loading capacity on the FPLC MonoQ ion-exchange column. Samples of adequate purity were obtained as evidenced by SDS-PAGE with Coomassie blue staining (Figure 8). The retention time of the Chimera2 differs substantially from the wild-type enzyme on the ion-exchange column. The wild-type enzyme typically elutes with 78mM NaCl while the Chimera2 typically elutes with 120mM NaCl. This difference was unexpected, given the differences of charged residues between the Chimera2 and the wild-type enzyme, and contrasts with earlier work in our laboratory which has shown that the MonoQ elution profiles of single-site mutant ß-lactamases differ only slightly from the profile of the wild-type RTEM-1 under these conditions. The greater affinity for the ion-exchange column suggests a structural difference between Chimera2 and RTEM-1. A structural difference was also suggested by the difference in the pI values of the two proteins. During the development of the purification protocol, chromatofocusing was performed using a Pharmacia MonoP column. While not characterized in detail, it was observed that Chimera2 eluted at a pH value approximately 2 pH units lower than the wild-type RTEM-1. As these pH measurements were not standardized with other known proteins, the absolute pI values of the enzymes cannot be reported, but the observed difference is striking. This cannot be explained by differences in the number of charged residues between the proteins.

Circular dichroism (CD) is a sensitive indicator of secondary structural elements in peptides or proteins[55]. The CD spectra of RTEM-1 and Chimera2 were measured under

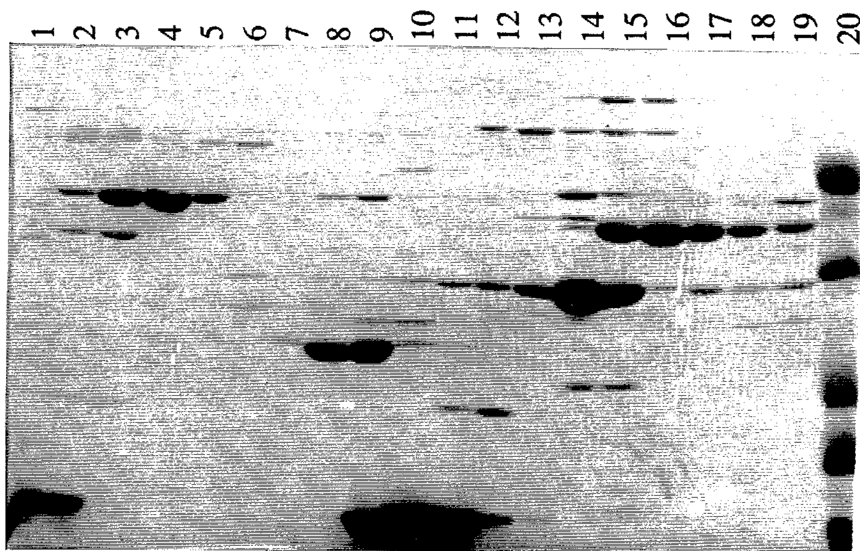

Figure 8. SDS-PAGE of fractions from a MonoQ column purification of Chimera2 stained with Coomassie blue. Lanes 1-19, fractions 25-43; lane 20, prestained molecular weight standards (BRL) of sizes 3kDa, 6kDa, 15kDa, 18kDa, 25kDa, 43kDa. The large band in lane 14 is Chimera2.

similar conditions. The spectra of single-site mutants have been found to be nearly identical to the wild-type enzyme spectrum[55,56]. In contrast, the spectrum of Chimera2 is substantially different from RTEM-1 (Figure 9). To analyze this difference, the program SSEAX was used to correlate the observed spectra with predicted secondary structures. While the method is not quantitative and requires many assumptions, it is used here to compare qualitatively the results obtained for two closely related proteins. The results obtained for the wild-type RTEM-1 correlated well with the expected structure based on the crystal structure of the homologous ß-lactamase from *S. aureus* PC1 (Calculated: 41% α-helix, 17% ß-sheet. From the crystal structure of the *S. aureus* PC1 ß-lactamase: 41% α-helix, 19% ß-sheet.[14]). When the spectrum of the Chimera2 was subjected to this analysis, the results suggested differing amounts of secondary structural elements (Calculated: 36% α-helix, 10% ß-sheet.). Though these numbers cannot be considered to reflect the exact structural features of Chimera2, they do show that a difference exists in the secondary structural features of the wild-type and chimeric ß-lactamase. The complexity of the contribution of differing secondary structural elements to the total protein CD spectrum makes it impossible to say with certainty whether the active site helix is formed or not in the chimera. The difference in the spectra of the two proteins does demonstrate a significant difference in the overall secondary structure.

CD was used to measure the thermal denaturation of Chimera2. The ellipticity at 222nm was measured as a function of temperature (Figure 10). A conformational change of Chimera2 that is reflected in a change in the CD signal occurs between 30-35°C in comparison with a change of wild-type RTEM-1 found to occur between 47-50°C in this work or 55-60°C as reported by others[25]. The observation of the thermal instability of Chimera2 correlates well with the results of the Western blot analysis that demonstrated the significantly increased proteolytic susceptibility of Chimera2 at 37°C vs. room temperature.

The purified Chimera2 showed no ß-lactamase activity against benzylpenicillin. The use of the CD to monitor reaction progress allowed high concentrations of enzyme to be

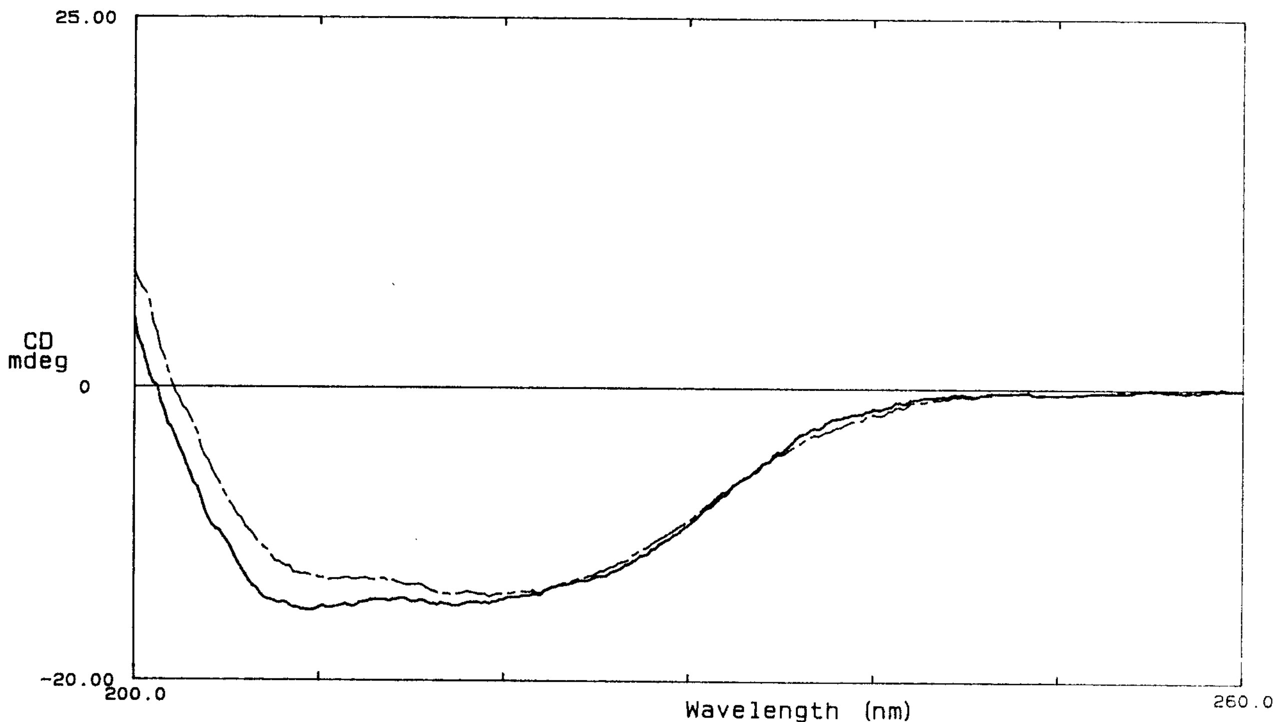

Figure 9. CD spectra of RTEM-1 (Solid line) and Chimera2 (dashed line). Spectra were normalized for differing concentration by the ellipticity at 222nm. Conditions: Protein concentration of ~3μM in 100mM potassium phosphate pH 7.0, path length 1mm.

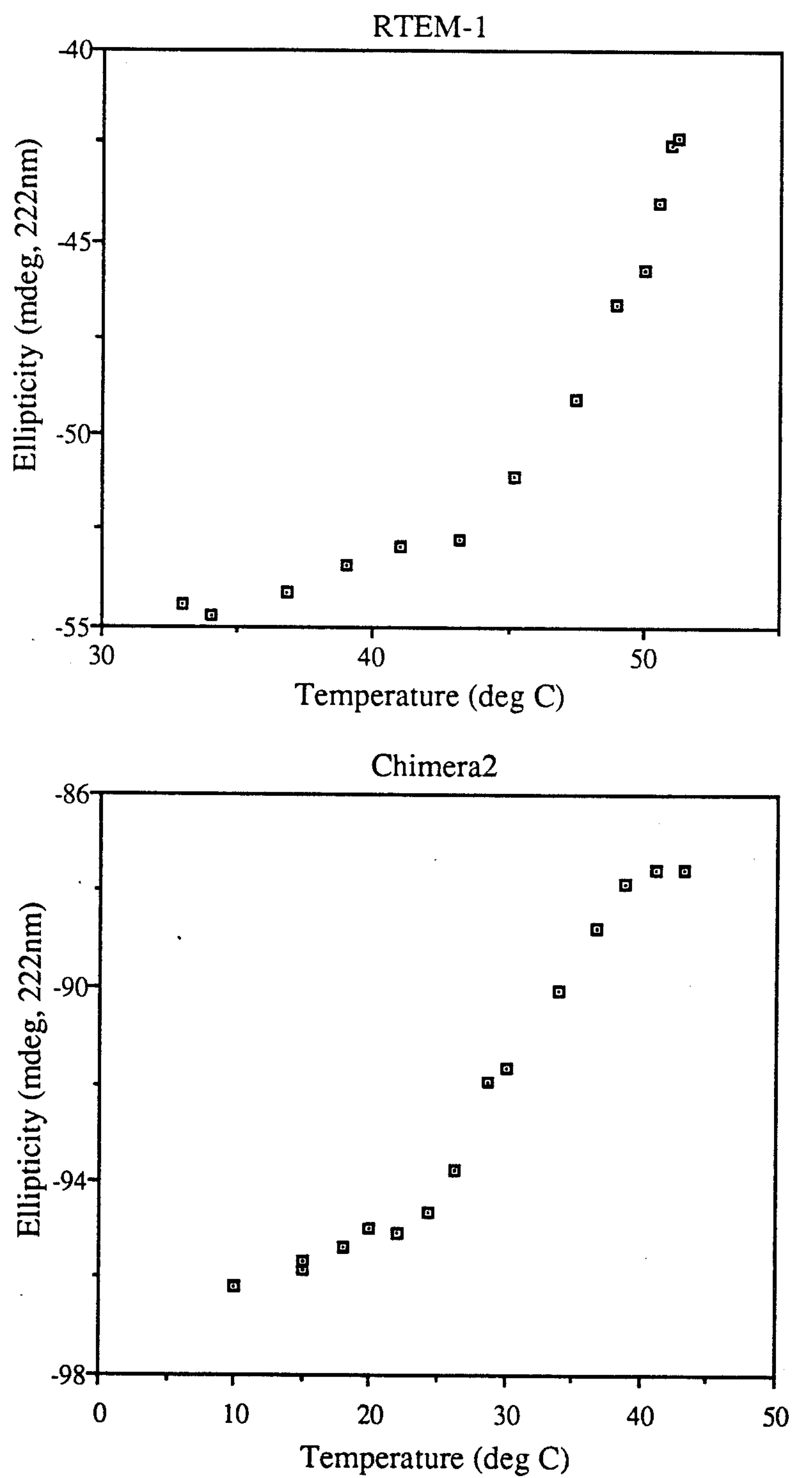

Figure 10. Thermal melting curves of RTEM-1 and Chimera2 in 100mM potassium phosphate pH 7.0 monitored by CD at 222nm.

used and activity as low as $3x10^{-3}$ sec^{-1} could be easily observed. An acyl-enzyme could not be detected using radiolabelled benzylpenicillin. The Chimera2 had no detectable depsipeptidase activity in an assay able to detect activity as low as $3x10^{-3}$ sec^{-1}.

In earlier work in our laboratory, an RTEM-1/PBP5 chimera was constructed, which replaced the nineteen nonconserved residues between residues 56 and 77 in the RTEM-1 sequence with the corresponding sequence from PBP5 with an unintentional insertion of a glutamate residue between residues 59 and 60 (Chimera Y.H.C., Figure 3). The resulting chimera was active with turnover numbers of $3.5x10^{-3}$ min^{-1} on the carboxypeptidase substrate ^{3}H-diacetyl-Lys-D-Ala-D-Ala (1.3% of the activity of the wild-type PBP5), and 1.3sec^{-1} on the ß-lactamase substrate benzylpenicillin[12]. More recently, the glutamate insertion was removed and the resulting chimera was active as both a ß-lactamase and carboxypeptidase. (Turnover numbers were $5x10^{-2}$ sec^{-1} for benzylpenicillin and $3.7x10^{-3}$ sec^{-1} for diacetyl-Lys-D-Ala-D-lactate[13].) The design of these chimeras was based solely on homologies as they were constructed before high-resolution structural data were available for the ß-lactamases. The activity found in these chimeras is expected to arise from alterations of residues near the binding site.

Conclusions

The exchange of the active site helix of RTEM-1 ß-lactamase for the analogous helix of PBP5 did not produce an active chimera with reduced specificity. The chimeras produced were substantially less stable than the wild-type RTEM-1, though the presence of the disulfide contributed a stabilizing effect. Chimera2 was shown to have altered secondary structural features and the structure created had no observed catalytic properties. These results show that while it is possible to obtain a protein with significant changes in amino acid sequence in purified form, the substituted sequence may not adopt the same structure as the wild-type sequence or as the structure formed in the native protein from which the sequence was derived. The secondary structural element of an α-helix could not be exchanged between two closely related proteins.

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Chapter 3

Relevance of Residues 233, 234, 235 and 237 in RTEM-1 ß-lactamase Towards Substrate Specificity

Introduction

The penicillin-recognizing enzymes have a wide range of activities towards the ß-lactam antibiotics. The ß-lactams are suicide substrates of the PBPs, forming stable acyl-enzymes that covalently inhibit the PBPs. The analogous acyl-enzymes of the ß-lactamases are rapidly hydrolyzed. The ß-lactamases have been classified by their preference for different types of substrates, and a high degree of sequence homology is found among members of the same class. The amino acid sequences of the penicillin-recognizing enzymes with differing activities may be compared to suggest conserved or nonconserved residues that may be important in the functional similarities and differences.

The sequences of the penicillin-recognizing enzymes have several regions of homology[1]. One such region is the so-called HTG box at position 234-236. (Numbering is of the class A ß-lactamases according to Ambler[2].) These residues are conserved or replaced conservatively among all the serine ß-lactamases and PBPs. As shown in Figure 1, the sequence is KSG or KTG in the class A ß-lactamases, KTG in the class C and D ß-lactamases and the low molecular weight PBPs, and HTG in the carboxypeptidase of *Streptomyces* R61. Residue 233, which is adjacent to the HTG box, is conserved as Asp233 within the class A ß-lactamases, which have greater activity as penicillinases than cephalosporinases, and is conserved as His233 within the class C ß-lactamases where the specificity is reversed.

Previous studies have suggested the importance of this region to substrate specificity. A mutant RTEM-1, created by chemical mutagenesis and selected for increased cephalosporinase activity, was found to carry the substitution A237T[3]. The residue was subjected to site saturation mutagenesis, and A237N was found to have even higher cephalosporinase activity[4]. Mutations in this region have also been found in *E. coli* PBP3,

Class A ß-lactamases	*B. licheniformis,S. albus G*	D K T G A
	B. cereus	D K S G A
	S. aureus PC1	D K S G Q
	RTEM, SHV	D K S G A
	Streptomyces cocaoi	D K S G Q
Class C ß-lactamases	*E.coli*	H K T G A
	C. freundi	H K T G S
Class D ß-lactamase	OXA2	A K T G W
Carboxypeptidases/ Transpeptidases	*Streptomyces* R61	G H T G T
	E. coli PBP 1A,1B	G K T G T
	E. coli PBP 2	A K S G T
	E. coli PBP 3	I K T G T
	E. coli PBP 5	I K T G H

Figure 1. Sequences of penicillin-recognizing enzymes around the HTG box. Sequence given is residues 233-237 in the numbering of the class A ß-lactamases. (Ref. 1,2,7)

where either a T235I or a T237I mutation gave rise to physiologically nonfunctional PBP3's, which were still able to bind penicillin[5].

The structure of the class A ß-lactamase of *B. licheniformis* 749/C has been determined by X-ray crystallography at a resolution of 2Å[6]. This enzyme is homologous to RTEM-1 ß-lactamase (33% [7]), and it is expected that the structure of the folded RTEM-1 enzyme is nearly identical with that of the *B. licheniformis* ß-lactamase. The putative structural homology is supported by the high degree of similarity between the structure of the *B. licheniformis* enzyme and the structure of the *S. aureus* PC1 enzyme, which has been solved to 2.5Å resolution[8]. These two enzymes have 41% sequence identity[7].

The structure of the enzyme-substrate complex of ß-lactamase has not been solved. Instead, all ß-lactamase structures determined to date are of unbound enzyme, and the location and orientation of substrate binding have been predicted by molecular modeling. The putative ß-lactam antibiotic binding site is a crevice on the protein surface which is closed at one end. The floor of the binding site is made up of residues 68-73 and residues 164-170. These include the nucleophilic Ser70 used to form the acyl-enzyme intermediate. Residues 71-72 are part of the first turn of the active site helix and allow the conserved Lys73 to be positioned near the substrate and Ser70. Substitution of residue 71 has been shown to have an effect on the stability of the protein but no effect on catalysis or binding[9]. One wall of the crevice is made up of two loops, residues 103-106 and residues 130-132, with the former near the variable side chain of the antibiotic and the latter near the lactam portion of the substrate in the enzyme-substrate complex. Within these sequences, the conserved residues Asn104, Ser130 and Asn132 are positioned such that their side chains may interact with the substrate. The other wall of the crevice is made up of residues 233-240. These residues form a strand of antiparallel ß-sheet with the backbone amide of 237 forming an antiparallel sheet structure with the carbonyl of the lactam and the nitrogen of the antibiotic side chain. The backbone amides of 237 and 70 and the active site helix dipole form the oxyanion hole, whose presence has been demonstrated by the kinetic

behavior of the enzyme towards modified substrates[10]. If a side chain were present on Gly236, which is conserved among all the serine ß-lactamases and PBPs, the side chain would be directed into the space occupied by Ser70. Lys234 is near the carboxylate common to virtually all ß-lactamase substrates, and the pair is expected to form a salt bridge in the enzyme-substrate complex. Removal of Lys234 by site directed mutagenesis of RTEM-1 has shown this residue to be involved primarily in substrate binding[11]. The closed end of the crevice is formed by residues 215-217. The side chain of residue 216 lies approximately between the methyl groups and the carboxyl group of the penam ring and near residues 234 and 235.

In this work, the relevance of residues 233, 234 235 and 237 to the substrate selectivity of RTEM-1 ß-lactamase was studied by mutagenesis. These four residues were changed in RTEM-1 to the sequences found in the other penicillin-recognizing enzymes and the RTEM-1 A237N mutant discussed above. Mutants were generated to yield all possible combinations of the four substitutions, D233H, K234H, S235T, and A237N. Because of the overlap of the genetic code and the method of mutagenesis, mutants K234N and K234Q were also obtained. Thus thirty-two possible sequences, including the wild-type sequence, were generated. These mutants were screened for resistance to several ß-lactam antibiotics.

Materials and Methods

Enzymes and Chemicals

Restriction enzyme BglI was purchased from New England Biolabs. Restriction enzymes BamHI and AvaI, and T4 DNA ligase were purchased from Boehringer Mannheim Biochemicals. All antibiotics were purchased from Sigma. All bacterial culture media were purchased from Difco Laboratories.

DNA

Oligonucleotides were synthesized by the Caltech Microchemical Facility using an Applied Biosystems automated DNA synthesizer. One third of a 0.2μM scale synthesis product was resuspended in 20μl loading buffer (80% formamide, 50 mM Tris-borate, 1mM EDTA, 0.1% (w/v) each xylene cyanol and bromophenol blue), and purified by polyacrylamide gel electrophoresis (15% 19:1 acrylamide : N,N'-methylenebisacrylamide, 40% (w/v) urea), in TBE buffer (89mM Tris, 89mM boric acid, 0.2mM EDTA). DNA was visualized by UV shadowing using a fluorescent TLC indicator plate, and the bands were excised, crushed, and soaked overnight in 200mM NaCl, 10mM Tris-HCl pH 7.6, 1mM EDTA at 37°C. Oligonucleotides used in the cassette mutagenesis construction were then loaded onto a NACS PrePac™ column (Bethesda Research Laboratories), washed and eluted in 600μl high-salt buffer (2M NaCl, 10mM Tris-HCl pH 7.2, 1mM EDTA) as per manufacturer's instructions, followed by desalting using a Sephadex G-25 spin column (Pharmacia, 3mls gel volume in 10mM Tris-HCl pH 7.5, 1mM EDTA) For oligonucleotides used as primers in DNA sequencing, supernatant from soaked gel slices was treated using a Sephadex G-25 spin column only, without the NACS PrePac™ column purification. Plasmid pBR322 was purchased from International Biotechnologies Inc. (IBI) A modified plasmid pBR322, constructed by K. O'Connor and W.J.Healey to contain a silent mutation introducing an XhoI restriction site at position 3426[12], was

prepared by standard alkaline lysis methods followed by CsCl gradient purification[13]. DNA concentrations were estimated from absorbance at 260nm.

Restriction digests were performed according to manufacturers' recommendations. Restriction fragments were isolated by 1.2% agarose gel electrophoresis (IBI) in TAE buffer (4.84g/L Tris base, 1.14ml/L acetic acid, 1mM EDTA) and isolated using the EluTrap (Schleicher and Scheull) according to the manufacturer's instructions.

DNA Sequencing

DNA sequencing was carried out using the Sequenase™ sequencing system of U. S. Biochemical Co. as recommended[14] with the following modifications: Double stranded DNA was prepared by standard mini-prep alkaline lysis of 1.5ml overnight cultures[13]. After removal of cellular debris, the supernatant was extracted twice with 1:1 phenol/chloroform. The DNA was precipitated with ethanol and washed twice with cold 70% ethanol. The template DNA (1-3μg) was mixed with the primer (100ng) in 10μl buffer, heated in a boiling water bath for four minutes, and frozen rapidly in a dry ice/ethanol bath to denature the plasmid and to anneal the primer. The remainder of the protocol is the same. Oligonucleotide primers were 17-18 bases in length. Gel electrophoresis was performed using 5% polyacrylamide gels (19:1 acrylamide: N,N'-methylenebisacrylamide, 7M urea) run with a modified TBE buffer (133mM Tris, 45mM boric acid, 2.5mM EDTA)

Construction of Mutants by Cassette Mutagenesis

Mutants were constructed by inserting a synthetic cassette between a BglI and a constructed XhoI site in the RTEM-1 ß-lactamase gene of pBR322. In one set, all 16 possibilities at the sites 233-235 were generated. In another set, the same mutants were generated with the addition of the A237N mutation. The presence of other BglI and XhoI restriction sites in the pBR322 plasmid necessitated the use of a third enzyme and a three piece ligation. The enzyme BamHI was used for this purpose. Two oligonucleotides (49

and 56 bases in length, 0.2 pmol/μl each in 10mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 50mM NaCl, 1mM dithioerythritol) were heated to 95°C in a water bath and allowed to cool slowly to room temperature to anneal the complementary strands. The ligation was performed under the following conditions: 20μg/ml DNA (1:10 molar ratio of vector to synthetic insert) in 50mM Tris-HCl pH 7.6, 10mM $MgCl_2$, 1mM spermidine, 10mM dithiothreitol, 0.4mM ATP and 5U T4 DNA ligase in a total volume of 25μl at 15°C overnight. Ligation mixtures were used to transform LS-1 *E. coli* to tetracycline resistance by the method of Hanahan[15]. Mutants were identified by DNA sequencing. After all 32 mutants were identified, plasmid was prepared for each by standard alkaline lysis followed by CsCl gradient purification[13], and the DNA sequences were verified. Oligonucleotides used in the mutagenesis had the following sequences:

Set 1

CGGCTGGCTGGTTTATTGCT(G/C)AT(C/A)A(C/G)(A/T)CTGGCGCCGGTG-AGCGTGGGTC

and

TCGAGACCCACGCTCACCGGCGCCAG(A/T)(G/C)T(G/T)AT(G/C)AGCAATAAACCAGCCAGCCG-GAA

Set 2

CGGCTGGCTGGTTTATTGCT(G/C)AT(C/A)A(C/G)(A/T)CTGGCAATGGTG-AGCGTGGGTC

and

TCGAGACCCACGCTCACCATTGCCAG(A/T)(C/G)T(G/T)AT(C/G)AGCAATAAACCAGCCAGCCG-GAA

(X/X) indicates a 50:50 mixture of the bases in that synthesis step.

Phenotypic Screening

L-agar plates (10g/L tryptone, 5g/L yeast extract, 10g/L NaCl and 15g/L bactoagar) containing the appropriate concentration of antibiotic were prepared no more than one day in advance. Overnight cultures of the mutants, which were grown in tetracyline (15mg/L),

were diluted by a factor of 10^5 in L-broth. 50μl of these diluted cultures were plated on one half of each plate and incubated overnight at the given temperature. Cells carrying wild-type pBR322 and cells without plasmid were plated as a control to verify the absence of cross-contamination.

Results and Discussion

The use of the ß-lactamase RTEM-1 in substrate specificity studies allows rapid access to the activity of generated mutants by phenotypic screening. The differences in phenotypic resistance as tested in this study will reflect two properties of the ß-lactamase produced. The first is the stability of the enzyme to periplasmic proteases, a factor whose effect is reflected in differences in resistance as a function of temperature. The second factor is the apparent second-order rate constant of the enzyme, k_{cat}/K_M, which reflects both the affinity of the enzyme for the substrate and the catalytic efficiency. Because all mutants are tested in the same strain of *E. coli* and are expressed from the same vector, any factors particular to the organism, such as the rate of production of ß-lactamases and the level and types of periplasmic proteases, will be constant in these experiments.

The mutants constructed in this work were screened for their ability to confer resistance to any of three ß-lactam antibiotics, ampicillin, cephalothin and cefotaxime at two different temperatures. Results are shown in Figure 2.

Any mutant with a substitution of Lys234 was phenotypically inactive. Mutants with the substitutions D233H and S235T were active. From phenotypic analyses of *E. coli* harboring these active mutants, the following results were obtained: 1) The D233H mutation led to a temperature-sensitive enzyme as seen by a difference in the resistance to ampicillin at 30°C vs. 37°C. Because the D233H and D233H/S235T mutants conferred no resistance to cephalothin, no comparison of the ampicillin vs. cephalothin activities can be made. 2) The S235T mutation led to an increased resistance to cephalothin without an observed reduction in the resistance to ampicillin. With the double mutant S235T/A237N the ampicillin resistance is reduced slightly, yet the cephalothin resistance is greater than the single mutant A237N and much greater than the single mutant S235T.

Residues 233-237	Ampicillin 30	37	Cephalothin 30	37	Cefotaxime 30	37
DKSGA (wt)	> 2000	> 2000	> 20	20	-	-
DKTGA	> 2000	> 2000	> 20	50	-	-
DHSGA	-	-	-	-	-	-
DHTGA	-	-	-	-	-	-
DQSGA	-	-	-	-	-	-
DQTGA	-	-	-	-	-	-
DNSGA	-	-	-	-	-	-
DNTGA	-	-	-	-	-	-
HKSGA	500	400	-	-	-	-
HKTGA	400	400	-	-	-	-
HHSGA	-	-	-	-	-	-
HHTGA	-	-	-	-	-	-
HQSGA	-	-	-	-	-	-
HQTGA	-	-	-	-	-	-
HNSGA	-	-	-	-	-	-
HNTGA	-	-	-	-	-	-
DKSGN	> 20	1000	> 20	150	-	-
DKTGN	> 20	1000	> 20	> 200	-	-
DHSGN	-	-	-	-	-	-
DHTGN	-	-	-	-	-	-
DQSGN	-	-	-	-	-	-
DQTGN	-	-	-	-	-	-
DNSGN	-	-	-	-	-	-
DNTGN	-	-	-	-	-	-
HKSGN	> 20	12	-	-	-	-
HKTGN	> 20	12	-	-	-	-
HHSGN	-	-	-	-	-	-
HHTGN	-	-	-	-	-	-
HQSGN	-	-	-	-	-	-
HQTGN	-	-	-	-	-	-
HNSGN	-	-	-	-	-	-
HNTGN	-	-	-	-	-	-

Figure 2. Phenotypic resistance of E. coli carrying mutant ß-lactamases to ß-lactam antibiotics at 30 and 37 deg.C. (>) indicates growth at the highest value tested. (-) indicates no growth at the lowest value tested (12mg/L ampicillin, 10mg/L). cephalothin and 0.07 mg/L cefotaxime.

The possible structural effects of these mutations can be modeled using the structural data available for the *B. licheniformis* enzyme. The D233H mutation is the substitution of a conserved residue of the class A ß-lactamases with a residue conserved among the class C ß-lactamases. In the three-dimensional structure, residue Asp233 is surrounded by residues 222, 246, 231 and 214. The space is very crowded and the accommodation of the five-membered ring of histidine may contribute to the instability of the D233H mutants. In addition, the ε-nitrogen of Arg222 is in a position to form a salt bridge with the carboxylate of Asp233, a contact that would be lost in the D233H mutation. This arginine is conserved in the class A ß-lactamases with the exception of the conservative replacement of Lys222 in the *S. aureus* PC1 enzyme, but it is not conserved in the class C ß-lactamases. Asp233 is also very near Asp246 in what might be an unfavorable contact. Asp246 is not a conserved residue, and both aspartate and isoleucine are found at this position in the class A ß-lactamases. Asn214 is also in a position to hydrogen bond with Asp233, but this residue is not conserved, and RTEM-1 has aspartate at 214. Val231 contacts the methylene group of the aspartate side chain, a function that is likely to be served in the RTEM-1 enzyme by the isoleucine found at this position. An analysis of these structural considerations suggests that the accommodation of the five-membered ring and the loss of the D233-R222 contact may be the primary factors in the destabilization of the D233H mutants.

The effect of the S235T mutation is simply the addition of one methyl group on the side chain. The *B. licheniformis* ß-lactamase has threonine at position 235. The hydroxyl group of the threonine is in a position to hydrogen bond with Lys234 and the carboxylate of the substrate, a contact that may be necessary in catalysis based on the conservation of a hydroxyl-containing group at this site. The methyl group of the threonine is located near two residues, Gly217 and Leu220, in the three-dimensional structure. Leu220 is conserved among the class A ß-lactamases with only two exceptions. The contact with Gly217 is more interesting because if a larger side chain were placed on the glycine, it would occupy the same space as the methyl group of the threonine. Gly217 is not

conserved among the class A ß-lactamases, and alanine or threonine may be found at this position. Structural analysis suggests that the presence of a methyl group or other larger functionality in this space, whether from residue 235 or 217, may somehow alter the position of the hydroxyl group of residue 235 to increase the enzyme activity on the cephalosporin substrate. The reported activities of known ß-lactamases with differing combinations of residues at positions 235 and 217 were compared but the result was inconclusive. The lack of correlation between sequences at 235 and 217 and the penam vs. cephem preference of the enzyme may be due to the contributions of other sequence differences among the enzymes as well as inconsistencies in the reported values of the kinetic parameters of these enzymes. (For example, the k_{cat} and K_M values for the *S. albus* G ß-lactamase on the substrate cephalothin have been reported as $260s^{-1}$ and $720\mu M$, or as $12s^{-1}$ and $1330\mu M$, respectively, by different authors[16,17].)

Several limitations must be considered when modeling the structural effects of mutations in RTEM-1 based on the structure of the *B. licheniformis* ß-lactamase. While the RTEM-1 and *B. licheniformis* ß-lactamases are homologous and the overall structures are expected to be very similar, the sequences are not identical, and significant structural differences may exist. These differences cannot be predicted nor fully accounted for by the modeling methods. In addition, the structure, determined in the absence of substrate, is that of an unbound conformation of the enzyme, not the enzyme-substrate complex. The differences in structure between unbound and active forms of ß-lactamase may be significant. There is considerable evidence that the class A ß-lactamases undergo conformational changes when substrate binds, as demonstrated by biphasic kinetic behavior, differing protease susceptibility profiles in the presence of different substrates and the effects of bound antibodies on activity[18-21].

Conclusions

The results presented here lead to two general conclusions. First, the substitution of a conserved residue Asp233 of the class A ß-lactamases in RTEM-1 with the corresponding residue from the class C ß-lactamases led to less stable enzymes. Molecular modeling suggests the accommodation of the five-membered ring and a loss of a favorable salt bridge are responsible for the decreased stability. Second, the substitution S235T led to an increase in the activity of RTEM-1 ß-lactamase on cephalothin, both as a single mutation and as an additive effect in the double mutation S235T/A237N. The cause of this effect is suggested by molecular modeling to lie in the subtle orientation of the hydroxyl group by the crowding of the neighboring space occupied by the methyl group of the threonine. This possibility can be tested by mutagenesis of residue 217. The effects of the A237N mutation cannot be explained by analysis of the structure. It is the backbone of this residue that interacts with the substrate. The limitations of the structural information and modeling methods preclude explanations based on currently available models.

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Chapter 4

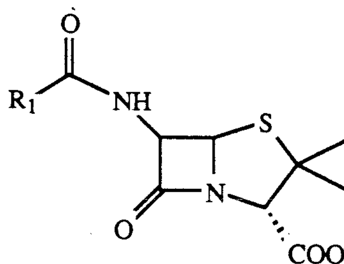
Origin of Resistance to the Third Generation Cephems: Simultaneous Randomization of Three Residues of RTEM-1 ß-lactamase

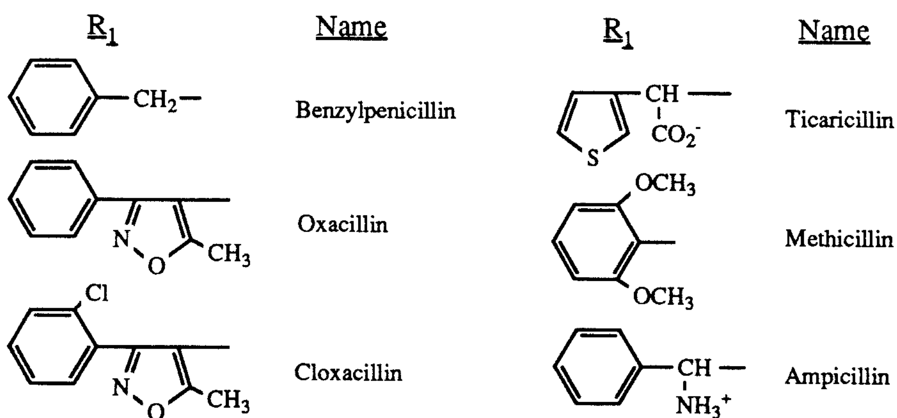
Introduction

Since Fleming's observation in 1929 of the inhibition of bacterial growth by mold cultures and the development of penicillin for clinical use by Florey and Chain in the early 1940's, many ß-lactam antibiotics have been developed and are in use. With the spread of ß-lactamase-mediated resistance to new hosts in the 1970's and 1980's, the need for broad-spectrum, ß-lactamase resistant antibiotics grew. With the availability of 6-aminopenicillanic acid in 1958[1], novel semi-synthetic penicillins were developed. These included methicillin, cloxicillin, carbenicillin and ticaricillin, which are less susceptible to hydrolysis by the ß-lactamases. During this time, the search for antibiotic-producing organisms continued. From cultures of the mold *Cephalosporium acremonium*, cephalosporin C was isolated. With the development of industrial production of 7-aminocephalosporanic acid, semi-synthetic antibiotics could be developed based on the cephem nucleus. This resulted in the development of the second generation cephalosporins including cephalothin, cephaloridine and cephalexin. These newer cephem antibiotics have a wide spectrum of activity, including the control of Gram negative and some penicillin resistant organisms. Yet, there remained some pathogens resistant to these antibiotics. A third generation of antibiotics, notably cefotaxime, ceftazidime and cefsulodins, are highly effective against these pathogens and are resistant to hydrolysis by the ß-lactamases[2]. The structures of some of these antibiotics are shown in Figure 1. This vast range of potential substrates, combined with the ease of phenotypic selection, has provided a tool for the study of the specificity determinants of ß-lactamase.

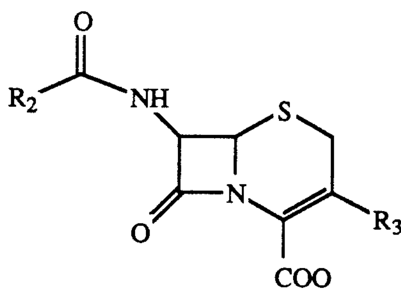
The extensive clinical use of the third generation cephems has selected for naturally occurring ß-lactamase mutants able to hydrolyze them. In 1983, *Klebsiella* isolates with plasmid mediated resistance to broad-spectrum cephalosporins were found in the Federal Republic of Germany[3]. The ß-lactamase produced was found to be a class A ß-lactamase

Penams:





Cephems:



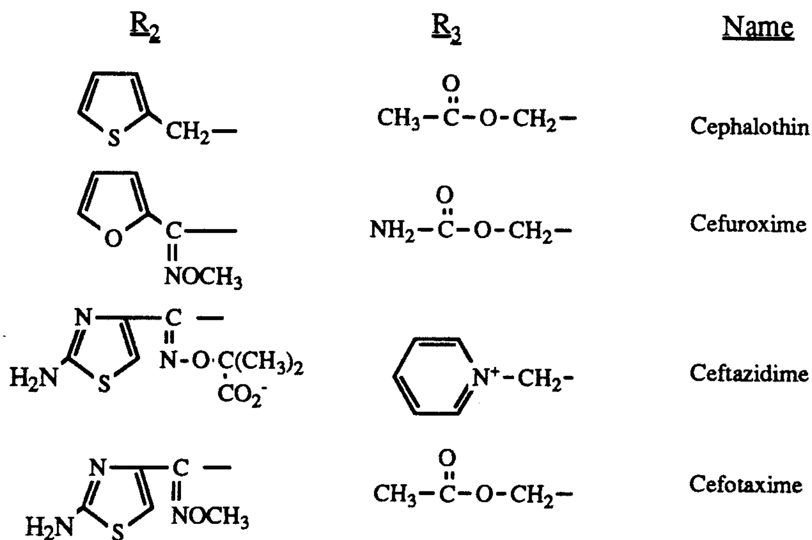


Figure 1. Structures of some common ß-lactam antibiotics.

closely related to SHV-1[4]. Shortly after, another ß-lactamase hydrolyzing these cephalosporins was found in resistant *Klebsiella* isolates in France and was found to be a derivative of RTEM-1. (RTEM and SHV are homologous with 68% sequence identity[5].) Since then, many more extended-spectrum ß-lactamases have emerged in clinical isolates of pathogenic bacterial species. Often these enzymes were named based on the resistance phenotype they conferred or the location from which they were isolated, and were later renamed when they were found to be related to SHV or RTEM. These extended-spectrum ß-lactamases are now found around the world and can be expected to emerge wherever the ß-lactam antibiotics are used.

The extended-spectrum ß-lactamases have been characterized by the resistance profiles they confer, by amino acid sequence and to a limited extent, by the kinetic parameters relative V_{max} and K_M. The resistance profiles of an isogenic strain of *E. coli* carrying these ß-lactamases are shown in Table 1. The minimum inhibitory concentrations (MICs) of a given drug are a function of the activity and quantity of ß-lactamase expressed by the bacteria. When the MICs of different antibiotics are compared, the relative permeability of the antibiotic through the outer membrane may be a factor. As an example, the MIC values of cefotaxime vs. ceftazidime for *E. coli* carrying RTEM-3 ß-lactamase do not accurately represent the relative activity of the purified enzyme on these two antibiotics because of the slower diffusion rate of ceftazidime compared to cefotaxime into the periplasm. However, because the diffusion effect will be the same for bacteria carrying different ß-lactamases, comparisons can be made between the relative resistance conferred by an enzyme on two antibiotics with the relative resistance conferred by another enzyme on the same two antibiotics. The expression of a naturally occurring extended-spectrum ß-lactamase can increase by over two orders of magnitude the concentration of ß-lactam antibiotic necessary to inhibit cell growth. The concentrations of the antibiotic necessary for therapeutic effectiveness are then greater than clinically achievable.

Table 1. Resistance Profiles of *E. coli* Expressing Some Naturally Occurring ß-lactamases[a]

	Minimum Inhibitory Concentration (mg/L)			
Mutant	Cephalothin	Cefotaxime	Ceftazidime	Aztreonam
R⁻	4	0.02	0.2	0.06
RTEM-1	32	0.05	0.2	0.06
RTEM-2	32	0.03	0.2	0.06
RTEM-3 (CTX-1)	64	2	8	1
RTEM-4	256	8	16	2
RTEM-5 (CAZ-1)	128	2	32	1
RTEM-6	8	0.5	128	32
RTEM-7	16	0.5	32	1
RTEM-9 (RHH-1)	32	1	128	32
SHV-1 (PIT-2)	16	0.01	0.2	0.06
SHV-2	256	4	2	1
SHV-3	128	2	2	0.5
SHV-4 (CAZ-5)	256	4	64	32
SHV-5 (CAZ-4)	-	4	32	-

[a]Ref. 6, 7

In all of the naturally occurring mutants sequenced to date, substitutions are found at one or more of a set of nine specific residues (Table 2). One of these residues, Leu19, is in the leader sequence and is not present in the mature enzyme. The location of the altered residues in the three-dimensional structure can be modeled based on the structure of the homologous class A ß-lactamase from *B. licheniformis* determined by X-ray crystallography to 2Å resolution[9]. The structure of the enzyme has been determined in the absence of substrate, and the enzyme-substrate complex has been modeled as discussed in Chapter 1. Three of the residues substituted in the extended-spectrum ß-lactamases, 37, 205 and 264, are found to be well removed from the active site. Analysis of the naturally occurring mutants of RTEM and SHV demonstrates that these residues are not involved in the specificity of the ß-lactamases. RTEM-1 and RTEM-2 differ only by a mutation from Gln to Lys at position 37, located in the amino terminal helix. The resistance conferred by these two enzymes is identical[10]. The second residue substituted that is distant from the active site, residue 264, is located along the ß-strand at the center of the ß-sheet domain of the enzyme. The identity of residue 264 may have an effect on the level of antibiotic resistance conferred by the enzyme as suggested by comparison of RTEM-3 and RTEM-4. Both RTEM-3 and RTEM-4 have the substitutions E104K and G238S, but they differ at positions 37 and 264 (see Table 2). Since the single mutation K37Q between RTEM-1 and RTEM-2 is functionally silent, the differences in activity between RTEM-3 and RTEM-4 are attributed primarily to the T264M substitution[6]. The profiles of resistance conferred by these two enzymes are the same. The increased resistance conferred by RTEM-4 may be due to increased levels of enzyme present because of a greater stability rather than to a change in the enzymatic activity. The third residue substituted that is distant from the active site, residue 205, is located along helix 9 and the nonconserved side chain projects into the solvent. The substitution of this residue is the only difference between SHV-2 and SHV-3. As in the case of the RTEM-3/RTEM-4 comparison, the profiles of resistance are alike, and the differences in MICs of cultures carrying SHV-2 or SHV-3 may be due to differences in

Table 2. Sequences of Some Naturally Occurring Extended-Spectrum ß-lactamases[a]

	Amino Acid Position								
Mutant	19	37	104	164	205	237	238	240	264
RTEM-1	Leu	Gln	Glu	Arg	Gln	Ala	Gly	Glu	Thr
RTEM-4	Phe	Gln	Lys				Ser		Met
RTEM-5		Gln		Ser		Thr		Lys	
RTEM-6		Gln	Lys	His					Met
RTEM-9	Phe	Gln	Lys	Ser					Met
RTEM-2		Lys							
RTEM-3		Lys	Lys				Ser		
RTEM-7		Lys		Ser					
SHV-1		Gln	Asp	Arg	Arg	Ala	Gly	Glu	Leu
SHV-2							Ser		
SHV-3					Leu		Ser		
SHV-4					Leu		Ser	Lys	
SHV-5							Ser	Lys	

[a]Ref. 6, 7. Numbering is according to the scheme of Ambler, Ref. 8.

protein stability. Substitution of these three residues, 37, 205 and 264, does not alter the substrate profile of ß-lactamase.

The remaining sites of substitution, residues 104, 164, 237, 238 and 240, are located in or near the substrate binding site. The substitution of each of these five residues alters the substrate specificity of the extended-spectrum ß-lactamases as shown by phenotypic resistance profiles. Residue 104 is located in a loop along one wall of the antibiotic binding site near the expected position of the variable side chain of a bound antibiotic. This residue is not conserved, and Glu, Asp, Thr, Asn, Ser, Pro, Ala, or Gln are found at this position. The *B. licheniformis* enzyme is Asn104. In the modeled enzyme-substrate complex, the asparagine side chain is hydrogen-bonded to the carbonyl of the substrate amide linkage. Each of the two mutations from the RTEM-2 sequence to the RTEM-3 sequence, E104K and G238S, has been constructed as a single site mutant. While both single mutants increased resistance to ceftazidime and cefotaxime, the E104K mutation had a greater effect on ceftazidime resistance and the G238S mutation had a greater effect on cefotaxime resistance[11]. A comparison of the MICs of *E. coli* harboring SHV-4 or SHV-5, which differ only at position 104, demonstrates the same effect. Residue 164 is across the crevice from 104 and is at the end of a loop that forms a part of the floor of the crevice. The side chain of residue 164, conserved as arginine among the class A ß-lactamases, projects into the solvent. Residues 237, 238 and 240 are consecutive in the RTEM-1 sequence, with the apparent discrepancy arising because the numbering is based on the sequence of the ß-lactamase of *S. aureus* PC1, which has an insertion at residue 239[8]. While residue 237 is most often found to be Ala, it is not conserved in the class A ß-lactamases, and Gln or Gly is also found at this position. Residue 238 is a Gly or Ala in all the class A ß-lactamases with the single exception of the chromosomal ß-lactamase of *Streptomyces cacaoi* KCC-SO352, which has a Val at this position. Residue 240 is not conserved and is found as Glu, Gly, Ser, Lys, Thr, Gln or Arg, with the *S. aureus* PC1 ß-lactamase possessing an inserted isoleucine at this site. Residues 237-240 are part of an

antiparallel ß-sheet. The backbone amide of residue 237 forms an antiparallel ß-sheet structure with the carbonyl of the lactam ring and the nitrogen of the antibiotic side chain amide linkage. The side chains of residues 237 and 238 are directed in opposite directions approximately perpendicular to and angled slightly away from the plane of the lactam ring. Residue 240 is the first residue of the turn at the end of the ß-strand. In the ß-lactamase of *B. licheniformis*, this residue is serine and the hydroxyl group is hydrogen-bonded to the backbone nitrogen amide of Val172. Substitutions at these five sites, residues 104, 164, 237, 238 and 240, are responsible for the altered substrate specificity of the extended-spectrum ß-lactamases.

The mechanisms by which mutations of residues 237, 238 and 240 alter substrate specificity are not understood. It has been suggested that the hydroxyl group of the serine introduced by the G238S mutation interacts with the nitrogen of the oxime side chain of the third generation cephems. It has also been suggested that the positive charge introduced by the mutation of 240 to Lys interacts with the carboxylate of the ceftazidime oxime function[7,12].

In this Chapter, I describe studies of residues 237, 238 and 240 of the RTEM-1 ß-lactamase. The three residues were simultaneously randomized by cassette mutagenesis, and transformants were selected on the antibiotic cefotaxime. These residues were chosen to assess a possible pattern of substitutions that increase the activity of the enzyme on the third generation cephems. The roles these residues play in the specificities of the broad-spectrum ß-lactamases may be elucidated by the analysis of the substitutions found and the properties of the various mutants obtained. Since these residues are consecutive in the linear sequence, the simultaneous randomization of all three was possible in a single mutagenesis step. Cefotaxime was chosen as the selecting ß-lactam to screen the mutant activity. I chose this cephem antibiotic because it is the least structurally complex of the third generation cephems, because it is a commonly prescribed and clinically important antibiotic, and finally, because the constructed mutant at residue 238 demonstrated a greater

impact on the activity of this enzyme towards cefotaxime as compared with ceftazidime. Further, RTEM-1 has no activity on cefotaxime and does not bind or confer resistance to this antibiotic[11,13]. (K_i of cefotaxime has been reported as > 3mM for RTEM-1[13].)

By screening over 30,000 colonies, an estimated 95% of the 8000 possible sequence combinations were sampled. The plasmids encoding ß-lactamases that conferred resistance to cefotaxime were isolated and the sequences at positions 237, 238 and 240 were determined. The colonies carrying the mutant ß-lactamases were screened for antibiotic resistance to a range of ß-lactam antibiotics. Three of the active mutants were assayed to determine the kinetic parameters k_{cat} and K_M on the substrates cefotaxime and benzylpenicillin. In this way, the range of potential substrates and the ease of phenotypic selection are exploited to determine the effect of differing sequences in this region.

Materials and Methods

Enzymes and Chemicals

Restriction enzyme BglI was purchased from New England Biolabs. Restriction enzymes BamHI and AvaI, T4 polynucleotide kinase and T4 DNA ligase were purchased from Boehringer Mannheim Biochemicals. All antibiotics were purchased from Sigma. All bacterial culture media and antimicrobic susceptibility disks were purchased from Difco Laboratories.

DNA

Oligonucleotides were synthesized by the Caltech Microchemical Facility using an Applied Biosystems automated DNA synthesizer. Fresh solutions of bases were used in the synthesis of oligonucleotides used in the cassette mutagenesis to obtain the best possible distribution of incorporation of mixed bases. The trityl protecting groups were not removed and the oligonucleotides were purified using Oligonucleotide Purification Cartridges (Applied Biosystems) according to the manufacturer's instructions. Oligonucleotides used as primers in DNA sequencing were 17-18 bases in length. One fifth of a 1μm scale synthesis product was resuspended in 1ml TE (10mM Tris-HCl pH 8, 1mM EDTA) and purified using a Sephadex G-10 spin column. 1ml TE was added to yield a final oligonucleotide concentration of ~10pmole/μl. Plasmid pBR322 was purchased from International Biotechnologies Inc. (IBI) A modified plasmid pBR322, constructed by K. O'Connor and W.J. Healey to contain a silent mutation that introduces an XhoI restriction site at position 3426[14], was prepared by standard alkaline lysis methods followed by CsCl gradient purification[15]. UV absorbance at 260nm was used to estimate DNA concentrations.

Restriction digests were performed according to manufacturers' recommendations. Restriction fragments were isolated by 1.2% agarose gel electrophoresis using NuSieve

GTG low melting agarose (FMC Bioproducts) in TAE buffer (4.84g/L Tris base, 1.14ml/L acetic acid, 1mM EDTA), and fragments were used in ligation reactions without the removal of the agarose.

DNA Sequencing

DNA sequencing was carried out using the Sequenase™ sequencing system of United States Biochemical Co. as recommended[16] with the following modifications: Double stranded DNA was prepared by standard mini-prep alkaline lysis of 1.5ml overnight cultures[15]. After removal of cellular debris, the supernatant was extracted twice with 1:1 phenol/chloroform. The DNA was precipitated with ethanol and washed twice with cold 70% ethanol. The pellet was resuspended in 20μl TE. 5μl of the template DNA were mixed with 5μl primer, heated in a boiling water bath for four minutes to denature the plasmid and frozen rapidly in a dry ice/ethanol bath to anneal the primer. Samples were placed on ice and 6.5μl of a Sequenase™ cocktail were added (2μl DTT, 2μl Sequenase™ buffer, 0.5μl Sequenase™ Labelling Mix, 1μl $\alpha[^{35}S]$ATP, 1μl Sequenase™ Mg^{++} buffer). 2μl Sequenase™ enzyme, diluted 1:7 with ice cold Sequenase™ dilution buffer were added. Tubes were spun in a microcentrifuge to mix the reagents and incubated 2-5 minutes at room temperature. 3μl of this reaction mixture was added to 2.5μl of each termination mix in separate wells of a 96-well microtiter tray. The dish was tapped to mix the reagents and incubated at 37°C for 5 minutes. 4μl of gel loading buffer were added to each reaction and the tray was placed in a boiling water bath for two minutes immediately before gel electrophoresis. Gel electrophoresis was performed using 5% polyacrylamide gels (19:1 acrylamide : N,N'-methylenebisacrylamide) run with a modified TBE buffer (133mM Tris, 45mM boric acid, 2.5mM EDTA). 3μl of each sample were loaded using a Bethesda Research Laboratories doublefine sharkstooth comb.

Construction of Mutants by Cassette Mutagenesis

Mutants were constructed by inserting a synthetic cassette between the BglI and the constructed XhoI site in the RTEM-1 ß-lactamase gene of pBR322. The presence of other BglI and XhoI restriction sites in the pBR322 plasmid necessitated the use of a third enzyme and a three piece ligation. The enzyme BamHI was used for this purpose. Two oligonucleotides, 49 and 56 bases in length, were kinased at a concentration of 0.2 pmol/µl each in 50mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 0.1mM EDTA, 5mM dithiothreitol (DTT), 0.5mM ATP and 2.5µl T4 polynucleotide kinase at 37°C for 45 minutes. The oligonucleotides were then heated to 95°C in a water bath and allowed to cool slowly to room temperature to anneal the complementary strands. The ligation was performed under the following conditions: To estimate the DNA concentration, the volumes of the gel slices containing the restriction fragments were estimated by weight, and a recovery of 80% was assumed. Gel slices were melted at 65°C for ~10 minutes. Aliquots of restriction fragments were mixed in prewarmed (37°C) tubes in the appropriate ratios and incubated at 37°C for 10-15 minutes. Concentrated buffer and water were prewarmed to 37°C and added to the fragments. Samples were moved to room temperature and allowed to equilibrate for ~2 minutes before the kinased and annealed oligonucleotides were added. Ligase was then added and samples were transferred to 15°C for incubation overnight. Final concentrations in the ligation reactions were: 20µg/ml DNA (1:10 molar ratio of vector to synthetic insert) in IBI ligase buffer (International Biotechnologies Inc., 25mM Tris-HCl pH 7.8, 10mM $MgCl_2$, 4mM ß-mercaptoethanol, 0.4mM ATP) with 0.25U/µl T4 DNA ligase.

Oligonucleotides used in the mutagenesis had the following sequences:

CGGCTGGCTGGTTTATTGCTGATAAATCTGGANN(G/C)NN(G/C)NN(G/C)CGTGGGTC

and

TCGAGACCCACG(G/C)II(G/C)II(G/C)IITCCAGATTTATCAGCAATAAACCAGCCAGCCGGAA.

Sequences are given in the 5'-3' direction. N represents an equal mixture of all four bases added in the synthesis step, (G/C) represents a 1:1 mixture of these two bases added in the synthesis step, and I represents the base inosine.

Competent TG-1 *E. coli* were prepared by the method of Hanahan[17]. Ligation mixtures were heated to 65°C for ~10 minutes and cooled to 37°C before addition to competent *E. coli* on ice (10μl ligation mixture/ml cell suspension). Aliquots of the transformed *E. coli* were plated on L-agar (10g/L tryptone, 5g/L yeast extract, 5g/L NaCl, 15g/L bacto agar) containing 0.15 mg/L cefotaxime. Aliquots were also plated on tetracycline-containing L-agar (15mg/L tetracycline) to estimate the numbers of colonies screened on cefotaxime.

Western Blot Analysis

The levels of mutant ß-lactamases present in the cells were assayed by Western blot analysis of whole-cell lysates prepared in the following manner: *E. coli* were grown in L-broth containing tetracycline (15mg/L) to late log phase ($ODU_{600nm} = 1$) at 37°C. 1.5ml of each culture were centrifuged to pellet the cells, which were then resuspended in a volume of loading buffer proportional to the measured absorbance. (100μl buffer/ODU. Buffer is 10% (v/v) glycerol, 3% (w/v) sodium dodecylsulfate (SDS), 31.2mM Tris-HCl pH 6.8, 5% (v/v) ß-mercaptoethanol and 0.05% (w/v) bromophenol blue) Samples were heated to 95°C for five minutes and vortexed briefly. Samples were loaded (15μl each) on a 12% polyacrylamide gel (12% 37.5:1 acrylamide : N,N'-methylenebisacrylamide, 375mM Tris-HCl pH 8.8, 0.1% SDS) with a 4% polyacrylamide stacking gel (4% 37.5:1 acrylamide : N,N'-methylenebisacrylamide, 125mM Tris-HCl pH 6.8, 0.1% SDS) and electrophoresed overnight at 5mA. (Running buffer is 25mM Tris, 192mM glycine pH 8.3, 0.1% SDS.) Proteins were transferred to nitrocellulose (Schleicher and Scheull BA85, 0.45 micron pore size) using a Bio-Rad TransBlot apparatus with platinum plate electrodes in the following manner: The gel was soaked in the blotting buffer for 30

minutes (25mM Tris, 192mM glycine pH 8.3, 20% (v/v) methanol). The gel was then sandwiched between nitrocellulose and blotting paper, placed in the TransBlot chamber containing blot buffer and electrophoresed at 150 mA for one and one-half hours. The nitrocellulose was washed, incubated in a solution containing normal goat serum to block nonspecific interactions and then incubated in a 1:1000 dilution of rabbit anti-ß-lactamase raised against denatured ß-lactamase as described[18]. The bound antibody was stained using the VectastainABC goat anti-rabbit IgG immunoperoxidase kit (Vector Laboratories) as recommended by the manufacturer.

Phenotypic Activity by Disk Diffusion

The antimicrobic susceptibility testing system of Difco was used to measure the *in vivo* activity of the mutants as described by the manufacturer. Bacto Mueller-Hinton plates were prepared according to the manufacturer's directions one day in advance using 25mls media in 100mm sterile plates. A turbidity of 0.1 ODU was measured for a $BaCl_2$ turbidity standard prepared as described by the manufacturer. A dilution of 25µl saturated cell culture in 1ml media was found to produce this turbidity. A sterile cotton-tipped wooden applicator was dipped in the diluted cell culture, and the excess liquid was expressed by pressing against the sides of the tube. The plates were inoculated by streaking the swab over the entire surface of the plate to produce an even lawn of bacterial growth. Plates were allowed to dry 5-10 minutes. Susceptibility disks were applied to the surface of the plate using the Dispens-O-Disc system (Difco), and each disk was gently pressed with a sterile forceps to insure complete contact with the agar surface. Plates were inverted and incubated at 37°C for 16-18 hours. Plates were placed on a dark background and the diameter of the zone of growth inhibition was measured with a ruler. Disks tested were benzylpenicillin (10U), oxacillin (1µg), cephalothin (30µg), cefoxitin (30µg), cefuroxime (30µg) and cefotaxime (30µg). Controls of TG-1 *E. coli* without plasmid and extremes of inoculation were also tested.

Minimum Inhibitory Concentration Determination

Minimum inhibitory concentrations (MICs) were determined by a broth microdilution susceptibility assay. Mueller-Hinton broth (Difco) was prepared as described by the manufacturer. 100μl of the media were placed in each well of a 96-well microtiter plate using a multitip pippetor. 100μl of the concentrated drug solutions were added to the first well, and each drug was serially diluted in the Mueller-Hinton broth. For each ß-lactamase tested, 10μl of fresh saturated cell culture were added to 10ml L-broth (10g/L tryptone, 5g/L yeast extract, 10g/L NaCl). 5μl of diluted cell culture were added to each well using an automatic pipettor. Plates were incubated 18 hours at 37°C in a large tray with wet paper towels in the bottom and foil covering to form a humid chamber. Drugs tested were ampicillin, benzylpenicillin, cephalothin, cefotaxime, cloxacillin and cefoxitin.

Expression and Purification of Mutants

Protein was isolated from cells harboring the plasmid pBR322 encoding the mutant ß-lactamases by osmotic extrusion as described[18,19], with some modification. Cells were grown to OD_{600}=1.6 at 30°C in XB-media (25g/L tryptone, 7.5g/L yeast extract, 20mM $MgSO_4$, 50mM Tris-HCl pH 7.5) containing tetracycline (15mg/L). The remainder of the procedure was carried out at 4°C. Cells were pelleted (GSA, 5Krpm, 10 min.), resuspended in cold sucrose solution (20ml/g wet cells, 450g/L sucrose, 0.5g/L EDTA, 25mM Tris-HCl pH 7.0) and shaken for 30 minutes. Cells were pelleted again (GSA, 10Krpm, 30 min.), resuspended in ice-cold water (20mls/g wet cells) and shaken vigorously for 30 minutes. Cells were pelleted (GSA, 10Krpm, 20min.) and supernatant was removed and filtered through a 0.2micron sterile filter. The volume of the protein solution was reduced by ultrafiltration (Amicon, YM10 membrane). Protein was purified by FPLC ion-exchange chromatography (Pharmacia) as described in Chapter 2. Fractions containing the ß-lactamases were identified by activity against benzylpenicillin as measured by a decrease in UV absorbance of a benzylpenicillin solution at 232nm. Purity was

determined by SDS-PAGE of 100μl aliquots of the fractions as described in Chapter 2. Protein concentrations were estimated by UV absorbance at 280nm using the extinction coefficient of wild-type ß-lactamase of 29,400 $M^{-1}cm^{-1}$ at this wavelength[19].

Activity Assays

Samples were assayed for ß-lactamase activity on the substrates benzylpenicillin and cefotaxime using the circular dichroism (CD) method developed by D.M. Long[20], with parameters for the measurement of cefotaxime developed here. A known amount of enzyme was added to 2ml substrate in 100mM potassium phosphate pH 7.0. The sample was mixed and transferred to a water jacketted cell, and the ellipticity was measured over time. (Slit width = 1500μm, time constant = 0.5 sec, 231.8nm for benzylpenicillin, 260nm for cefotaxime) The ellipticity of the hydrolyzed product is zero at this wavelength, allowing the concentration of the substrate to be obtained directly from the CD signal. (Ellipticity of benzylpenicillin is 393deg $cm^{-1}M^{-1}$ at 231.8nm, and the ellipticity of cefotaxime is 355deg $cm^{-1}M^{-1}$ at 260nm.) The high absorbance of cefotaxime limits the experimentally useful concentration to < 0.5mM for this substrate. Initial rates were determined by a linear regression fit of the time-course data. Kinetic constants were determined using the Hanes-Woolf replot method[21].

Molecular Modeling

Modeling of structures was based on the structure of the class A ß-lactamase of *B. licheniformis* as determined by X-ray crystallography to 2Å resolution[9]. All modeling was done using the program Biograf version 2.1 by BioDesign Inc. on a DEC VAX station, and graphics were displayed on an Evans & Sutherland terminal. Coordinates of the putative location of the substrate benzylpenicillin in the active site were provided by D. M. Long[20]. The structure of the substrate cefotaxime was modeled using the Biograf software. Conformational searches were performed and the energies of the "best" structures were minimized. The cefotaxime was modeled in the active site by superposition

of the lactam ring with the modeled location of benzylpenicillin. Mutant enzymes were modeled by the replacement of the native residue. Structures were then optimized by conformational searches followed by energy minimizations.

Results and Discussion

In the sequences of the extended-spectrum ß-lactamases, substitutions occur at a limited number of residues. To probe the importance of three of these residues, the functionally acceptable substitutions at these sites have been determined by simultaneous randomization. A cassette was synthesized with the triplet NN(G/C) at each of the three positions on one strand with the triplet II(G/C) in the complementary position of the other strand. The base inosine was used because it would pair with any of the four nucleotides[22,23]. By using only G/C in the third position, all possible amino acids can be encoded, while the probability of encoding the least likely amino acid is increased[24]. *E. coli* harboring plasmids encoding the generated mutants were selected on the antibiotic cefotaxime. Aliquots of transformed cell suspensions were plated on tetracycline-containing agar to estimate the number of colonies screened. Plasmids were isolated from each of the two hundred eighty-seven colonies that were resistant to cefotaxime, and the sequences were determined. An autoradiograph of a representative sequencing gel is shown in Figure 2. Sixty-eight different amino acid sequences were found at the three residues mutagenized (Figure 3). Sequences of thirty-nine mutants that were not selected on cefotaxime were determined to verify the random incorporation of bases at the mutagenized sites (data not shown.).

The mutations at the three residues may be considered individually or as potentially compensating triplets. When each residue is considered individually, an overall pattern appears. Residue 237 was generally a small residue, and the amino acids Ser, Gly, Ala and Thr were found in this position. Seven residues were found in position 238: Ala, Asn, Arg, Ser, Thr, Asp and Gly. The Ser238 substitution appeared to be the most favorable at this position. A positively charged residue was commonly found in position 240. When the sequences obtained are considered as triplets, some compensatory changes appear. A

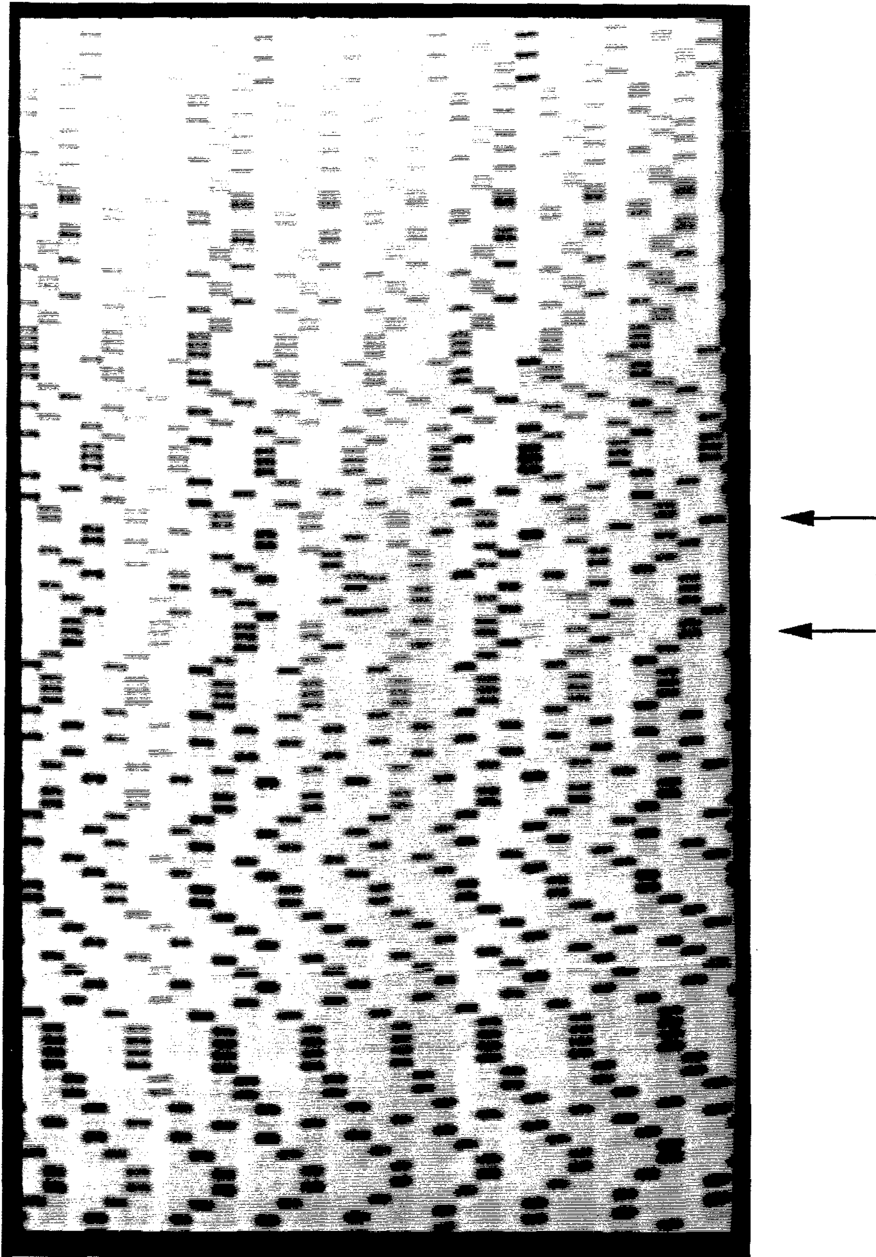


Figure 2. An autoradiograph of a representative sequencing gel. The arrows flank the mutagenized region.

237	238	240
A	A	H
A	A	K
A	A	R
A	A	V
G	A	H
G	A	K
G	A	R
S	A	K
A	N	H
A	N	K
A	N	R
A	N	T
A	N	Y
G	N	H
G	N	K
G	N	R
G	N	T
S	N	A
S	N	K
S	N	T
A	R	T

237	238	240
A	S	H
A	S	K
A	S	R
A	S	N
A	S	Q
A	S	E
A	S	M
A	S	S
A	S	T
A	S	Y
A	S	A
A	S	I
A	S	L
A	S	V
S	S	H
S	S	K
S	S	R
S	S	N
S	S	Q
S	S	E
S	S	M
S	S	S
S	S	T
S	S	Y
S	S	A
S	S	I
S	S	L
S	S	V

237	238	240
G	S	H
G	S	K
G	S	L
G	S	R
G	S	T
G	S	V
T	S	K
T	S	Q
T	S	R
T	S	T
R	S	R
R	S	T
A	T	H
A	T	K
S	T	K
A	D	K
A	D	R
G	G	P
S	G	P

Figure 3. Sequences of RTEM-1 derivatives at residues 237-240 that confer resistance to cefotaxime. The wild-type sequence is A G E.

glycine at position 238, the wild-type residue, was found if and only if it was followed by a proline at position 240. Arginine was found at position 238 only once in the sequence ART and aspartate was found only twice, in the sequences ADK and ADR.

Western blot analysis of whole-cell lysates was performed to assess the levels of the ß-lactamases present in the cells. Eight of the inactive mutants that were not selected against cefotaxime were included to verify comparable levels of protein present. This experiment demonstrated that there was little variation in the amount of enzyme present (Figure 4).

E. coli carrying the mutant ß-lactamases were tested for resistance to ß-lactam antibiotics by a disk diffusion susceptibility assay. Use of this technique allowed the assay of all sixty-eight mutants in an efficient manner. Results are shown in Table 3. The commercially available disks containing benzylpenicillin and oxacillin are not of a high enough drug concentration to affect the growth of *E. coli,* and all cultures displayed no inhibition of growth in the vicinity of the disks. The assay was inconclusive for three of the mutants, SSQ, SST and SSV.

The thirty-eight most resistant colonies were then characterized by a broth microdilution susceptibility assay to determine the minimum inhibitory concentration of the antibiotics cephalothin, ampicillin, benzylpenicillin, cefotaxime, cloxacillin and cefoxitin. These results are shown in Table 4. The values reported are the highest concentration of antibiotic in which growth was observed. All of the mutants confer a level of resistance to the penams ampicillin and benzylpenicillin that is reduced compared to the resistance conferred by the wild-type RTEM-1. However, the majority of the mutants confer increased resistance to cephalothin and the penam cloxacillin when compared to the wild-type RTEM-1.

Three of the mutants were further characterized to determine the kinetic constants k_{cat} and K_M. These data are shown in Table 5. The values for k_{cat} on the substrate

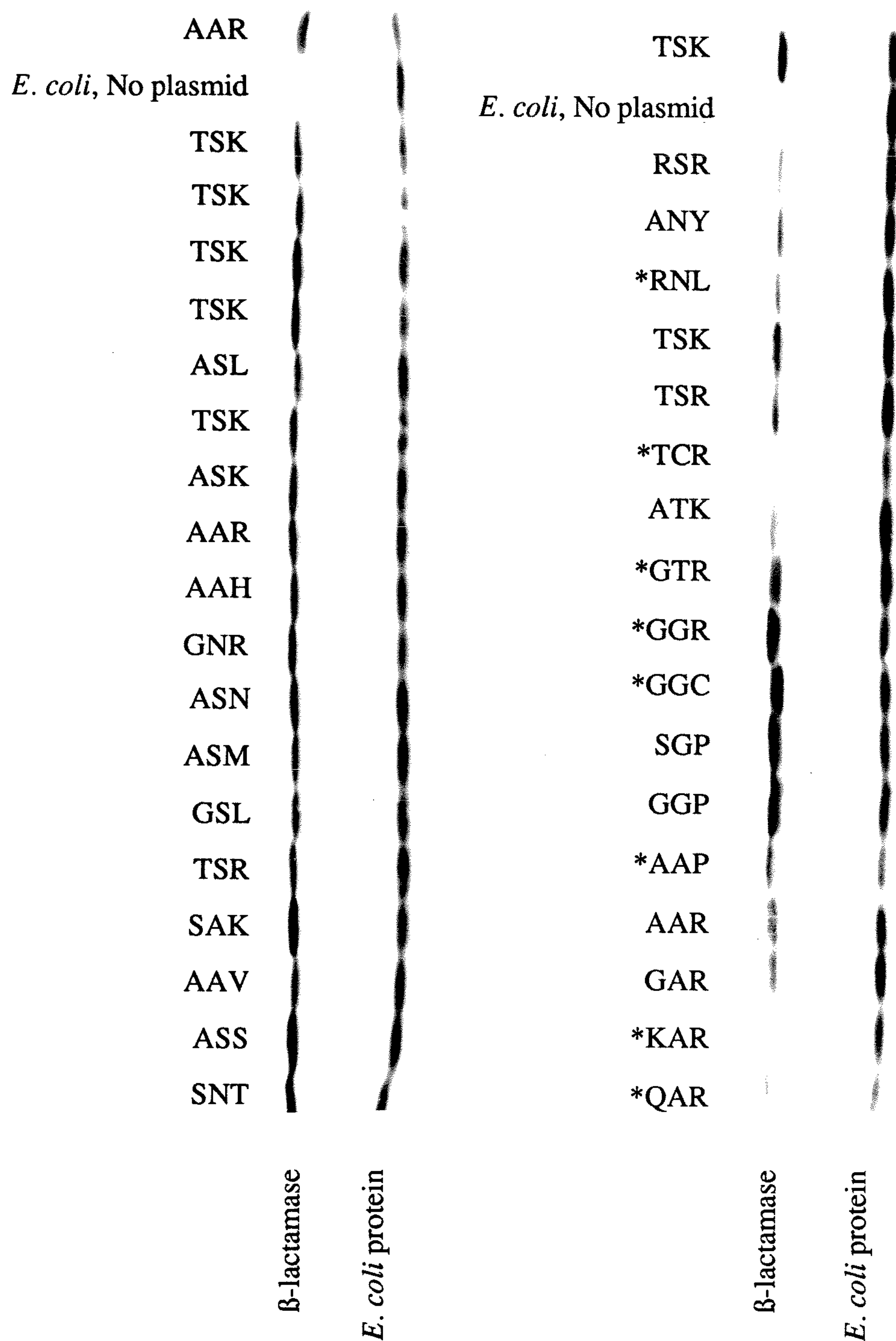


Figure 4. Western blot analysis of RTEM-1 mutants at sites 237, 238 and 240. Mutants marked with an asterisk (*) were not selected for activity on cefotaxime.

Table 3. Zone of Inhibition of Growth in a Disk Susceptibility Assay

	LOT	TAX	CXM	FOX		LOT	TAX	CXM	FOX
A S K	7	22	17	26	G G P	12	30	18	25
A S R	7	25	20	27	R S R	12	30	22	24
S S K	7	25	18	22	A A V	13	30	22	27
A A K	8	25	22	28	S S I	13	30	21	25
A S H	8	25	17	26	S S N	13	30	21	25
A N K	10	25	15	25	A S N	14	30	22	26
A N H	12	25	15	25	T S T	15	30	22	24
A R T	13	26	20	26	A N T	17	30	20	24
A S T	13	26	21	26	A N Y	18	30	20	23
A A R	9	27	22	27	G N T	18	30	20	26
S N K	10	27	16	25	G S T	19	30	23	24
A S E	10	27	21	26	S G P	7	31	17	26
A S L	10	27	22	27	S S A	10	31	21	25
A A H	11	27	20	28	S N A	11	31	21	26
S S H	11	27	18	25	G N R	14	31	20	24
G A K	12	27	21	26	S S E	14	31	22	25
A D K	9	28	17	25	S S L	14	31	21	25
G G P	11	28	18	25	S S M	16	31	22	24
A N R	11	28	18	25	G A H	17	31	23	28
A S M	11	28	22	24	G S R	17	31	22	24
G S K	11	28	21	27	S S Y	17	31	21	23
A S I	12	28	22	25	G A R	15	32	23	27
G S K	12	28	21	24	S T K	17	32	23	24
G N K	13	28	19	25	G S V	19	32	21	24
A S Y	13	28	21	26	G G R	15	33	21	25
R S T	14	28	20	25	A A P	15	33	22	23
G N H	15	28	17	24	A T H	19	33	23	24
G S H	15	28	20	27	wild type	11	34	21	23
S A K	10	29	21	26	wild type	12	34	21	22
S S R	10	29	20	23	G G C	13	34	21	25
A S Q	11	29	22	26	T S Q	14	34	25	24
A S V	11	29	22	26	S S S	19	34	22	25
T S K	12	29	23	24	T R A	20	34	23	24
A S S	13	29	21	27	R N L	20	34	23	23
G S L	13	29	22	25	G T R	21	34	23	25
S N T	14	29	19	23	wild type	12	35	21	23
A T K	14	29	22	24	T S R	15	35	27	24
A S A	10	30	22	25	T C R	20	35	24	26
A D R	11	30	20	26	K A R	20	35	23	25
A A V	12	30	21	28	G S L	11	-	-	25
G G P	12	30	18	26	R-	18	35	22	24

Value given is diameter of inhibition zone in mm. Abbreviations are : LOT, cephalothin; CTX, cefotaxime; CXM, cefuroxime; FOX, cefoxitin. Discs are 7mm in diameter.

Table 4. Growth of Colonies Harboring Mutant ß-lactamases in a Broth Microdilution Susceptibility Assay

			TAX	LOT	AMP	PEN	CLOX	FOX
A	S	K	10	270	3100	3200	1600	0.56
A	S	R	5	180	1600	3200	1600	0.56
S	S	K	3.8	180	800	1600	400	0.56
A	A	K	2.5	90	3100	3200	1600	0.56
A	S	H	5	90	1600	1600	800	0.56
A	N	K	3.8	90	3200	3200	1600	0.56
A	N	H	5	45	3100	3100	1600	1.1
A	R	T	3.8	45	1600	3100	800	0.56
A	S	T	3.8	45	1600	3100	800	0.56
A	A	R	1.2	90	3100	3200	1600	0.56
S	N	K	0.96	90	800	1600	400	0.56
A	S	E	1.2	90	1600	3200	800	0.56
A	S	L	1.9	90	1600	3200	800	0.56
A	A	H	3.8	90	3200	3200	1600	0.56
S	S	H	2.5	90	800	1500	200	0.56
G	A	K	1.2	45	1600	1600	1600	0.56
A	D	K	0.48	90	800	1600	800	0.56
G	G	P	0.24	45	3200	3200	1600	0.56
A	N	R	1.9	90	3100	3200	1600	0.56
A	S	M	1.2	90	1600	3200	800	0.56
G	S	K	2.5	90	3100	3100	800	0.56
A	S	I	1.9	90	1600	3100	1600	0.56
G	N	K	0.96	45	1600	3100	1600	0.56
A	S	Y	2.5	90	1600	3200	800	0.56
R	S	T	1.9	45	800	1600	100	0.56
G	N	H	0.96	22	1600	1600	800	1.1
G	S	H	1.2	22	800	800	400	0.56
S	A	K	0.96	90	1600	3200	400	1.1
S	S	R	1.9	90	800	1600	200	0.56
A	S	Q	1.9	90	1600	3200	1600	0.56
A	S	V	1.2	90	1600	3200	800	0.56
T	S	K	1.2	180	400	800	100	0.56
A	S	S	0.96	45	1600	3200	400	0.56
G	S	L	1.9	90	1600	3200	800	0.56
S	N	T	0.48	22	800	1600	400	1.1
A	T	K	2.5	90	1600	3200	1600	0.56
A	S	A	0.48	90	3100	3200	1600	0.56
S	G	P	0.48	270	1600	3200	400	1.1
	WT		0.03	45	6250	6250	800	0.56
	R-		0.03	5.6	6.2	12	100	0.56

Sequence given is the sequence found at residues 237-240. Value given is the highest concentration , in mg/L, of antibiotic in which growth was observed. Abbreviations are: TAX, cefotaxime; LOT, cephalothin; AMP, ampicillin; PEN, benzylpenicillin; CLOX, cloxacillin; FOX, cefoxitin.

benzylpenicillin are only slightly decreased, while the values of K_M are below what can be accurately measured by the spectroscopic technique.

Table 5. Kinetic Constants of Selected Mutant ß-lactamases

	Benzylpenicillin		Cefotaxime
Sequence	k_{cat} (s^{-1})	K_M (μM)	K_M (μM)
AGE (wt)	980[a]	42[a]	> 3000[b]
AAK	400	< 10	>500
ASK	50	< 5	120 (130[c])
ASE	d	d	260[c]

[a] Ref. 25. [b] K_i, Ref. 13. [c] Measured using crude preparations of enzyme. [d] Not measured.

The possible structural effects of the functionally acceptable substitutions were modeled based on the homologous ß-lactamase of *B. licheniformis*. The orientation of benzylpenicillin in the binding site has been modeled previously based on the known roles of certain residues and by comparison with the structure of the D-alanyl-D-alanyl carboxypeptidase of Streptomyces R61[9,20]. In this model of the enzyme-substrate complex, the side chains of residues 237-240 are directed away from, and have little or no contact with, the substrate. A structure of the substrate cefotaxime was constructed using the Biograf software. Several conformational searches were performed, followed by energy minimization to determine the best model of the structure. The cefotaxime side chain is highly conjugated and is expected to be a rigid planar structure. The modeled cefotaxime structure was docked in the putative binding site by superimposing the lactam ring onto the corresponding structure of benzylpenicillin previously modeled into the active site[20]. Contacts between the enzyme and the substrate were analyzed, but no conclusions could be made. It is not known if the *B. licheniformis* ß-lactamase binds or hydrolyzes cefotaxime.

Ala238 (Gly238 in the RTEM-1 sequence) was analyzed to identify residues with which it might have contacts. It was found that the side chains of residues Ala67, Ala69 and Asn170 are near the methyl group of Ala238. Ala238 was then replaced with serine, the possible conformations were searched and the energy of the structure was minimized. In all of the conformations obtained, the hydroxyl group of the serine was too far from the oxime group of the substrate for this contact to be the cause of the altered activity as previously proposed[7,12]. Instead, there was a possible contact between the serine hydroxyl and the side chain of the conserved Asn170.

The substitution of Ser240 with Lys in the enzyme-substrate model also resulted in no obvious contacts between the residue and substrate. Instead, it was found that a salt bridge could be formed between the modeled lysine and Glu171. In order to form this contact, a salt bridge between Glu171 and Lys270 would have to be removed. An analogous salt bridge between Glu171 and Lys 270 is not expected to be present in the structure of RTEM-1, as residue 270 is not conserved among the class A ß-lactamases and the RTEM-1 sequence has a methionine at this position.

Several other functionally active substitutions were modeled in the *B. licheniformis* ß-lactamase structure, but it was not possible to explain the effects of these substitutions. The lack of conclusions from these modeling studies is most likely due to the limitations of the available structural information. The *B. licheniformis* ß-lactamase, though homologous with RTEM-1, is a different enzyme with differing catalytic properties. While the presence and orientation of secondary structural elements are expected to be alike or very similar in the RTEM-1 structure, the sequence differences may lead to changes in the locations of side chains that may preclude explanation of mutagenesis results based on the structural data. In addition, the structure was determined in the absence of substrate, and as such, is the conformation of unbound enzyme. The structure of an enzyme-substrate complex of a ß-lactamase has not yet been determined. There is considerable evidence that the class A ß-lactamases undergo conformational changes in the presence of various

substrates[26-28]. The limitation of the unbound enzyme structure as a model of the active enzyme is further demonstrated by the location of the side chain of Arg164. This residue is conserved among all class A ß-lactamases, and its substitution in RTEM-2 yields the extended-spectrum ß-lactamase RTEM-7. However, in the X-ray determined structure, it is a surface residue with no apparent function and would not be expected to be conserved.

There are several possible effects of the substitutions that may lead to the altered activity. In the simplest case, substituted residues may contact the substrate directly and introduce new stabilizing contacts. Or conversely, the substitutions may remove a destabilizing contact between a residue side chain and a potential substrate. These two possibilities are not likely due to the range of structures of substrates for which the activity of the enzyme is altered. If it were a simple case of introducing or removing a specific contact between a residue and the substrate, then the activity change would be limited to substrates of a similar structure. This is not the observed result. Instead, the K_M's of such disparate structures as benzylpenicillin and cefotaxime are decreased substantially in the mutants (see Figure 1 and Table 5).

During the enzymatic hydrolysis of cephems by ß-lactamase, the acyl-enzyme bound substrate may undergo elimination of the 3'-substituent leading to a second acyl-enzyme structure (Figure 5). This second acyl-enzyme intermediate hydrolyzes slowly and transiently inhibits the enzyme. The extent of the elimination reaction and its contribution to the observed steady-state rate constants are dependent upon the nature of the 3'-leaving group and the particular ß-lactamase studied[29-31]. The possibility of an elimination reaction affecting the observed rates of hydrolysis must be considered when analyzing ß-lactamase activity on the cephem antibiotics. A mutation that slows the rate of deacylation may allow a greater partitioning of the initial acyl-enzyme to the 3'-eliminated acyl-enzyme. However, the increased activities of the mutants on the cephem antibiotics studied here are not due to a change in an elimination reaction. Hydrolysis of benzylpenicillin by the wild-type enzyme is not inhibited by cefotaxime, even at high cefotaxime concentrations[13], which would be

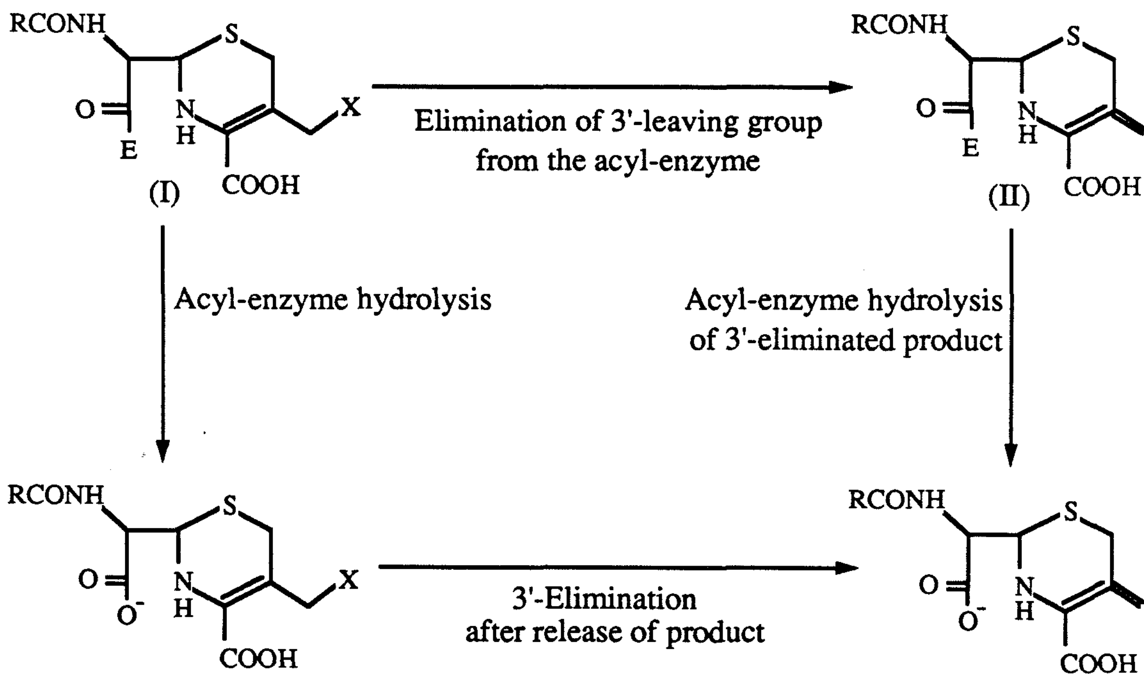


Figure 5. 3'-Elimination reaction of cephem antibiotics during ß-lactamase catalyzed hydrolysis. A decrease in the deacylation rate of the acyl-enzyme intermediate (I) increases partitioning to the 3'-eliminated acyl-enzyme (II), which is hydrolyzed slowly.

expected if the inactivity of the wild-type enzyme towards hydrolysis of cefotaxime were due to the blocked deacylation of a 3'-eliminated intermediate. Also, the mutants studied here had substantially decreased K_M values for benzylpenicillin. This could not be due to an elimination reaction because there is no such analogous reaction possible in the penams.

Another possible effect of the mutations may be an enlargement of the binding site to accommodate the rigid ring structure of the cefotaxime. This is not expected to be the primary effect for two reasons. First, some of the mutants show significant increases in activity on cephalothin, a second generation cephem with a flexible side chain, while others show a decrease on this substrate, with no apparent correlation to sequence. Second, a comparison of the activities on the rigid penam cloxacillin and cefotaxime showed no correlation. While many of the mutants showed an increased activity against cloxacillin, a penam with a rigid side chain, this increase was not correlated with increased activity against cefotaxime (Figure 6).

The mutations may allow the enzyme to adopt conformations active on cefotaxime that are not accessible to the wild-type enzyme. Often the results of mutagenesis studies are analyzed by treating the enzyme as a rigid peptide backbone with various side chains dangling from it. The presumption is that a change in one of the side chains affects the side chains of other residues without affecting the backbone. Application of the current molecular modeling methods often require this assumption because of the limitations of computing ability when many atoms are moved in a conformational search. While the simplification may hold true for some systems, the assumption should not be applied to all enzymes or mutations without consideration of its validity. The class A ß-lactamases have been shown to exist in altered conformational states induced by the presence of different substrates, and these differing conformations display differing activities. This known characteristic of the class A ß-lactamases suggests that the naturally occurring and generated mutants are able to hydrolyze the substrate cefotaxime through a conformational change to an active conformation not accessible to the wild-type enzyme.

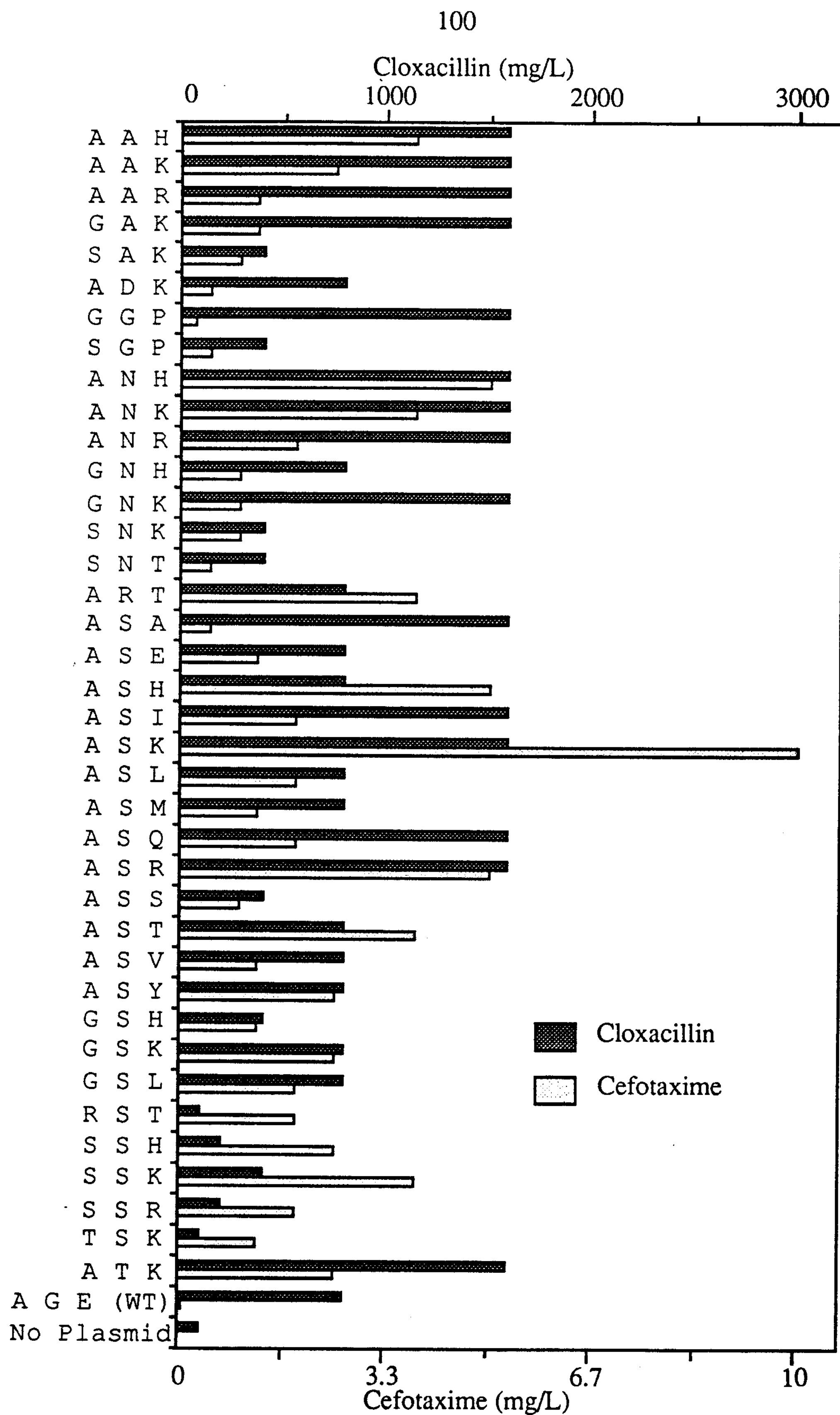


Figure 6. Bar graph of the minimum inhibitory concentrations of cloxacillin and cefotaxime against *E. coli* carrying mutant ß-lactamases.

Conclusions

By simultaneous randomization of residues 237, 238 and 240 of RTEM-1 ß-lactamase, sixty-eight mutant ß-lactamases were identified that were able to hydrolyze the ß-lactam antibiotic cefotaxime. The wild-type RTEM-1 does not bind, and has no activity against this antibiotic. Analysis of available structural information yields no explanation of the activity of these mutants. The side chains of these residues do not appear to contact the substrate directly. The effect of the mutations is not a simple enzyme-substrate contact as was previously proposed. Instead, the mutations allow a subtle reorganization of the binding site to increase the affinity for the substrate. The alteration of binding properties is expected to arise through a conformational change of the enzyme that cannot be predicted using current modeling techniques and currently available structural data.

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