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19. ABSTRACT (Continue on reverse if necessary and identify by block number)
This work focuses on the creation of new catalytic activities by modification of existing enzymes. To this end, techniques for the synthesis of large genes have been developed together with a complementation system for the expression and correct folding of proteins that are normally self-processing but that have been mutated so they can no longer process themselves. (Concurrent expression of the pro-sequence and the mature protein sequence encoded on separate but compatible plasmids accomplishes this purpose; the pro-sequence apparently performs the role of a chaperonin in this case.)
The enzymes studied are two serine hydrolases, α-lytic protease and β-lactamase. For α-lytic protease the new activity sought was the ability to hydrolyze organophosphates rather than being irreversibly inhibited by these substances. For β-lactamase we created a structural variant that had a dramatically different catalytic role on the normal β-lactam substrates. Rather than catalyzing the normal β-lactam ring opening hydrolysis reaction, the mutant catalyzed the cleavage of the substrate into two fragments, a characteristic catalysis
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FINAL REPORT June 17, 1991

CONTRACT N00014-83-K-0487

PRINCIPAL INVESTIGATOR: Dr. John H. Richards

CONTRACTOR: Dr. Michael T. Marron

CONTRACT TITLE: Mutant Proteins--Enzymes to Hydrolyze Toxic Organophosphates

START DATE: 15 June 1983

RESEARCH OBJECTIVE: To alter the catalytic activity of native enzymes by random and site-specific mutagenesis.

PROGRESS:

A. SUMMARY

i. Synthesis of a gene for a bacterial amide hydrolase (α-lytic protease) by a novel, general method that allows the synthesis of very large genes.

ii. Development of an approach for expression that allows production of correctly folded mutants of native enzyme as well as both catalytically active and inactive mutants.

iii. Preliminary study of the properties of mutants as catalysts for the hydrolysis of organophosphates.

iv. Alteration in the substrate specificity of β-lactamases by mutagenesis.

v. Alteration in the fundamental nature of catalysis by β-lactamases.

B. TRAINING

The training of the following individuals has been substantially advanced by their support from this ONR award. Their present positions are also given.

Postdoctoral Fellows

Frances Arnold	Assistant Professor of Chemical Engineering, California Institute of Technology
John Tomich	Assistant Professor of Biochemistry and Pediatrics, University of Southern California School of Medicine and Childrens' Hospital of Los Angeles

Graduate Students

Steven Carroll	Research Scientist, Merck Sharpe and Dohme
Yie-Hwa Chang	Assistant Professor of Biochemistry, University of St Louis

Diane Hollenbaugh	Postdoctoral Fellow, Bristol Myers Co., Seattle
Sheila Iverson	Senior Research Associate, University of Texas, Austin
Robert Kaiser	Senior Research Fellow, Division of Biology, California Institute of Technology
David Long	Postdoctoral Fellow, Department of Biochemistry, University of Colorado, Boulder

C. DETAILED REPORT OF PROGRESS

(i) Gene Synthesis

Our approach involves synthesis of the gene in a stepwise fashion. The construction of the gene by this method proceeds from the ends toward the middle. Segments of the gene are sequentially cloned into an appropriate vector that allows amplification of the growing gene at intermediate stages of synthesis. After a segment has been inserted and the plasmid amplified, the resulting intermediate, containing two unique restriction sites within the segment last inserted, is opened at these sites, which then act as recipients for the next segment of the gene. Importantly, although all bases that define one of the particular sites used for opening must, of course, be present in that intermediate, the site(s) need not be reconstituted for incorporation of the next cassette; only those bases necessary to provide compatible overhangs or located upstream of the upstream site and downstream of the downstream site need to be retained in the final gene. Thus, any particular restriction endonuclease may in principle be used multiple times in a given synthesis.

Though a very conservative approach for the synthesis of a gene that encodes α-lytic protease (597 base pairs), this general strategy has considerable flexibility and should prove particularly suited to the synthesis of larger genes that might be difficult to prepare by the consecutive annealing of segments followed by cloning. The approach also permits editing at intermediate stages. The strategy is outlined in Fig. 1 and the sequence of the synthetic gene shown in Fig. 2.

(iia) Expression of Synthetic Gene and Catalytically Inactive Mutants

Active Enzymes. With the cloning of the wild-type α-lytic protease gene, and its subsequent expression using the promoter and leader sequence from the *E. coli* PhoA gene (1), the clear choice for expression of the synthetic gene was to substitute the protease coding portion of the wild type gene with the synthetic gene. Using cassette mutagenesis, a unique Sty I restriction site was added to the synthetic α-lytic protease gene by introducing a single silent mutation at the 88th base in the gene. This Sty I site and an existing Bgl II site downstream of the synthetic gene were used to excise the majority of the gene. The excised fragment was then used as a cassette replacement for the corresponding region of the wild-type α-lytic protease gene in the PhoA, pro-α-lytic protease expression vector, pALP5. The presence of a second Sty I site in the pALP5 vector precluded using a simple two-fragment replacement scheme. (The Bgl II and BamH I restriction sites possess complementary cohesive ends, allowing the two sites to be ligated together, yet removing both sites in the produce plasmid.) This chimeric gene contained only

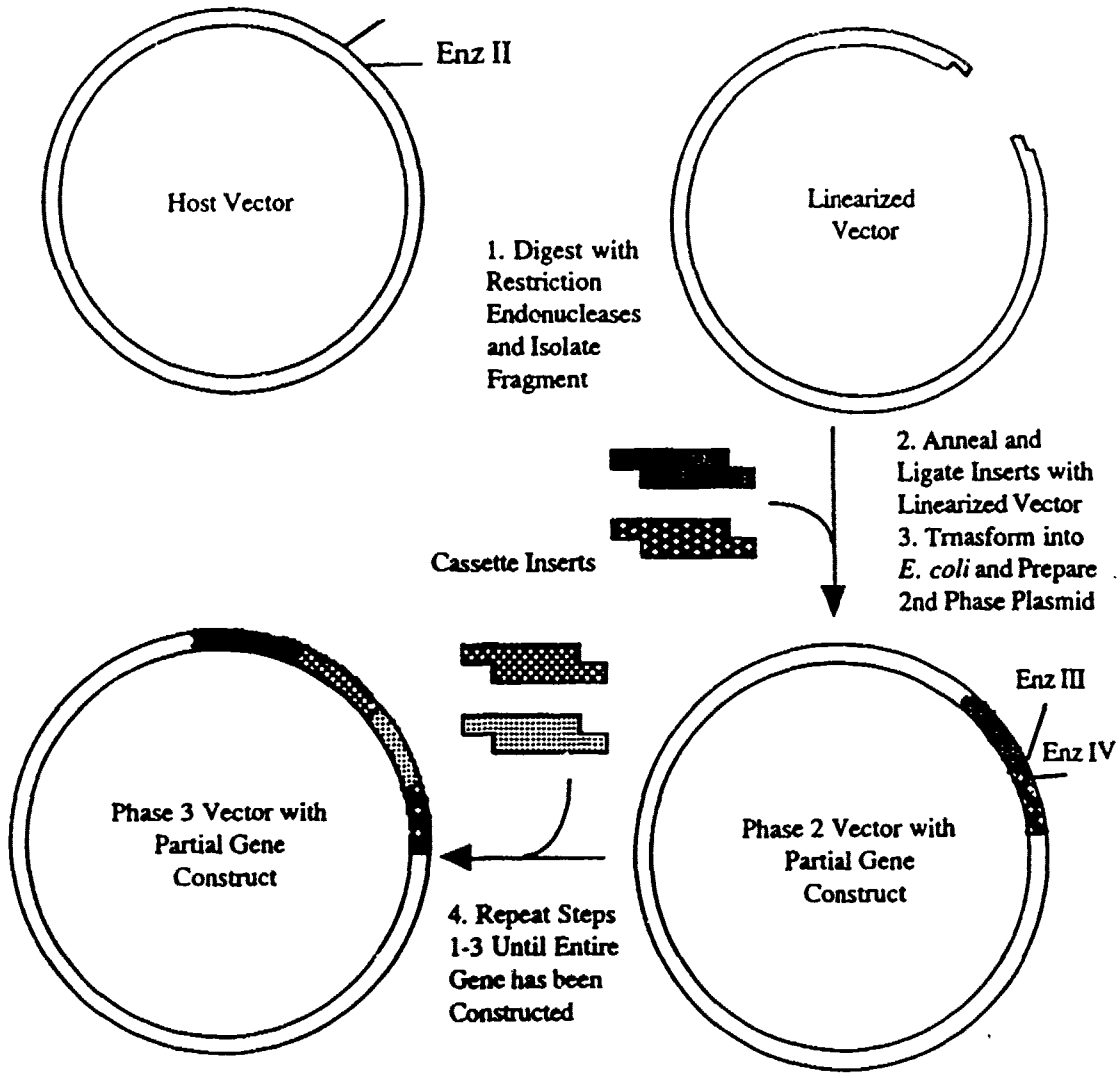

Figure 1. A general overview of the gene synthesis technique using cassette mutagenesis.

fourteen of the sixteen unique restriction sites present in the original synthetic gene, due to the loss of the two sites upstream of the Sty I site.

α-Lytic protease was expressed through the induction of the PhoA promoter by the depletion of phosphate in the media. The pro-enzyme is translocated into the periplasm of the cell, where it self-cleaves into the mature enzymatic form. Growths of wild-type α-lytic protease accumulate large amounts of the enzyme in the media, apparently from leakage of the enzyme through the periplasmic membrane (2).

Activity was screened using the membrane-bound protease activity assay (3). *E. coli* cells harboring either the pALPE or pALPE-S195A plasmids were grown on membrane-agar plates over a two-day period, allowing the enzyme expressed and secreted into the extracellular space to become bound to the nitrocellulose membrane. Bound α-lytic activity was assayed by immersing membranes in a solution of the

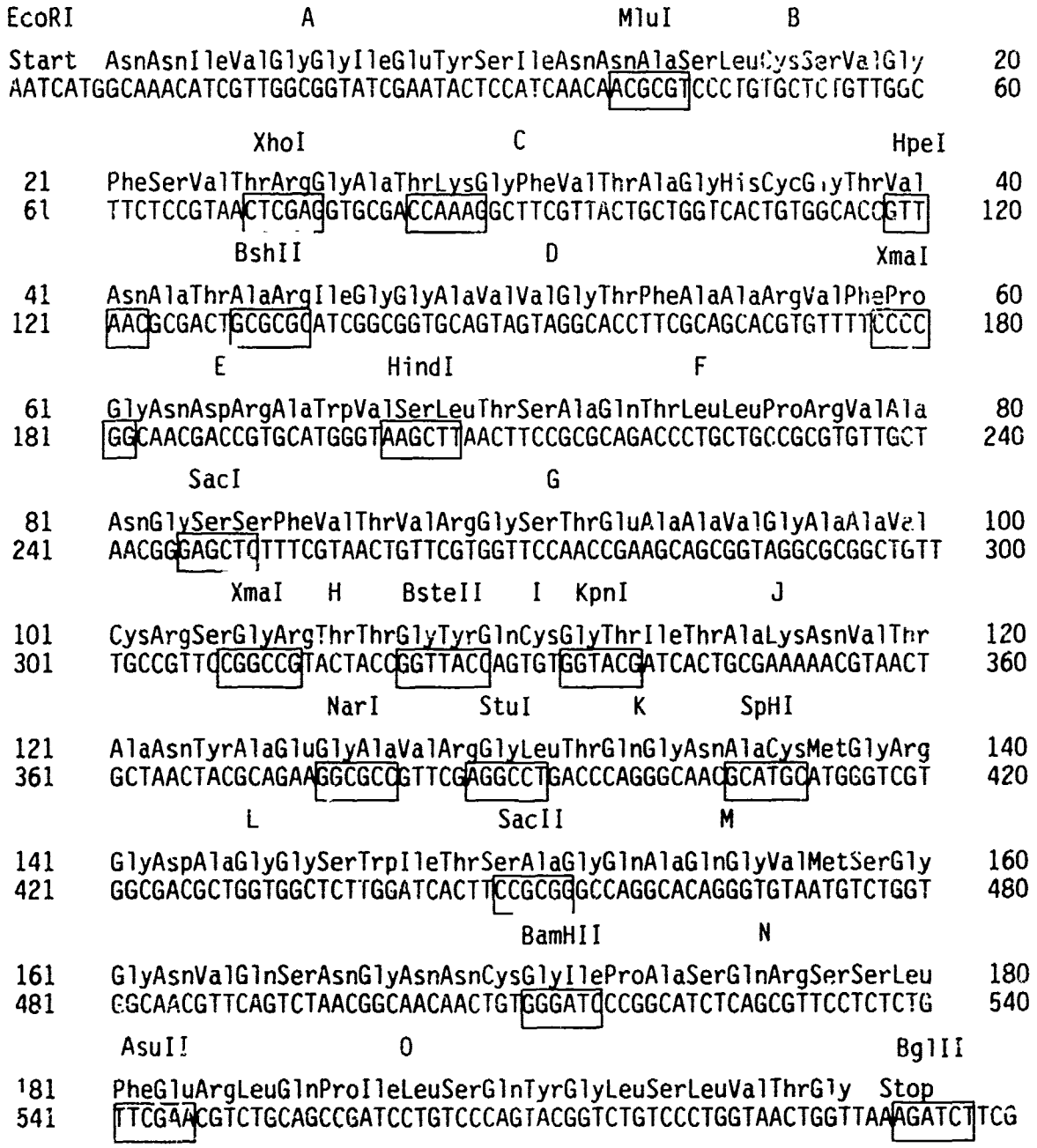

Figure 2. A synthetic gene for α-lytic protease. Restriction enzyme sites are boxed. The synthesized oligonucleotides are labeled A through O.

protease substrate, N-acetyl-Ala-Ala-Ala-methyl ester, and phenol red; the localized increase in acidity from cleavage of the substrate produces a colorimetric change in the vicinity of those colonies producing active enzyme. As expected, the assay displayed active α-lytic protease expressed from cells containing the pALPE (wild-type coding gene) plasmid. However, cells harboring the pALPE-S195A plasmid, as well as control cells without either of the α-lytic protease vectors, displayed no activity.

To determine whether expression of the mutant α-lytic enzyme was taking place and to what extent the enzyme was being post-processed, Western analyses were performed on supernatants from 4 day growths in MOPS media. Cells harboring the wild-type coding plasmid revealed a band at ~20 kD, indicative of the mature enzyme, and traces of a ~40 kD band, indicating presence of small amounts of the pre-enzyme precursor, in supernatant samples (Fig. 3) . However, cells expressing the Ser

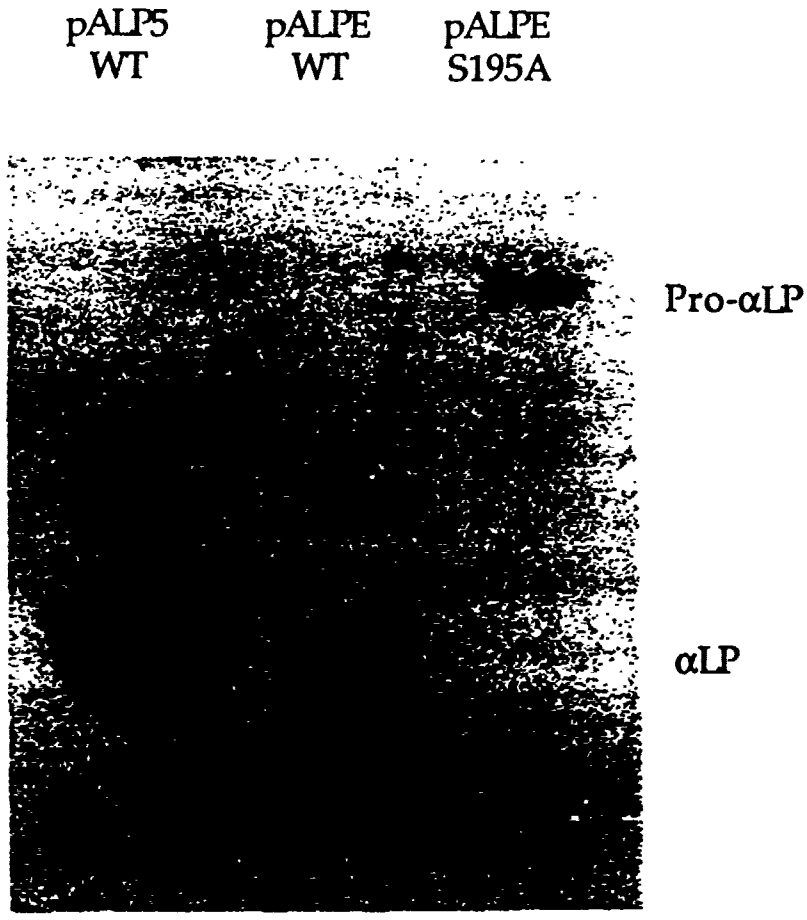

Figure 3. Western blot of α-lytic protease samples. Lane 1 is wild-type α-lytic protease expressed from the pALP5 vector. Lane 2 is wild-type α-lytic protease expressed from the pALPE vector. Lane 3 is the Ser 195 Ala mutant of α-lytic protease expressed from the pALPE-S195A vector. Trace amounts of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

195 Ala gene (pALPE-S195A) displayed only the ~40 kD band in the supernatant samples. The lack of the 20 kD band in Western stains of pALPE-S195A harboring cells demonstrates that the Ser 195 Ala active-site mutant is incapable of self-processing, resulting in its expression only as the pro-enzyme.

(iib) The Complementation Expression System

Inactive Mutants. Initial experiments with the wild-type α-lytic protease gene demonstrated that expression of the protease portion of the gene alone resulted in the production of an inactive, improperly folded, α-lytic protease enzyme (4). By separating the pro-coding the protease-coding regions of the wild-type gene, and placing each region, along with a leader sequence, under the control of an independent promoter, we demonstrated that, though the proper folding (activation) of α-lytic protease required the presence of the pro-region of the protein, it was not necessary that the pro-peptide be covalently attached to the protease portion of the enzyme for activation, as it is in the naturally occurring zymogen. The α-lytic protease pro-region thus functions as both an inhibitor of protease activity while in the cytosol of the cell, and as a chaperonin (5) for the protease domain in arranging the unfolded protease into a state appropriate for activity.

We therefore chose to create a complementation expression system to obtain properly folded mutants, inactive of the α-lytic protease domain. This system consists of the two domains of the α-lytic protease gene cloned into and expressed from separate plasmids. Plasmids from compatible groups were chosen, allowing the two vectors to co-exist in the same cell line. The PhoA promoter and leader sequence from the recombinant α-lytic protease expression vector were chosen as the operon and upstream sequenes for both sub-genes, since they had been shown to produce high yields in the expression and translocation of the wild-type enzyme.

Construction of the two sub-genes from the wild-type gene and the synthetic gene was performed in four steps. Initially the PhoA promoter and leader coding sequence were isolated from the pALP5 vector and cloned into pBR322, interrupting the *Tet* gene. Transformants were screened for sensitivity to tetracycline, indicating the presence of the insert in the *Tet* gene. A three-fragment ligation of the synthetic gene, from pBR32LP-STYI, a synthetic oligonucleotide linker, and the described pBR322-PhoA plasmid was employed to construct a vector (pALP32P1M) for expression of the α-lytic protease domain alone. The use of the original synthetic α-'ytic protease gene meant that the pALP32P1M construct contained the full complement of restriction sites in the synthetic gene. The product plasmids were screened by restriction mapping.

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

A plasmid (pCTERM-2n3) containing a modified α-lytic pro-coding region, already isolated from the mature coding region, became available from the Agard laboratory. A three amino acid change made in the pro-coding domain was shown to enhance the specific activity of α-lytic protease isolated from a complementation system using this modified pro-peptide (6). It is believed that the modified pro-protein is better able to properly fold the mature α-lytic protease enzyme, thus producing a higher percentage of active enzyme in the protein preparations. We reconstructed our pro-peptide expression vector using this modified gene.

The maintenance of the two sub-genes on separate plasmids in a single cell required that the two plasmids have compatible origins or replication. Accordingly, the pro-coding domain gene from the pCTERM-2N3 plasmid was cloned into the

pACYC184 plasmid. This plasmid contains the p15A origin of replication and the chloramphenicol resistance gene, allowing it to co-exist and be maintained with vectors possessing the Col E1 origin and ampicillin resistance, such as pALP32P1M. With the two sub-genes in place on compatible vectors, an expression cell line was created through a double transformation and selection of colonies resistant to both ampicillin and chloramphenicol. Agarose electrophoresis of plasmid DNA isolated from ampicillin/chloramphenicol resistant colonies displays the two distinct vectors harbored in such cell lines. These double-vector cell lines will be referred to as "the complementation expression system."

(iic) Expression of α-lytic protease Protease Mutants

Mutant α-lytic protease enzymes were expressed from cells harboring the "complementation" expression plasmids. With both the pro-domain and the protease domain genes under PhoA control, expression was induced by phosphate depletion of cells grown in MOPS media. A quick and crude isolation scheme took advantage of the excretion of the enzyme into the growth media, by centrifuging the cells out of solution, followed by the removal of the low molecular weight constituents of the media by dialysis. The remaining solution, containing the enzyme, was lyophilized to dryness.

Western blots of lyophilized samples (Fig. 4) displayed a ~20 kD band for both

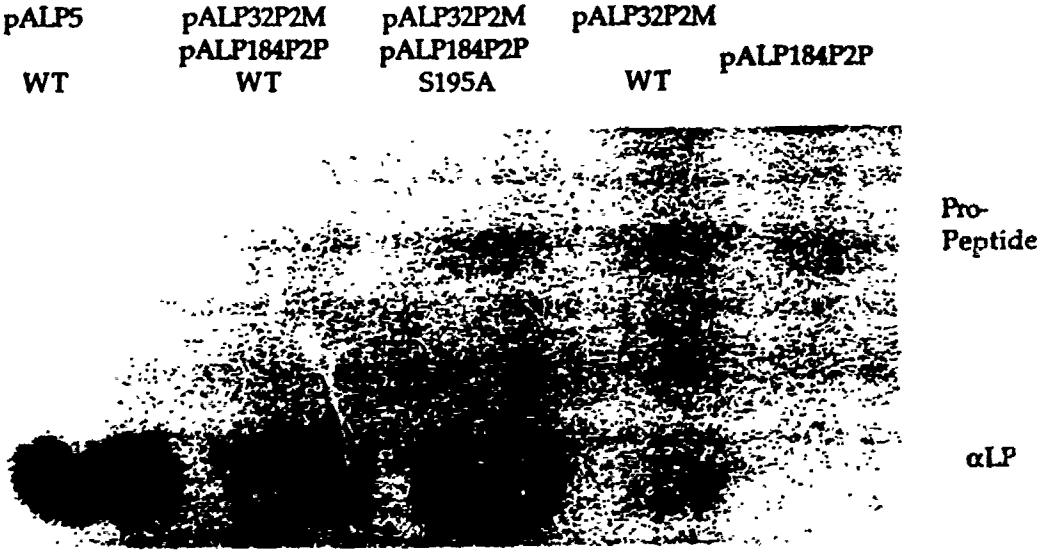

Figure 4. Western blots of supernatant samples from α-lytic protease complementation expression cell lines. Lane 1 is wild-type α-lytic protease from the pALP5 vector. Lane 2 is wild-type α-lytic protease from complementation cells harboring the wild-type protease domain and the pro-domain expression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

expression of the wild-type enzyme and the Ser 195 Ala mutant, indicating that the protease domain is being produced and exported. Since the protease domain is expressed separately, the existence of Western staining material in the growth media

is not proof that the enzyme is properly folded. However, expression of the protease domain in the absence of the pro-peptide results in very little of the 20 kD peptide being detected in the media; the improperly folded protease domain is unlikely to be stable to the other proteolytic enzymes produced by the cells.

(iid) Activity of α-Lytic Protease Mutants

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

(iii) Assaying Phosphatase Activity of Mutants

The use of phosphate esters, such as diethyl *p*-nitrophenyl phosphate (DNP), as substrates for proteolytic enzymes exhibit biphasic kinetics. This kinetic behavior is a result of the rapid acylation of the enzyme with the substrate, followed by a slow deacylation. For DNP, these kinetics are manifest as a burst of *p*-nitrophenol, from the acylation, proportional to the enzyme concentration and the degree of enzyme saturation by the substrate. After the initial burst, enzyme active sites are occupied by the phosphate-enzyme complex, and the rate of *p*-nitrophenol production becomes proportional to the rate of enzyme deacylation (turnover). For the wild-type α-lytic protease, the deacylation rate is nearly zero, with the half-life of the complex being on the order of hours (7). In addition to the biphasic enzymatic kinetics, diethyl *p*-nitrophenyl phosphate undergoes a continuous second order with hydroxyide self-hydrolysis reaction, and as such, reaction profile plots of wild-type α-lytic protease display a steady linear increase in *p*-nitrophenol concentration, both prior to the addition of enzyme, and after the inital burst acylation of the enzyme (Fig. 5).

If one assumes that the dissociation of the product from the serine 195 mutants is faster than the rate of hydrolysis of the substrate, organo-phosphatase activity would be expected to be seen as an increase in the rate of *p*-nitrophenol production, without the presence of the initial biphasic burst, since the mutants would be incapable of forming a covalent complex with the substrate. A typical plot of the Ser 195 Ala mutant (Fig. 6) does in fact demonstrate the lack of the burst kinetic phase. However, linear regression analysis of the pre-enzymatic and enzymatic reactions displayed no increase in the rate of DNP hydrolysis, with the measured rates being within 10% of each other. Various concentrations of DNP, above and below the wild-type K_M for the compound, displayed the same results.

The lack of detection of organo-phosphatase activity in the alanine mutant is not entirely unexpected. Though the replacement of the active-site serine with an alanine would provide a sufficient volume in the active site to accommodate a water molecule for use as the "activated" nucleophile, the methyl side chain of the alanine might tend to exclude the highly polar water molecule from close association in the pocket (8). Mutagenesis experiments on subtilisin, involving the substitution of the

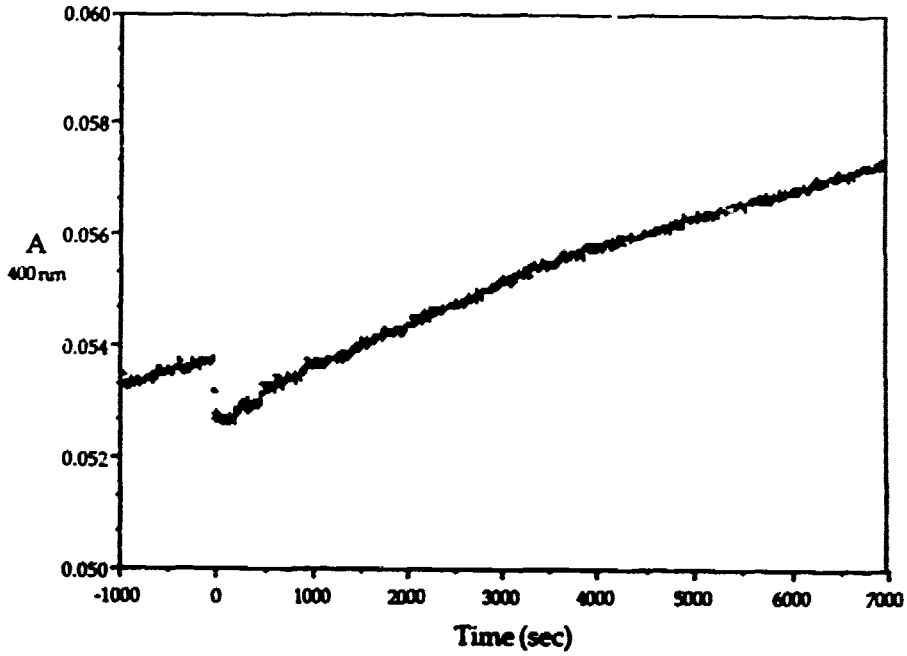

Figure 5. Typical reaction profile of α-lytic protease and diethyl *p*-nitrophenyl phosphate. $E_0 = \sim 2 \times 10^{-6}$M; $S_0 = 91$ μM; 10% (v/v) acetonitrile, 50 mM Tris, pH 7.75, 25°C. The reaction rate can be seen to be non-linear upon addition of enzyme and return to the linear self-hydrolysis rate of the inhibitor after the added enzyme has been acylated.

active-site serine 221 with an alanine (9), have demonstrated a measured decrease in the catalytic hydrolysis rate of peptide ester substrates on the order of 10^{-6}. An equivalent decrease in the rate of nucleophilic attack on the DNP substrate with respect to wild-type α-lytic protease, would be below the detection limits of the experimental methods employed.

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

(iva) Substrate Specificity of β-Lactamases

Lactamases (General). The β-lactam antibiotics are substrates of two large groups of bacterial enzymes. The first group, the penicillin binding proteins (PBPs), function as the D-Ala-D-Ala-carboxypeptidases and transpeptidases involved in the crosslinking of peptidoglycan in the final stage of cell wall biosynthesis (10). These enzymes are the natural target of the fungal antibiotics. The PBPs catalyze the

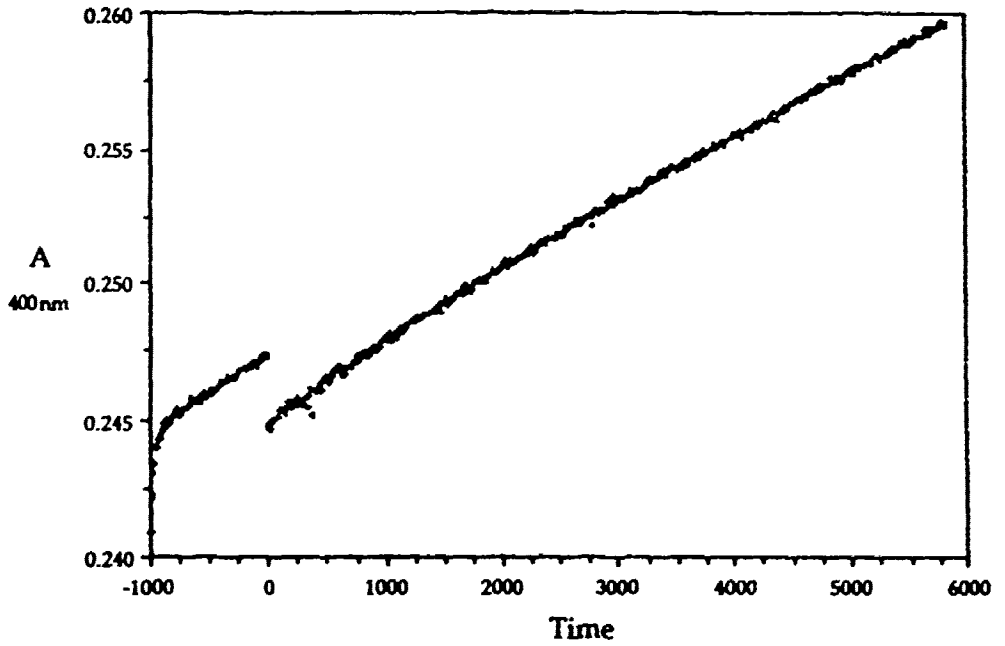

Figure 6. Typical reaction profile of the Ser 195 Ala mutant α-lytic protease and diethyl *p*-nitrophenyl phosphate. $E_0 = \sim 5 \times 10^{-6}$M; $S_0 = 430$ μM; 10% (v/v) acetonitrile, 50 mM Tris, pH 7.75, 25°C. The reaction rate can be seen to maintain the same linear self-hydrolysis rate of the substrate upon addition of the mutant enzyme.

hydrolysis of a C-terminal D-alanine-D-alanine dipeptide leading to a serine-ester-linked acyl-enzyme intermediate at the penultimate D-alanine residue. This step is then followed by either the hydrolysis of the intermediate to generate a free D-alanine terminus or transfer of the free amino terminus to another peptide chain. The latter reaction, known as transpeptidation, allows these enzymes to stabilize the bacterial cell wall against hypotonic lysis (Fig. 7). The PBPs are also able to bind the β-lactam antibiotics as structural analogues to the D-Ala-D-Ala dipeptide. However, reaction with these compounds, via nucleophilic attack on the lactam carbonyl group, results in a stable acyl-enzyme complex (Fig. 8). This competitive inhibition, characterized by the slow rate of antibiotic deacylation, prevents the cell wall crosslinking that is necessary for cell viability.

The second group of enzymes, the β-lactamases, are also acylated by the β-lactams. In contrast to the PBPs however, these enzymes rapidly deacylate the penicilloyl complex at rates nearing diffusion control (Fig. 9). The second-order rate constant (k_{cat}/K_M) for RTEM-1 β-lactamase utilizing benzyl penicillin as a substrate is on the order of 10^8 $M^{-1}sec^{-1}$ (11). Unlike the PBPs, the β-lactamases show no activity towards the D-Ala-D-Ala dipeptide (10), nor is the rate of β-lactam hydrolysis inhibited at any measurable level by the acyclic peptide substrates.

Tipper and Strominger (12) were the first to propose a possible evolutionary relationship between the two families of enzymes. The basis of their hypothesis was the observation that the D-Ala-D-Ala dipeptide and the penam antibiotic molecular structure are surprisingly analogous (Fig. 10). It is reasonable to assume that a mutation in the carboxypeptidase which would allow deacylation of the penicilloyl complex would have a genetic advantage that would surely be propagated.

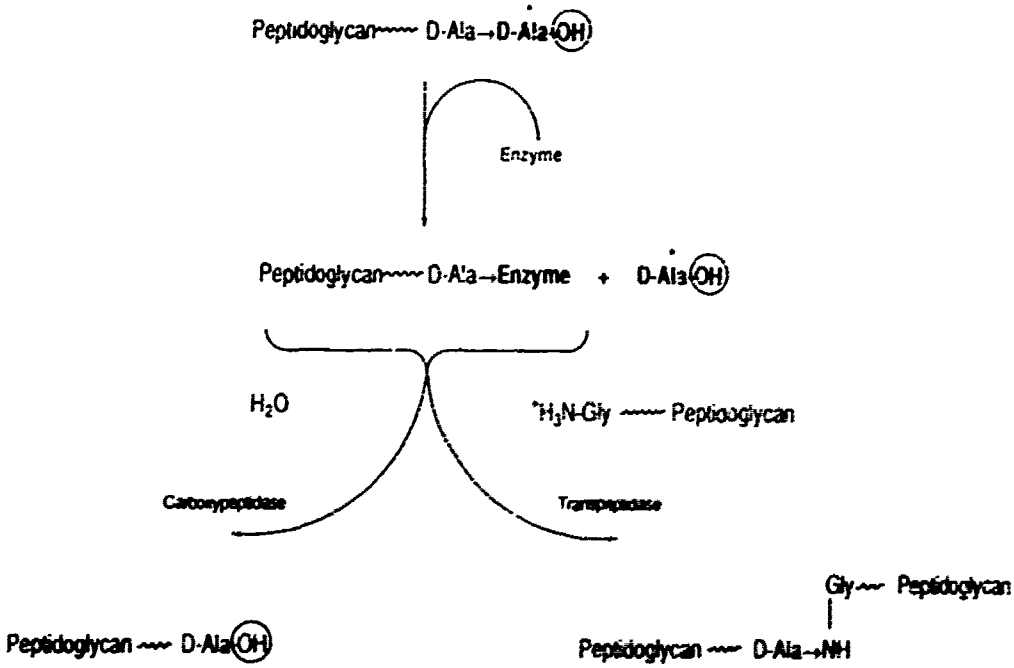

Figure 7. The process of bacterial cell wall biosynthesis. The strands of peptidoglycan are targeted by the D,D-carboxypeptidase, resulting in the hydrolysis of the terminal D-alanine residue. The acyl-enzyme intermediate can then be deacylated by water or an amino acceptor. the latter resulting in a stabilized crosslinked structure.

Examination of low resolution structures of the two enzymes reveals a conservation of secondary structural elements (13) (Fig.11). Further studies have shown that the amino acid sequences around the active-site serine in β-lactamases exhibit significant homology (14, 15) to those present in the PBPs (Table I). In both enzymes, the active-site serine is incorporated into a semiconserved triad: Ser-Thr-Xaa-Lys in the β-lactamases and Ser-Xaa-Thr-Lys in the PBPs. These factors combine to strengthen the initial hypothesis that the β-lactamases have evolved to enable the bacterial host to survive the selective pressure imposed by the β-lactam antibiotics.

A key argument against the proposed relationship is that the amino acid homology between the two enzymes is in fact low (>20%) (16). However, recent examination has revealed that the sequence homologies between individual class A β-lactamases are also relativeiy low, with an average homology of ~41% homology (17). Thus is would appear that a more macroscopic effect, secondary rather than primary structure, is dominant in the proteins' function.

(ivb) Carboxypeptidase/Lactamase Chimera

To further examine this relationship, we have created a chimeric enzyme consisting of the active-site sequence from PBP 5 of *E. coli*, cloned into RTEM-1 β-lactamase with the eventual goal of recruiting D,D-carboxypeptidase activity into β-lactamase. At the onset of this project there was no high resolution crystal structure available for a class A β-lactamase and thus the exact spatial arrangement of the

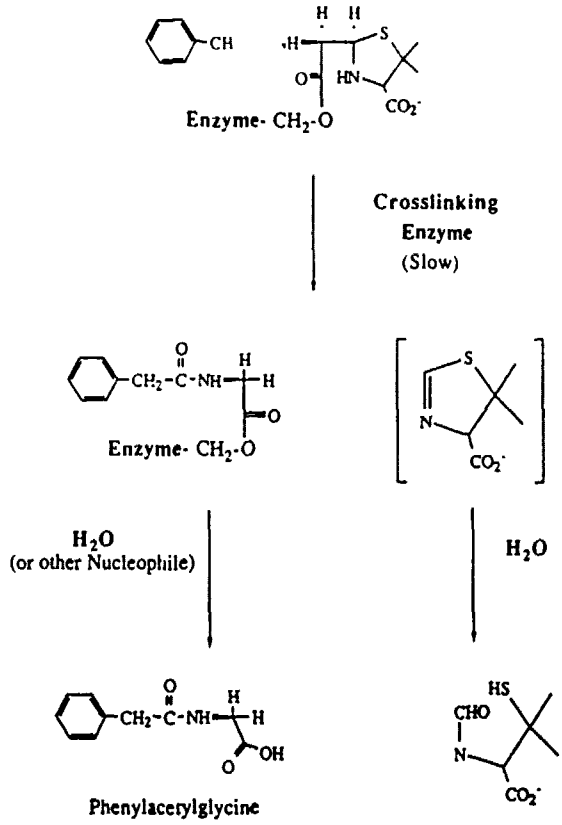

Figure 8. Attack of the lactam bond by the active-site serine in the D,D-carboxypeptidases resuls in the acyl-enzyme intermediate. The D,D-carboxypeptidases are unable to rapidly deacylate the complex, thereby the antibiotic inhibits cell wall crosslinking.

amino acids was not known. To select the specific amino acids which would be exchanged, sequence homology was examined. Among the PBPs whose amino acids sequences are known, PBP-5 of *E. coli* contains the most homologous (27%) amino acid sequence incorporating the active site serine, when compared with the class A β-lactamases (14, 18, 19). The capacity to inactive β-lactams is not absent in all PBPs; several can complete deacylation of the penicilloyl complex, thus displaying ineffectual levels of β-lactamase activity. However, PBPs tend to degrade the penicilloyl moiety into phenylacetylglycine (10, 20, 21). This is not true for PBP-5, which deacylates to form penicilloic acid at a higher rate than the majority of PBPs. This finding could be a possible implication of a more synchronous evolutionary relationship with the class A β-lactamases (18).

To attempt to alter in a major way the substrate specificity of a β-lactamase we therefore created a chimera between the RTEM-1 β-lactamase and PBP-5 of *E. coli*. This chimera involves changes of 18 residues and gives a protein that differs at 7% of the residues from the parent. Whereas RTEM β-lactamase has no D-Ala-D-Ala carboxypeptidase activity, that of the chimera is significant and is, in fact, about 1% the activity of PBP-5 on diacetyl-L-Lys-D-Ala-D-Ala; in terms of free energy of activation, the chimera stabilizes the transition state for the reaction to within about 2.7 kcal/mol of the stabilization achieved by PBP-5. Furthermore, the chimera catalyzes hydrolysis exclusively at the carboxyl-terminal amide bond which is the site of cleavage by D-Ala-D-Ala carboxypeptidase. Though containing all those

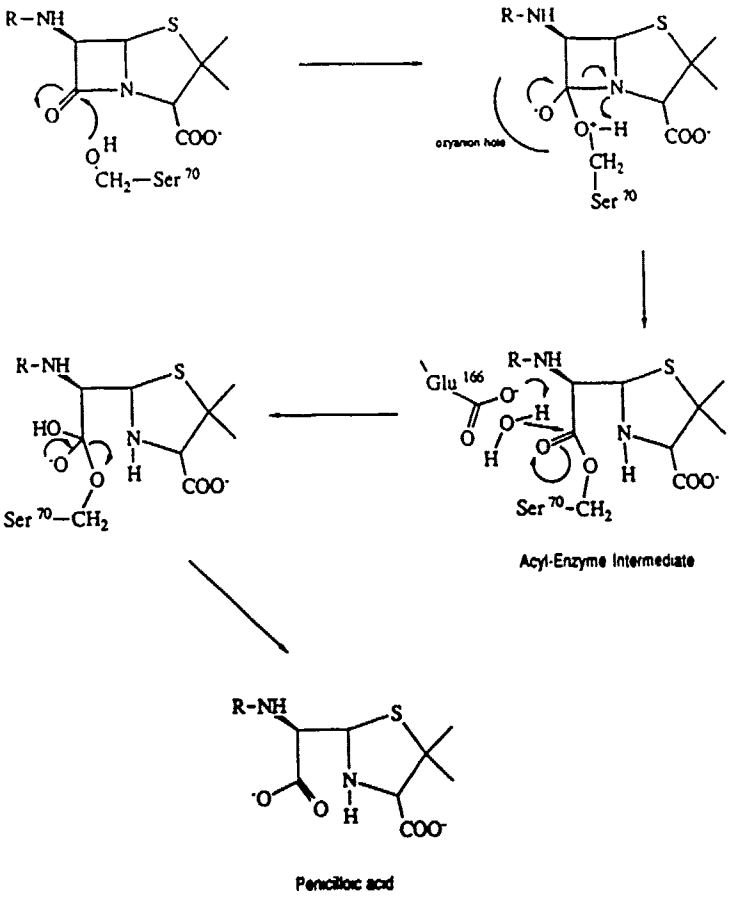

Figure 9. A mechanism for the hydrolysis of the β-lactam antibiotics by β-lactamase. The active-site serine forms an acyl-enzyme intermediate with the antibiotic which is subsequently hydrolyzed by the transfer of a water molecule from glutamate 166.

residues that are conserved throughout class A β-lactamases and are thought to be essential for β-lactamase activity, the chimera has considerably reduced activity ($\approx 10^{-5}$) on penams such as penicillins and ampicillins as substrates. As a catalyst, the chimera shows an induction period of ≈30 min, reflecting a slow conformation rearrangement from an inactive precursor to the active enzyme.

(v) Two high resolution crystal structures for class A β-lactamases (22, 23) have been published since the design and construction of the PBP-5/RTEM-1 chimera which reveal that the structural changes made in the chimera encompass the α-helix containing the active-site serine, the β2-strand of the five stranded antiparallel β-sheet, and the random coil connecting the two structures. Precisely which amino acid changes were responsible for the altered activity was not immediately evident.

With the insight gained from the PBP-5/RTEM-1 chimera and newly published crystal structures, we designed and constructed a series of chimeric enzymes involving RTEM-1 β-lactamase and the R61 carboxypeptidase of *Streptomyces* (10, 24). The R61 carboxypeptidase was chosen as the ca[illegible] peptidase donor because

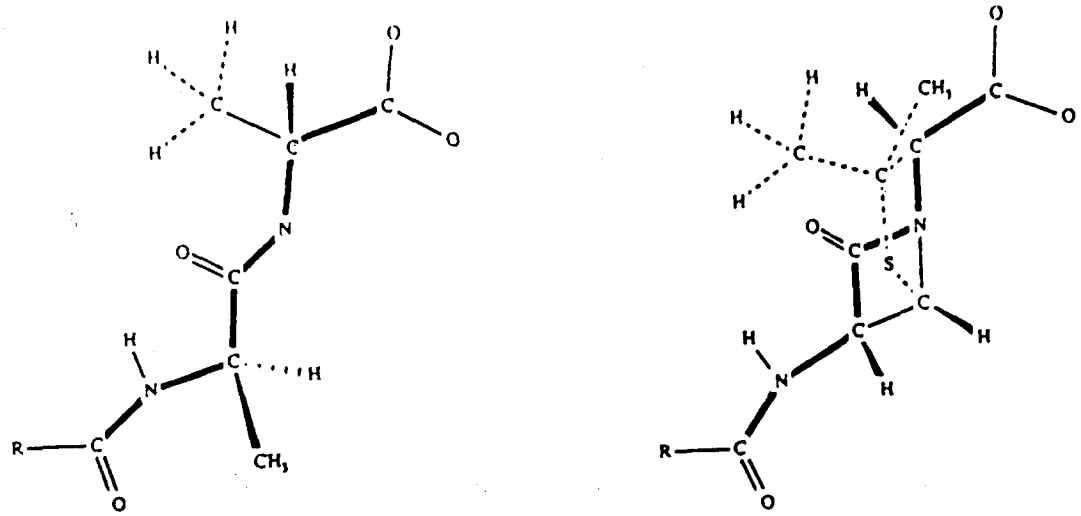

Figure 10. Chemical structures emphasizing the close structural analogy of the β-lactam antibiotics (right with the D-Ala-D-Ala depeptide (left). Hydrolysis of the lactam bond leads to a conformation which is nearly superimposable with the peptide (12).

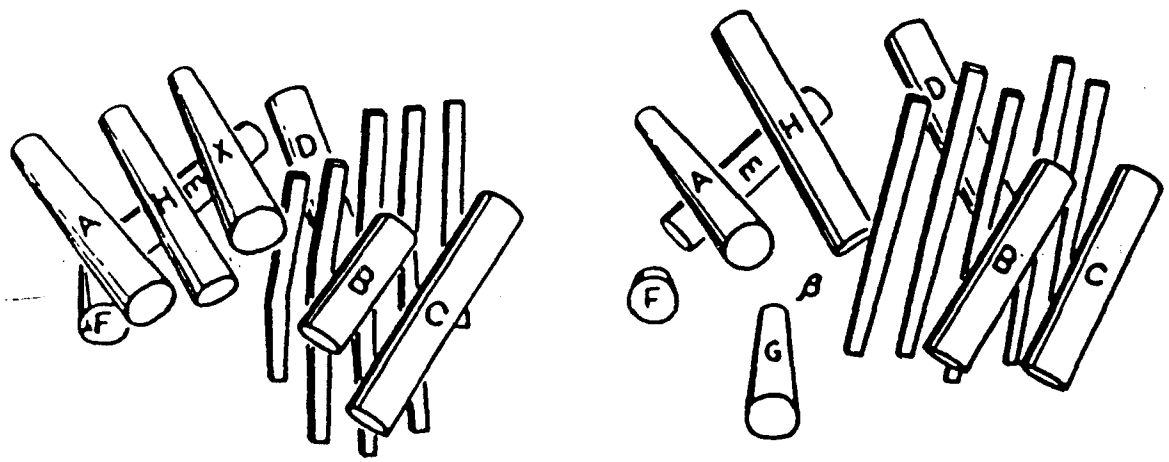

Figure 11. Examination of the low resolution crystal structures of the β-lactamase from *Bacillus licheniformis* and the R61 carboxypeptidase from *Streptomyces sp.* reveals a strong conservation of secondary structures between the two classes of enzymes (13).

both crystal coordinates and extensive kinetic data are available. An additional factor which influenced this choice was that R61 has a greatly enhanced turnover rate (10) of the synthetic substrate diacetyl-L-Lys-D-Ala-D-Ala, compared to the other PBP's (3300 min^{-1} for R61 vs. 174 min^{-1} for PBP-5). Unlike PBP-5 of *E. coli*, the R61 enzyme is not capable of simple β-lactam hydrolysis to the corresponding penicilloic

	70										
RTEM-1	R	F	P	M	M	***S***	***T***	F	***K***	V	L	L
B. licheniformis	R	F	A	F	A	***S***	***T***	I	***K***	A	L	T
B. cereus	R	F	A	F	A	***S***	***T***	Y	***K***	A	L	A
S. aureus	R	F	A	Y	A	***S***	***T***	S	***K***	A	I	N
• • •												
Streptomyces R61	R	F	R	V	G	***S***	V	I	***K***	S	F	S
PBP 5	R	R	D	P	A	***S***	L	T	***K***	P	M	V
PBP 6	R	L	P	I	A	***S***	M	T	***K***	M	M	T

Table I. The amino acid sequences, centered around the active-site serine of the class A β-lactamases compared with the R61 enzyme and the low molecular weight PBPs of *E. coli* (15). The residues are numbered by the Ambler convention for β-lactamases (14).

acid, but rather fragments benzyl penicillin into phenylacetyl glycine and N-formyl-D-penicillamine (20, 21) (Fig. 12). Whereas it was initially advantageous to use PBP-5 as the carboxypeptidase donor because it was capable of penicilloic acid production, use of the R61 enzyme could possibly alter the β-lactamase catalysis in the chimera to yield the fragmented product. Isolation of this product would be additional evidence of a truly altered catalysis pathway and proof that activity had been incorporated into the chimera from the carboxypeptidase enzyme.

The mutations in the PBP-5/RTEM-1 chimera were concentrated around the active-site serine. While these residues are undoubtedly important, the goal of the R61 project has been to create a chimeric binding cavity by mutation of residues that are not adjacent to the active serine in the linear amino acid sequence in addition to the mutations in the α-helix. As in the original chimera, these additional mutations would involve replacement of a particular amino acid with that present in R61, as determined by sequence alignments (15) (Fig. 13). Residues were chosen for mutagenesis on the basis of their: (i) known (or suspected) role in catalysis, (ii) location and orientation within the β-lactamase cavity, and (iii) homology to those residues present in the R61 carboxypeptidase/transpeptidase.

To generate the chimeric binding cavity, four general sites within the RTEM-1 cavity were chosen for mutation (Fig. 14). A synthetic scheme was designed in which a convergent stepwise synthesis would allow for the examination of the physical and catalytic effects of each mutation, both individually and in combination with each

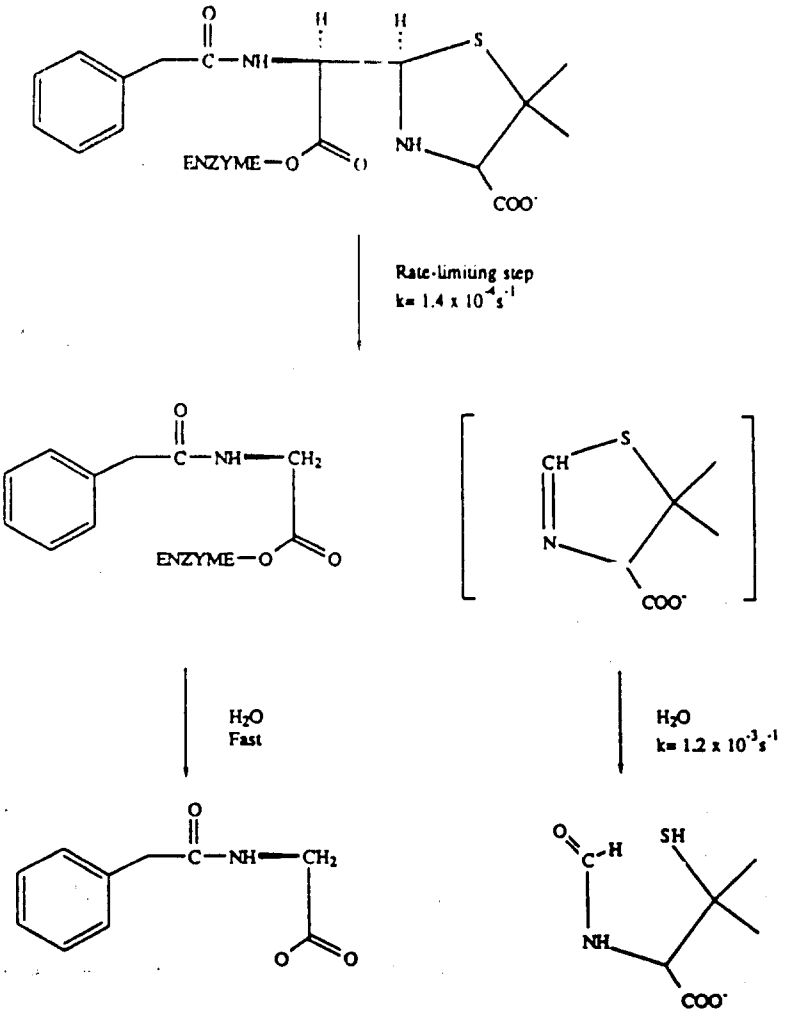

Figure 12. Degradation of benzyl penicillin by the R61 carboxypeptidase involves fragmentation of the penam's C_5-C_6 bond, followed by hydrolyis (or aminolysis).of the phenylacetylglycyl intermediate.

other. As with the PBP-5 chimera, the α-helix containing the active serine was targeted for cassette mutagenesis. However, unlike the PBP-5 chimera, the exchange of residues was designed to be limited primarily to those residues which would be exposed to solvent (Fig. 15). This was intended to minimize disruption of the hydrophobic packing and maintain intrinsic protein stability. The three remaining regions are: (i) Glu 166, (ii) Lys 234 and Ser 235, and (iii) Asp 131 and Asn 132 (Ambler convention of β-lactamase numbering (16).

Glutamate 166 was chosen because of this residue's known involvement in deacylation of the acyl-enzyme intermediate formed with β-lactam antibiotics (25). The corresponding residue in R61 is an aspartic acid. While this is a conservative mutation, it has been shown that the E166D mutant has a greatly reduced deacylation rate--presumably due to the decrease in chain length (~1 Å). Four out of five class A β-lactamases contain a glutamate at this position, while six out of seven PBPs which contain an aspartate (Fig. 13, Box V).

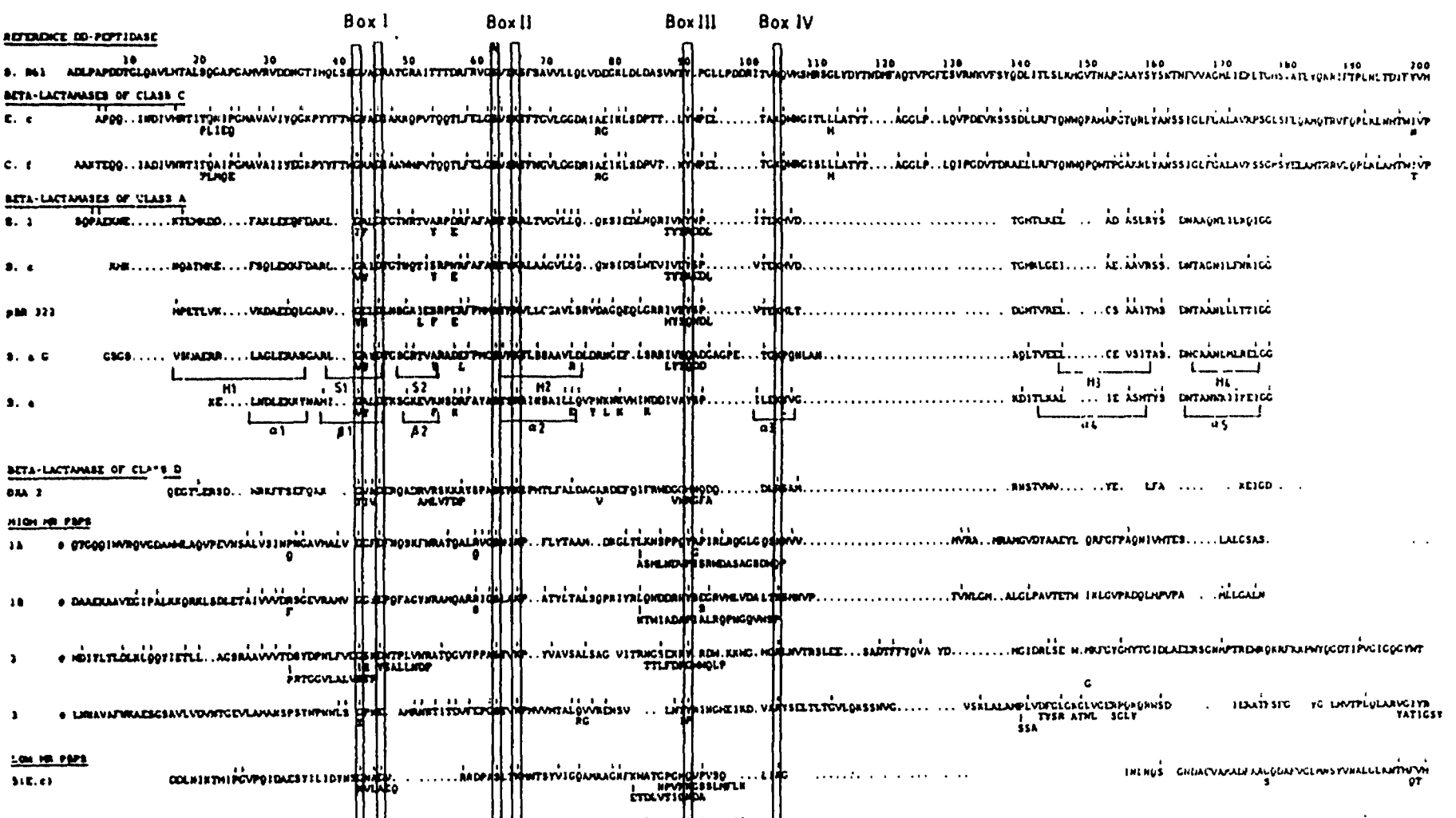

Figure 13. Proposed alignments of the amino acid sequences of the β lactamases and the penicillin binding proteins. Streptomyces R61 D,D-carboxypeptidase is the reference sequence (15).

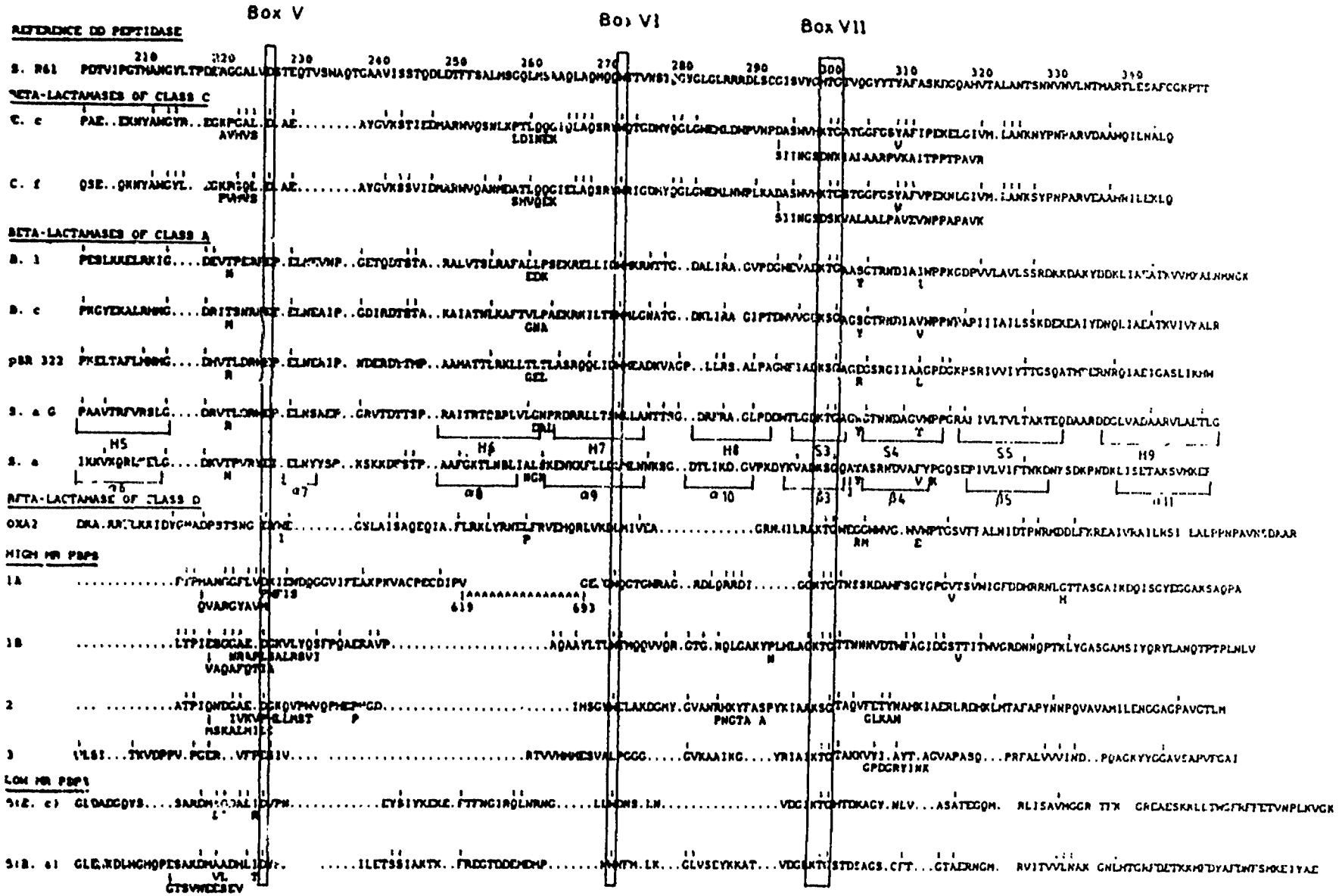

Figure 13. Continued

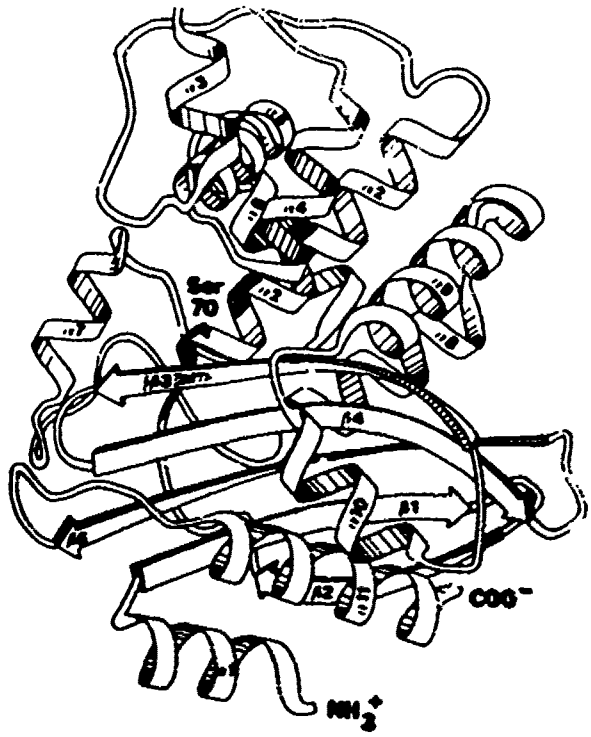

Figure 14. Four regions of the β-lactamase binding cavity were selected to be mutated: (i) the α-helix containing the active serine (P67R, M68V, M69G, F72I, V74S), (ii) Asp 131 and Asn 132, (iii) Glu 166, and (iv) Lys 234 and Ser 235.

RTEM	- R F p m m S t f K v l l c -
R61	- R F r v g S v i K s f s a -
Chimera	- R F **r v g** S t **i** K **s** l **s** c -

Figure 15. The sequences of RTEM β-lactamase, R61 carboxypeptidase and the resulting R61.1 chimera. Residues on the α-helix containing the active serine were chosen for mutagenesis because of their access to solvent or catalytic potential.

Lysine 234 and serine 235 were chosen because of their involvement in a semi-conserved triad (Fig. 13, Box VII) which is present in all class A and class C β-lactamases (Lys-Ser-Thr-Gly) in addition to all PBP's (His/Lys-Thr/Ser-Gly) (15).

Site saturation at residue 234 (26) has revealed that this residue is involved with substrate binding (K_M) while having little or no effect on catalysis (k_{cat}). The role of residue 235 has never been investigated. Directly following this triad in linear sequence, residue 237 has already been shown to be involved in altering β-lactamase substrate specificity (27). It is believed that the amide nitrogen of residue 237 participates in the stabilization of the oxyanion intermediate, in a motif similar to that observed in the serine proteases (28, 29).

The roles, either structurally or catalytically, of residues 131 and 132 have never been investigated. These sites follow a serine (Ser 130, in β-lactamase, Ser 160 in R61) conserved in all class A β-lactamases and low molecular weight PBPs (15). Two factors were dominant in the decision to mutate the 131-132 dyad. First, the side chain from residue 132 (Asn in RTEM-1, Phe in R61) is juxtaposed in the folded structure to the side chain from Glu 166. The tremendous disparity in hydrophobic character of asparagine and phenylalanine side chains could have an enormous effect on the ability of Glu 166 to access the water necessary to hydrolyse the acyl-enzyme intermediate. Second, while the side-chain of residue 131 (Asp in RTEM-1, Asn in R61) is not exposed to the solvent accessible cavity but projected into the hydrophobic core, superimposition of the α-carbon traces of R61 (30) and the PC1 β-lactamase (22) from *Staphylococcus aureus* (a closely related enzyme) shows nearly identical placement of residues 130, 131, and 132 (160, 163, and 164 in R61) despite the insertion of two additional residues in R61 between the serine and aspartate residues. For completeness, the single mutation at residue 132 (N132F) would also be made should it be determined that residue 131 was solely involved in protein stability.

In total, ten chimeras were constructed and are listed in Table II.

Of particular interest are the growth characteristics of cells harboring plasmids encoding β-lactamases that contain the D131N, N132F double mutation (R61.C, R61.S, and R61.5) multiplied at a markedly reduced rate compared to non-transformed cells. The doubling time of the chimeric cells was ~80 min vs. ~25 min for the normal cells. Examination of these cells by light microscopy revealed an altered ovoid cell morphology (Fig. 16). None of the other remaining chimeric genes induced this altered morphology, but rather were present in normal rod shaped cells. Work has been performed by Spratt and coworkers which overexpressed PBP-5 of *E. coli* as a soluble enzyme (31). A spherical cell morphology was observed in *E. coli* harboring the PBP-5 containing plasmid. The viability of these cells was greatly reduced by the overproduction of the PBP-5 gene product, even when cloned into the low copy number plasmid pSC105 (32). It is presumed that the presence of the external source of transpeptidase activity results in a hyper-crosslinked cell wall. The resulting cell wall structure impedes cell division, thereby resulting in an increased doubling time. Thus the increased doubling time and altered cell morphology would appear to be an indication of hypercrosslinking as catalyzed by the chimeric (D131N, N132F) proteins.

Interestingly, competent D1210 cells were transformed with the R61.C (N132F) plasmid with only slight ill effects on growth or morphology. A primary difference between the two strains is that the XL-1-Blue cells contain the gene responsible for tetracycline resistance, maintained on the F-plasmid. The tetracycline resistance factor is a membrane bound enzyme that functions by pumping the antibiotic out of the cells (32). To investigate the possibility that the presence of these proteins in the cell wall may initially destabilize the cell wall, thereby magnifying the subtle effect of the R61.C gene product, the gene was subcloned into pBR322 which contains the

Name	Description
pJN-XMS	Xho I @ 5032, Mlu I @ 4955, Sac I @ 4898
R61.A	pJN-XMS mutagenesis with: E166D
R61.B	pJN-XMS mutagenesis with: K234H, S235T
R61.C	pJN-XMS mutagenesis with: D131N, N132F
R61.D	pJN-XMS mutagenesis with: N132F
R61.1	**Base Hybrid.** Xho I --> Mlu I insert into active site. P67R, M68V, M69G, F72I, V74S, L76S
R61.2	R61.1 + R61.C: P67R, M68V, M69G, F72I, V74S, L76S, D131N, N132F
R61.3	R61.A + R61.B: E166D, K234H, S235T
R61.4	R61.A + R61.B + R61.C: D131N, N132F, E166D, K234H, S235T
R61.5	R61.1 + R61.4: P67R, M68V, M69G, F72I, V74S, L76S, D131N, N132F, E166D , K234H, S235T

Table II. The complete set of ten RTEM-1.R61 chimeras. Individual mutations were performed to produce the basic chimeras, then condensation ligation schemes were employed to build the complete chimeric binding cavity in a stepwise synthesis.

tetracycline resistance gene. The pBR322 R61.C plasmid was then propagated in both *E. coli* strains TG1 and D1210. Cells from both strains revealed a diminished growth rate and altered phenotype when transformed with the R61.C plasmid. It is important to note that the experiments involving the over-expression of PBP-5 were also performed in the presence of tetracycline.

E. coli cells containing the plasmid encoding the chimera exhibit greatly reduced or no resistance to penam antibiotics. Western blots (Fig. 17) performed to

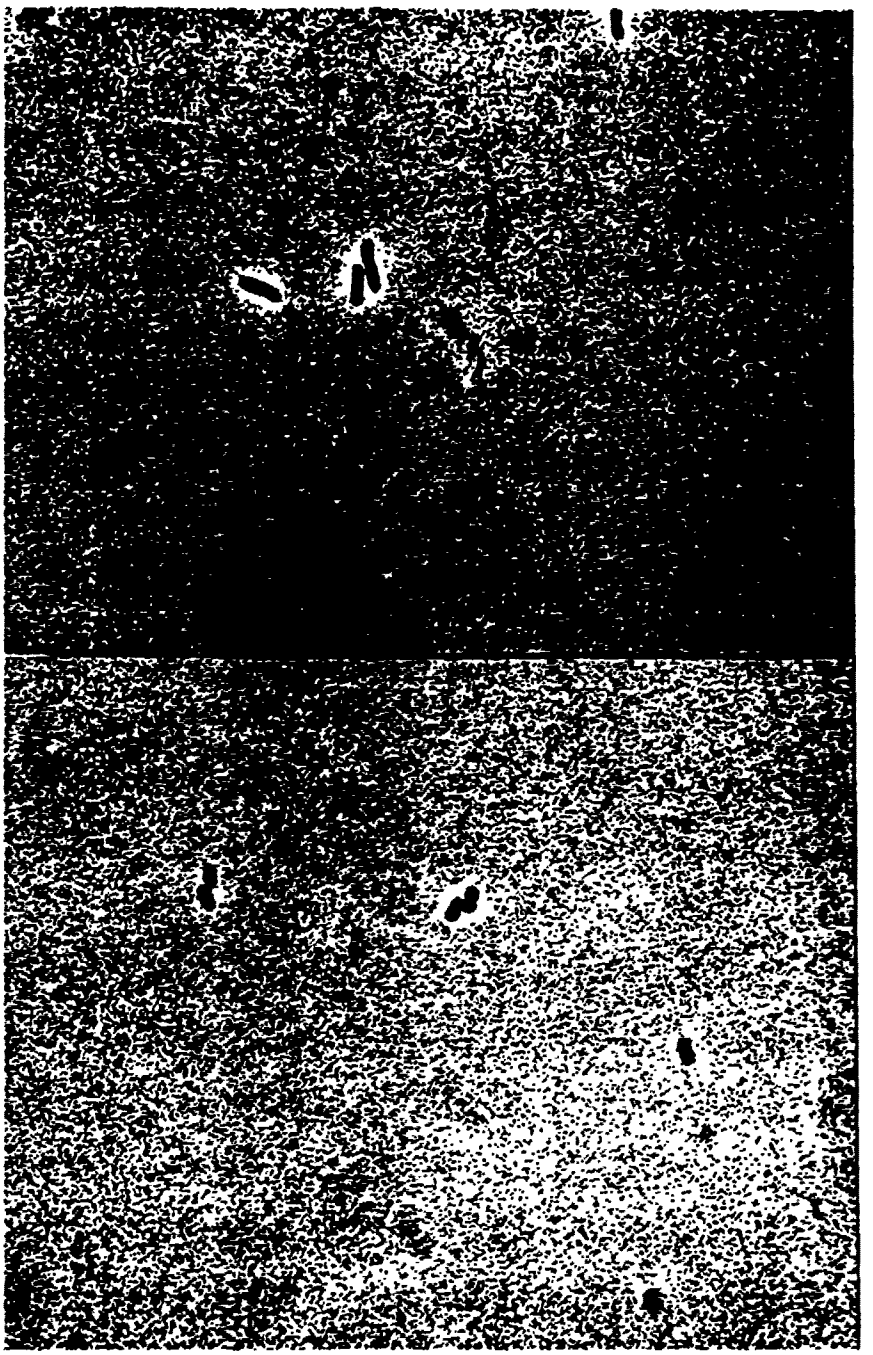

Figure 16. Expression of the D131N, N132F chimera in *E. coli* XL1-Blue cells resulted in an altered, ovoid, cell morphology. Normal rod-shaped *E. coli* at the same magnification are shown for comparison.

examine the *in vivo* stability of the chimeras revealed high levels of proteolytic degradation of several chimeras, especially the R61.5 chimera, which cannot be detected. No substrate induced stabilization was observed for cells grown in the presence of sub-lethal levels of ampicillin. The chimeric proteins (with the exceptions of R61.2 and R61.5) were isolated from the periplasm of the *E. coli* strain D1210 cells (grown in the presence of 50 μg/ml of kanamycin) by osmotic extrusion (19b). The

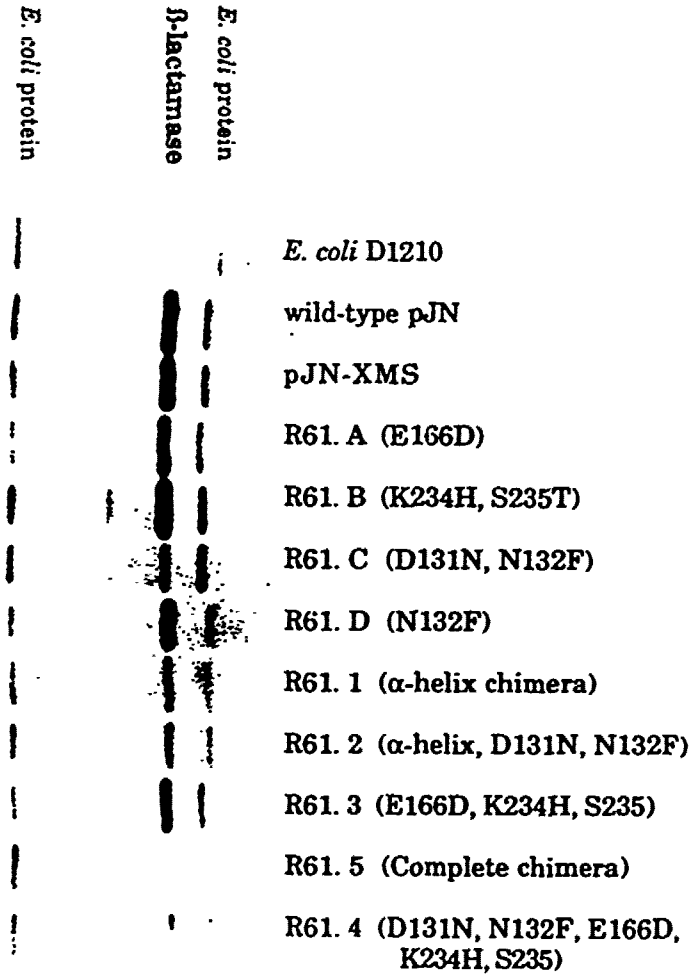

Figure 17. Western blots comparing the *in vivo* stability of the R61/RTEM-1 chimeras at 37°C to wild-type β-lactamase. The R61.5 chimera is not present in any measurable amount. The band corresponding to he R61.C (D131N, N132F) chimera is diminished compared to the R61.D (N132F) chimera, yet they both are thermally stable at 37°C as determined by melting experiments.

crude protein was first purified using an anion exchange gravity column with Q-Sepharose resin. This pre-purification is performed to protect the Mono-Q FPLC column from damage due to large concentrations of contaminating cellular proteins and lipids. The chimeric protein was further purified by FPLC purification using a Mono-Q anion exchange column, followed by removal of small amounts (>5% by weight) of contaminating proteins via gel filtration chromatography (Sephacryl HR-100). Purity was confirmed by analysis using 12% SDS-PAGE stained with Coomassie blue (R250). Protein concentrations for kinetic analyses were estimated at 281 nm using the β-lactamase extinction coefficient (11) of 29,400 $M^{-1}cm^{-1}$.

The purified chimeric proteins were partially characterized to facilitate identification of mutants which exhibit interesting catalytic or structural properties. The general structural characteristics of each chimera were examined by circular dichroism (CD). CD scans (180-300 nm) were performed to compare the gross structural content of the chimeras relative to the wild-type β-lactamase. All

enzymes, with the exclusion of the R61.C and R61.1 chimeras, showed CD spectra nearly superimposable with those of the wild-type ß-lactamase at 25°C. Due to a strong temperature instability, assessment of the R61.1 enzyme was performed at 15°C to insure accurate measurements. At this reduced temperature, while there was a mild decrease in the molar elipticity in the region of α-helix content, the resulting spectrum is remarkably similar to the wild-type. This demonstrates that the mutations performed were accommodated, albeit with reduced stability, within the native protein conformation.

The most striking results were obtained with the R61.C (D131N, N132F) chimera (Fig. 18). While an exact determination of the structural changes cannot be

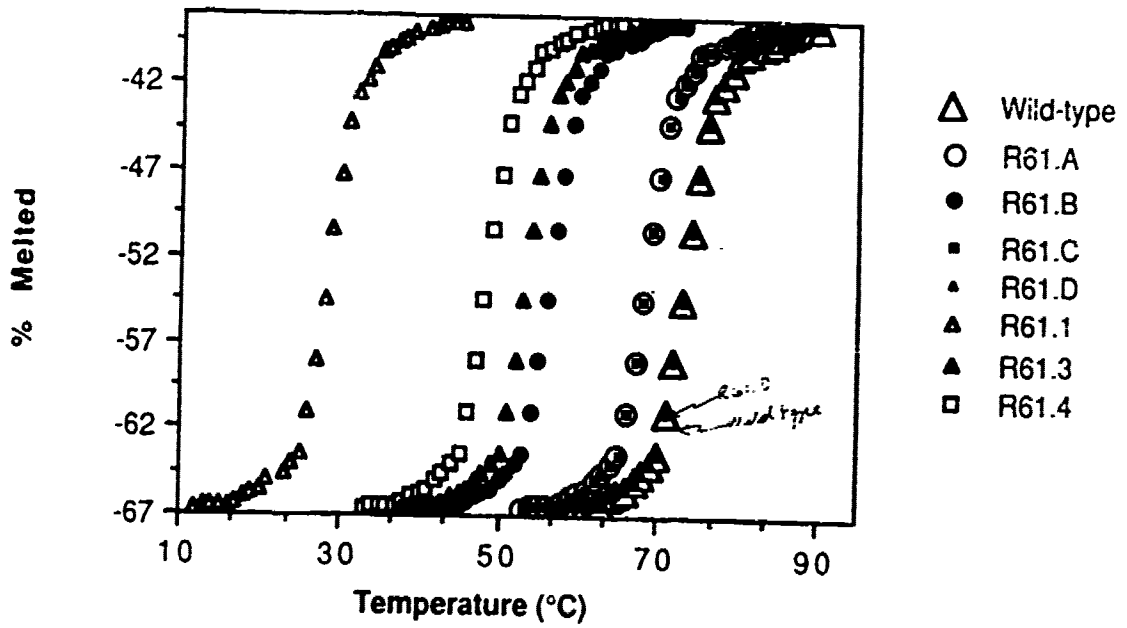

Figure 18. CD scans comparing the overall structural similarity between the wild-type ß-lactamase and the R61.C chimera. Only the R61.C chimera exhibited a CD spectra markedly different from the wild-type.

determined without X-ray crystal data, it appears that the chimera's overall structure is markedly altered compared to the RTEM-1 parent enzyme. Temperature dependent melting curves (Fig. 19) were also determined by CD spectropolarimetry. With the exception of the chimeras with the mutations at the active-site serine, the chimeras exhibited temperature melting points either equal to the wild-type or reduced by only 5-15°C. The R61.1 chimera melted substantially lower at about 30-35°C (Table III). The R61.C chimera melts at a temperature only ~5C lower than the wild-type RTEM-1 parent, but periplasmic levels of the chimera are low as determined by Western blots (Fig. 17). It would therefore appear that the D131N, N132F mutations induce a conformational change which does not decrease protein stability at 37°C, yet increases the rate of proteolysis.

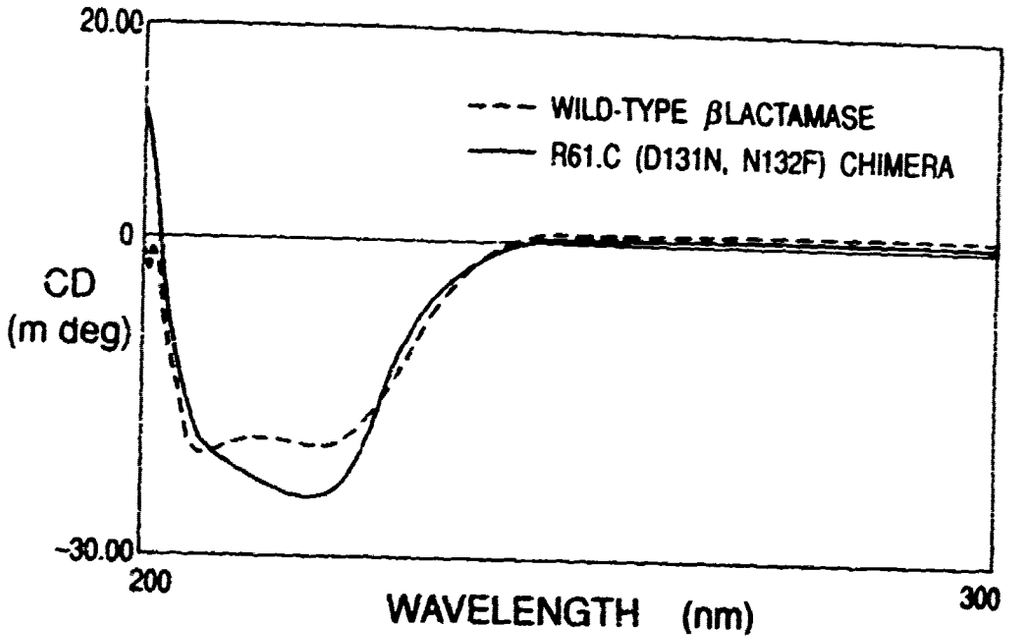

Figure 19. Thermal melting curves comparing the chimeras to the wild-type ß-lactamase. With the exception of the chimeras containing mutations in the active site α-helix (R61.1 shown, R61.2 and R61.5 were not determined), all the chimeras were thermally stable at 37°C, with melting points lowered only 5-15°C.

Chimera	Temperature (°C)
R61.1	30
R61.2	ND
R61.3	55
R61.4	50
R61.5	ND
R61.A	70
R61.B	58
R61.C	70
R61.D	75
ß-lactamase	75

Table III. Melting temperatures of the R61 RTEM-1 chimears as determined by decrease in molar elipticity at 222 nm. ND - not determined.

Cursory measurements of β-lactamase activities were determined (Table IV).

Chimera	K_M (mM)	k_{cat} (sec^{-1})	k_{cat}/K_M	Product	w/glycine
R61.1	ND	$>10^{-3}$	ND	pen	pen
R61.2	ND	ND	ND	.....	.....
R61.3	65.5 ± 2.2	$6.8 \times 10^{-3} \pm 8.2 \times 10^{-4}$	1.04×10^{2}	pen	pen
R61.4	ND	$>10^{-3}$	ND	pen	pen
R61.5	ND	ND	ND	.....	.. ..
R61.A	21 ± 9	1.1 ± 0.02	5.1×10^{4}	pen	pen
R61.B	342 ± 22	0.53 ± 0.1	1.56×10^{3}	pen	pen
R61.C	ND	$>10^{-3}$	ND	pag	pagg
R61.D	ND	$>10^{-3}$	ND	pen	pen
β-lactamase	20	2000	1×10^{8}	pen	pen
R61 Cpase		10^{-4}		pag	pagg

Table IV. Preliminary kinetic parameters of the R61/RTEM-1 chimeras utilizing benzyl penicillin as a substrate. The products of the substrate hydrolysis and the corresponding product with the reaction in the presence of glycine are given. Parameters were obtained by UV spectrophotometry. Due to extremely low levels of activities for some mutants, detailed parameters could not be obtained by this method. ND - not determined, *pen* - penicilloic acid, *pag* - pheylacetyl-glycine, *pagg* pehnylacetylglycylglycine.

The R61 carboxypeptidase does not hydrolyze benzyl penicillin to the corresponding penicilloic acid, the β-lactamase hydrolysis product, but rather generates phenylacetylglycine (10, 20, 21). This "altered" catalytic activity was a strong factor in the choice of the R61 carboxypeptidase. In the original PBP-5 chimera it was indeterminable whether the decreased activity of the chimera utilizing penam antibiotics was a result of enhanced carboxypeptidase character or whether the mutations simply destroyed β-lactamase catalysis. Reactions with excess benzyl penicillin were initiated with the purified chimeric enzymes at 37°C and incubated for 12 hr. Similar reactions were performed in the presence of 100 mM glycine. Once again, all enzymes yielded varying amounts of penicilloic acid with the exception of the R61.C enzyme. This chimera degraded benzyl penicillin to phenyl-acetylglycine and yielded the corresponding transpeptidase product, phenylacetylglycylglycine (33), when incubated in the presence of glycine. No penicilloic acid could be detected above background hydrolysis. The degradation products were isolated by thin-layer chromatography and verified by proton NMR. The phenylacetylglycine produced by the chimera was identical to synthetic phenylacetylglycine under all conditions examined.

Thus, the most prominent results were obtained from the chimeras containing the D131N, N132F double mutation. The first indication of a possible carboxypeptidase/transpeptidase activity was observed by changes in cell morphology and reduced rate of cellular division involved with the chimeras. It has been shown that overproduction of PBP-5 *E. coli* results in a spherical cell morphology and reduced growth rate due to hyper-crosslinking of the cell wall (31). Moreover, this chimera

catalyses the cleavage of β-lactams the reaction characteristic of transpeptidases /carboxypeptidases, the chimera does <u>not</u> catalyze hydrolysis, the reaction characteristic of β-lactamase.

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