STUDIES OF THE ROLES OF RESIDUES 71 AND 72 OF RTEM-1 BETA-LACTAMASE AND THE STRUCTURE-FUNCTION RELATIONSHIPS BETWEEN BETA-LACTAMASES AND D,D-CARBOXYPEPTIDASES BY MUTAGENESIS

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To

my mother and my wife

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ABSTRACT

The technique of site-directed mutagenesis, including oligonucleotide-directed mutagenesis and cassette mutagenesis, enables us to make any possible structural alterations at desired sites in proteins whose genes have been cloned. This technique when combined with biochemical and X-ray crystallographic analysis has been proved to be a very powerful tool for studying the structure-function relationships in proteins. Using this technique, I have studied some functional requirements of RTEM-1 beta-lactamase and the structure-function relationships between beta-lactamases and D,D-carboxypeptidases.

Beta-lactamases can catalyze the hydrolysis of the amide bond in the beta-lactam ring of penam and cephem antibiotics. The precise catalytic mechanism of these enzymes is still unclear; the specific amino acid residues, in addition to the active site Ser, involved in binding or catalysis remain unknown.

In Chapter I, to study the role of a conserved residue 71, Thr, of RTEM-1 beta-lactamase, I have changed this residue into Ile, Leu and Met, respectively, using oligonucleotide-directed mutagenesis. The results indicate

that although the Thr residue may not be directly involved in binding or catalysis, both the methyl and the hydroxyl group on the beta-carbon of Thr71 play a very important role in stabilizing the conformation of the wild-type RTEM-1 beta-lactamase; in this regard, the methyl group seems more important than the hydroxyl group.

It has been proposed that beta-lactamases may have evolved from D,D-carboxypeptidases. Recently, structural data became available for comparing a penicillin-binding-protein (or a D,D-carboxypeptidase) from Streptomyces R61 with class A beta-lactamases from B. licheniformis 749/C and B. cereus 561. The significant similarity found by X-ray crystallography in the spatial arrangement of the elements of secondary structure in these proteins strongly supports the hypothesis described above. Interestingly, there are conserved triads for both class A beta-lactamases and D,D-carboxypeptidases in the immediate vicinity of the active site Ser; the one for class A beta-lactamases is Ser-Thr-Xaa-Lys; however, the one for D,D-carboxypeptidases is Ser-Xaa-Thr-Lys.

In Chapter II, to further study the roles of residues 71 and 72 of RTEM-1 beta-lactamases and to study the possibility of creating a substantial D,D-carboxypeptidase activity within the background of beta-lactamase structure, I have changed the diad Thr71Phe72 of RTEM-1 beta-lactamase into Thr71thr72 and the D,D-carboxypeptidase-like

sequences, Leu71Thr72 and Ile71Thr72. The results indicate that although these two residues may not be directly involved in binding or catalysis, they may play a very important role in keeping the active site Ser and the conserved Lys residue in the correct orientation for efficiently catalyzing the hydrolysis of the beta-lactam antibiotics by beta-lactamases. Moreover, none of these mutant beta-lactamases shows detectable D,D-carboxypeptidase activity, suggesting that I may have to change more than two amino acid residues around the active site Ser of RTEM-1 beta-lactamase to generate a mutant beta-lactamase which can catalyze appreciable D,D-carboxypeptidase activity.

In Chapter III, to further study the structure-function relationships between beta-lactamases and D,D-carboxypeptidases, I constructed a hybrid protein by the replacement of a polypeptide chain containing 29 amino acid residues (from residue 47 to 75, in the number system of Ambler) of RTEM-1 beta-lactamase with the corresponding sequence containing 30 amino acid residues of PBP-5 of E. coli. The results indicate that beta-lactam antibiotics can not only induce the catalytic activity of this hybrid protein but also stabilize the conformation of this protein. The purified hybrid protein shows about 10^{-3} of the specific activity of the wild-type beta-lactamase against benzylpenicillin and, interestingly, given the

objective, this hybrid protein also shows about 1.8% of the D,D-carboxypeptidase activity of the wild-type PBP5 of *E. coli*. Furthermore, this hybrid protein shows no detectable transpeptidase activity, suggesting that the alpha-G helix in D,D-carboxypeptidase may play an important role in the transpeptidation reaction.

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NOMENCLATURE

The one-letter notation used is that recommended by the IUPAC-IUB Commission on Biochemical Nomenclature : A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; X, unknown or other amino acid; Y, tyrosine.

Expressions of the form T71I indicate that a mutation that replaces threonine at residue 71 with isoleucine has been introduced. Expressions of the form T71I, F72T indicate that a double mutation that replaces threonine at residue 71 with isoleucine and phenylalanine at residue 72 with threonine has been introduced.

INTRODUCTION

One of the most basic and interesting areas in protein biochemistry is to discover how structure at the molecular level is related to biological function. The utilization of the technology of protein crystallography and other physical techniques such as nuclear magnetic resonance, electron spin resonance, and circular dichroism, etc. has provided many insights into the structure-function relationships in proteins. However, since we can apply these techniques only to naturally occurring proteins, chemically modified proteins or mutant proteins obtained by random mutagenesis, many aspects of these problems could not previously be studied. When, however, one can generate structural variants in proteins at will, rational studies of these problems become possible; much more valuable information for discovering the basic principles that govern the structure-function relationships in proteins becomes available. Eventually, it may become possible to design new proteins that have novel and useful functional properties.

With the advent of the technology of molecular biology and the development of the oligonucleotide synthesis, it has become possible to do site-directed mutagenesis on cloned genes. When applying this technique on genes that encode proteins, one can generate any possible structure variants at any residue in those proteins.

Two general approaches for site-directed mutagenesis,

cassette mutagenesis and oligonucleotide-directed mutagenesis, have been described. Cassette mutagenesis involves the replacement of a short segment of a gene, bounded by unique restriction endonuclease sites with a synthetic mutant oligonucleotide duplex. Conveniently located restriction endonuclease sites may be present in the gene in its wild-type form. Alternatively, they may be introduced by oligonucleotide-directed mutagenesis. There are two general approaches for oligonucleotide-directed mutagenesis. The first uses single-stranded circular DNA vectors of the M13 or fd filamentous phages and the second uses double-stranded circular plasmid DNA vectors. Both approaches have their advantages and disadvantages. The site-directed mutagenesis techniques have been extensively reviewed (1, 2, 3,).

The site-directed mutagenesis technique was first applied on studying the structure-function problems in proteins in 1982 (4, 5). Since then, this approach has been applied to many enzymes including tyrosyl-tRNA synthetase (6, 7), trypsin (8), dihydrofolate reductase (9, 10), alkaline phosphatase (11), lac carrier protein (12), cytochrome C (13, 14), aspastate transcarbamoylase (15), triosephosphate isomerase (15, 16), lysozyme (17, 18), subtilisin (19), and beta-lactamase (20, 21).

Using the technique of site-directed mutagenesis, I have studied some functional requirements of RTEM-1 beta-

lactamase and the structure-function relationships between beta-lactamases and D,D-carboxypeptidases.

Beta-lactamases catalyze the hydrolysis of the amide bond in the beta-lactam ring of penam and cephem antibiotics, producing acidic derivatives that have no antibacterial properties. They are the major determinants of the resistance to beta-lactam antibiotics of most bacterial pathogens (22). Based on amino acid sequence data and enzymatic analysis, these enzymes have been classified into three groups, class A, B and C. Class A bata-lactamases that have molecular weights of around 29,000 show significant amino acid sequence homology among themselves (23, 24), and preferentially hydrolyze penam antibiotics. This group includes the RTEM-1 and RTEM-2 beta-lactamases, as well as those of S. aureus PC1, Bacillus licheniformis 749/C, and B. cereus 569/H1. Class B beta-lactamase is a metalloenzyme of molecular weight 23,000, which attacks cephem antibiotics and is produced only by Bacillus cereus (23). Class C beta-lactamases are chromosomally determined cephalosporinases of E. coli. They are larger proteins (mol. wt. 39,000) and share no sequence homology with the class A beta-lactamases (25, 26). The crystallographic structures of the class A beta-lactamases of B. licheniformis 749/C (27) and B. cereus 569 (28) have been solved to 3.5 Å and 2.5 Å resolution, respectively. The precise mechanism by which beta-

lactamases catalyze the hydrolysis of beta-lactam antibiotics remains uncertain, although there is considerable and persuasive evidence that the reaction intermediate is an acyl-enzyme (29, 30, 31, 32). The specific amino acid residues that are involved in catalysis, in addition to serine-70 [in the numbering system of Ambler (23)], are still unclear. In this work, the role of the conserved residue 71, Thr, of RTEM-1 beta-lactamase has been studied by oligonucleotide-directed mutagenesis.

Penicillin-binding proteins (PBPs) are defined as those bacterial proteins that bind penicillins and other beta-lactam antibiotics covalently. These enzymes can catalyze the final polymerization and cross-linkage of peptidoglycan (33, 34). In a given organism, PBPs are numbered in order of decreasing apparent molecular weight, which usually ranges from molecular weight 140,000 to 40,000. In general, high-molecular-weight PBPs (mol.wt. 60,000-140,000) are usually minor components, do not catalyze D,D-alanine carboxypeptidase (CPase) or transpeptidation reactions using model substrates, and are often essential for cell viability (34). By contrast, the low-molecular-weight PBPs (mol. wt. 40,000-50,000) are relatively abundant, catalyze D,D-alanine carboxypeptidase reactions <u>in vitro</u> and appear to be unnecessary for cell viability. Most PBPs are membrane proteins. Several exocellular D,D-

carboxypeptidases have also been purified and characterized, such as the D,D-carboxypeptidases secreted by Antinomadura R39, Streptomyces R61 and Streptomyces albus G (35, 36, 37). The crystallographic structure of the D,D-carboxypeptidase from Streptomyces R61 has been solved to 2.8 Å resolution (38).

The interaction of CPases with acyl-D-alanyl-D-alanine substrate is best described by a three-step mechanism (34). Enzyme E and substrate S first react to form a noncovalent complex E.S.. In the second step, a covalent acyl-enzyme intermediate E-P forms, with release of the terminal D-alanine. Reaction of E-P with either H_2O or RNH_2 regenerates free enzyme with formation of either the CPase product (P-OH) or the transpeptidase product (P-NH-R) (34), respectively.

The interaction of CPases with beta-lactam antibiotics, however, leads to the formation of a relatively stable covalent acyl-enzyme intermediate. By the formation of this intermediate, the antibiotics essentially inhibit the CPases irreversibly. It has been proposed that beta-lactam antibiotics bind to and inactivate these CPases by virtue of their structural similarities to the acyl-D-alanyl-D-alanine substrate (39). It has also been hypothesized that beta-lactamases may have evolved from penicillin-sensitive enzymes by developing an efficient catalytic mechanism for hydrolysis of the penicilloyl-enzyme

intermediates (39). The recent discoveries of the significant similarity between the three-dimensional structures of R61 D,D-carboxypeptidase and the class A beta-lactamases of B. licheniformis 749/C (27) as well as B. cereus 569 (28) strongly support the hypothesis described above.

In this work, to further understand the role of residue 71 and 72 of RTEM-1 beta-lactamase and to study the role of these two residues in the structure-function relationships between beta-lactamases and D,D-carboxypeptidases, I have prepared double mutants with D,D-carboxypeptidase-like sequences in the immediate vicinity of the catalytic serine 70. To continue studying the structure-function relationships between beta-lactamases and D,D-carboxypeptidases, I have constructed a hybrid protein of RTEM-1 beta-lactamase and PBP-5 of E. coli In short, these studies are focused on the use of mutagenesis to study some requirements for catalytic function of RTEM-1 beta-lactamase and the structure-function relationships between beta-lactamases and D,D-carboxypeptidases.

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CHAPTER I

STUDIES OF THE ROLES OF RESIDUE 71 OF RTEM-1 BETA-LACTAMASE BY OLIGONUCLEOTIDE-DIRECTED MUTAGENESIS

INTRODUCTION

Beta-lactamases can catalyze the hydrolysis of the amide bond in the beta-lactam ring of penam and cephem antibiotics (1). They are the major determinants of the resistance to beta-lactam antibiotics of most bacterial pathogens. Based on amino acid sequence data and enzamatic analysis, these enzymes have been classified into three groups, class A, B and C. Individual beta-lactamases vary considerably in their specificity toward different classes of beta-lactam antibiotics (2). These enzymes have not been found to hydrolyze acyclic peptides, involving D-Ala-D-Ala. However, they can catalyze the hydrolysis of acyclic depsipeptides which are ester analogues of D-Ala-D-Ala (3).

It is believed that there is a covalent acyl-enzyme intermediate for the beta-lactam hydrolysis reaction by beta-lactamases (4, 5, 6, 7). The existence of the covalent acyl-enzyme intermediate can be observed directly by the use of a poor substrate cefoxitin (4), or trapped by the use of low pH or subzero temperature for good substrates such as dansyl-penicillin and dansyl-cephalosporin (5, 8). Despite these findings, the precise catalytic mechanism of beta-lactamases remains unclear.

The structural data of the class A beta-lactamases of

B. licheniformis 749/C (9) and B. cereus 569 (10) are now available. However, the relatively low resolution complicates detailed interpretation of these structures; the knowledge we have of the essential catalytic groups is still limited. Early chemical modification studies, reviewed in Ref. (11), implicated almost every type of amino acid with a reactive side-chain. These investigations dramatically illustrate the limitation of this approach. In fact, the only residue for which there is strong evidence of its functional involvement is serine-70 [in the numbering system of Ambler (12)]. This residue is implicated by covalent modification, by inhibitors (13), by conservation of sequence homology, by analogy to the related D,D-carboxypeptidase crystallographic structure (14) and by site-specific mutagenesis (15, 16, 17).

The site-specific mutagenesis experiments have been undertaken on the RTEM-1 beta-lactamase. Replacement of the active-site Ser by Thr (15) leads to the absence of the catalytic activity in the mutant. The replacement of the active-site Ser by Cys leads to reduced catalytic activity (16, 17) and a different substrate specificity to that of the wild type (17). This technique, which enables us to make any possible structural alterations at desired sites in proteins, has also been used for studying the essentiality of specific residues in catalysis for tyrosyl-tRNA synthetase (18, 19, 20), alkaline phosphatase (21),

cytochrome C (22), aspartate transcarbamoylase (23), lac carrier protein (24), triose phosphate isomerase (25) and poliovirus 3C protease (26).

In this work, I have focused on a conserved residue 71, Thr, of class A beta-lactamases. In the beta-lactamase of S. aureus PC1, replacement of this Thr residue by Ile gives rise to a mutant that shows no catalytic activity (12); however, a mutant of the RTEM-1 beta-lactamase of E. coli in which Thr 71 has been replaced by Ser still shows appreciable catalytic activity (27). To further investigate the role of this residue in class A beta-lactamases, I have changed this residue Thr 71 of RTEM-1 beta-lactamase into Met, Ile and Leu, respectively, using oligonucleotide-directed mutagenesis. Interestingly, cells that produce any of these three mutant beta-lactamases show appreciably resistant phenotype to both ampicillin and benzylpenicillin. The mutant proteins with the replacement of the residue Thr 71 by Met or Leu are too unstable to be isolated and further characterized. Mutant beta-lactamase with replacement of Thr 71 by Ile shows dramatic changes in the kcat and Km values against penam and cephem antibiotics. At the same time, the role of this residue Thr 71 had also been studied by the use of site-saturation (28). Combining all the results described above, we suggest that the residue Thr 71 might not be directly involved in catalysis; however, it does play a very

important role in stabilizing the conformation of RTEM-1 beta-lactamase.

MATERIALS AND METHODS

CELLS

E. coli HB101 cells containing wild-type or mutant beta-lactamase genes in pBR 322 were grown in LB media (29). Mutagenesis experiments were done in *E. coli* JM103, which was served as a host for phage M13mp8 (30). For growth of plasmid containing the tac promoter, *E. coli* strain D1210 was used as a host. *E. coli* D1210 is a lac I^q derivation of HB101.

DNA

Plasmid DNA was prepared by the alkaline lysis method (29).

The plasmid pKK carrying the wild-type beta-lactamase gene under the control of the tac promoter was obtained from John Rossi.

Sequence determination was done by the dideoxy method (31) on a 700bp EcoRI-PstI fragment subcloned into M13mp9; three synthetic oligonucleotides, 5' - CAT TTC CGT GTC GCC C- 3', 5' - GTT GAG ACC GCG GCG - 3' and 5' - CAC AAC ATG GGG GGA TC -3', complementary to the insert were used as primers.

ENZYMES AND CHEMICALS

Restriction enzymes, T4 DNA ligase and T4 polynucleotide kinase, were obtained from BRL, DNA

polymerase (Klenow fragment) from Boehringer-Mannheim. All antibiotics were from Sigma Chemical Co.

MUTAGENESIS IN M13 VECTORS

The methods for growth of phage, and purification of phage DNA or RF DNA for sequencing, mutagenesis and cloning had been described in detail (30).

The following oligonucleotide were used as mutagenic primers :

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Met71   5'- G ATG AGC ATG TTT AAA G  - 3'
Ile71   5'- G ATG AGC ATT TTT AAA G  - 3'
leu71   5'- G ATG AGC CTA TTT AAA GT - 3' .
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Instead of using the M13 universal sequencing primer, I used a synthetic oligonucleotide (5'- G TTG AGA CCG CGG CG - 3') complementary to the insert at a site 50bp upstream from the sequence of interest as a second primer. The procedures used for the mutagenesis (Figure 1) are described below.

Single stranded template DNA (0.5 pmole) was combined with 20 pmoles of a phosphorylated mutagenic primer and 20 pmoles of the phosphorylated second primer in a 20 mM tris buffer solution of total volume 12 microliters at pH 7.5 containing 6mM NaCl, 8mM MgCl2 and 10mM DTT.

This solution was heated to 90oC for 5 minutes and allowed to stand at room temperature for 45 minutes. The synthesis of the second strand was initiated by adding 3.5 microliters of a solution containing 7.5 nanomoles of each of the four deoxynucleotide triphosphates, and four units

of the Klenow fragment of E. coli DNA polymerase I. After this mixture was incubated at 15°C for 2 hours, 20 nanomoles of ATP and 4 units of T4 DNA ligase (BRL) were added and the reaction mixture was allowed to incubate at 15°C for another 12 hours. The reaction mixture was then heated to 65°C for 10 minutes. Aliquots of this mixture were used to transform E. coli JM103.

SCREENING FOR MUTANTS

When an adequate number of independent transformants had been obtained, positive colonies were identified either by dot blots or by screening filter replicas prepared on Whatman 541 filter papers.

The synthetic oligonucleotides, used to direct mutagenesis, were phosphorylated with gamma-^{32}P-ATP by T4 phosphonucleotide kinase and used as hybridization probes.

For doing dot blots, cells from sixty single plaques were transferred into 2ml of broth, respectively, and incubated for 6 hours with good aeration, the cells were centrifuged and 3 microliters of each supernatant were spotted on dry, untreated nitrocellulose filters and baked for 90 minutes in a vacuum at 80°C. Prehybridization was done at room temperature for 30 minutes in a solution containing 0.2% SDS, 10X Denhardt's (29), 0.1 mg/ml sonicated and denatured salmon sperm DNA, 1 mM sodium orthophosphate, 1 mM sodium pyrophosphate, 0.5 mM ATP and 6XSSC buffer. Then the nitrocellulose filters were

hybridized for at least 2 hours at room temperature in a solution containing 0.2% SDS, 10X Denhardt's, 0.25 mg/ml yeast tRNA (Sigma, phenol extracted), 1 mM sodium orthophosphate, 1 mM sodium pyrophosphate, 0.5 mM ATP, 6XSSC buffer and 1 to 2 ng/ml of the labeled oligonucleotide.

The filters were washed in 6XSSC, three times for five minutes, and then at 1 to 3 degrees below the estimated Td; autoradiograms were taken after each wash.

The plaques carrying mutant phage can also be identified by hybridizing the mutagenic oligonucleotide, labeled with ^{32}P, to filter replicas that are prepared on Whatman 541 filter papers.

Whatman 541 filter replicas were first prehybridized in 6X SET, 0.5% NP-40 (Sigma), 100 mg/ml deproteinized, denatured salmon sperm DNA, 1 mM sodium orthophosphate, 1 mM sodium pyrophosphate, 0.5 mM ATP at 65 ^{o}C for 2 hours. The filters were then hybridized for at least 2 hours at room temperature in a solution containing 0.2% SDS, 10X Denhart's, 0.25 mg/ml yeast tRNA (Sigma, phenol extracted), 1 mM sodium orthophosphate, 1 mM sodium pyrophosphate, 0.5 mM ATP, 6XSSC buffer and 1 to 2 ng/ml of the labeled oligonucleotide. Washing was done in 6XSSC, first at room temperature (3 times for 5 minutes each time) and an autoradiogram was made. Additional washes were done at 1 to 3 degrees below the estimated Td for the oligomer;

autoradiograms were taken after each wash.

After positive colonies were identified, the mutant phages were plaque-purified and characterized by DNA sequencing.

PURIFICATION OF MUTANT BETA-LACTAMASE

The isolation procedure was basically that in the literature (27).

E. coli strain D1210 containing mutant RF DNA was grown to late log phase in ten liters of XB medium (25g tryptone, 7.5g yeast extract, 50 ml of 1M Tris-HCl, pH 7.5, 15mg ampicillin/liter) in a New England Biosystems fermenter with vigorous aeration at 30°C. The cells were collected by centrifugation and resuspended with 1 liter of 50 mM Tri-HCl buffer, pH 7.0. IPTG was then added to 1mM. After 4 to 6 hours at 4°C the cells were collected by centrifugation. The osmotic extrusion was done as described (27). Solid ammonium sulfate was added to the supernatant to reach 20% (w/v). After being stirred for 12 hours at room temperature, the ammonium sulfate solution was centrifuged; and the precipitate was collected and discarded. Additional ammonium sulfate was added to the supernatant to reach 60% (w/v). After being stirred at room temperature for another 12 hours, the ammonium sulfate solution was centrifuged at 16,000Xg for 45 minutes; and the precipitate was collected. This precipitate was redissolved in distilled water and the insoluble portion

removed by centrifugation at 10,000xg for 20 minutes. Activity recovered from the ammonium sulfate precipitation step was 80% to 100%. After removal of the ammonium sulfate residue by dialysis against 25 mM triethanolamine-HCl, pH 7.25, this solution was concentrated to 5 to 10 ml and applied to a 2.5 X 30cm DEAE-cellulose (DE-52) column equilibrated with the same buffer. The column was developed using a linear triethanolamine-HCl gradient (25 to 200 mM, pH 7.25). The active fractions were pooled and concentrated to 2 ml by ultralfiltration and applied in two equal portions to a 1 X 90 cm column of Ultragel 54 and eluted with 20 mM Tris, pH 7.0. About 10 to 15 mg of pure protein can be isolated from a 10-liter culture, representing an overall yield of approximately 23% to 35%. Only 1 - 3 mg of Ile71 mutant beta-lactamase and about 100 µg of Leu71 mutant beta-lactamase could be isolated by this procedure from a 10-liter culture.

ENZYME ASSAYS

After purification, the purity of mutant beta-lactamases was checked on 12% SDS-PAGE. Protein concentrations were determined according to the published conversion factors, $29{,}400\,M^{-1}$, and the absorbance at 281 nm (32). Protein activities were determined by measuring the change in absorbance with time at a specific wave length. All measurements were done at 25 °C in 50 mM potassium phosphate, pH 7.0, using a Beckman Acta CIII with 1 cm path

length cells. Hydrolytic cleavage of ampicillin was observed at 235 nm, benzylpenicillin at 240 nm, and cephalothin at 260 nm. The $E^{-1}_{M\ cm^{-1}}$ values used were 500 for benzylpenicillin (32), 1875 for ampicillin and 7,700 for cephalothin (4).

THERMAL STABILITY

Purified mutant beta-lactamases (0.1 mg/ml in 0.1 M potassium phosphate, pH 7.0) were incubated at the indicated temperature. After the various times indicated, aliquots of samples were removed and immediately assayed at 30 °C in 1 mM potassium phosphate buffer, pH 7.0, for remaining activity using benzylpenicillin as substrate.

RESULTS

Residue 71, Thr, of RTEM-1 beta-lactamase has been specifically changed into Met, Leu and Ile, respectively, by the use of oligonucleotide-directed mutagenesis. Table 1 shows the mutagenic primers used for directing each mutation. The scheme for two-primer mutagenesis is shown in Figure 1. Two methods have been used for screening the mutants as described in Methods and Materials. The autoradiograms obtained by screening the mutants with the ^{32}P-labeled mutagenic probe directing the change of Thr71 of RTEM-1 beta-lactamase into Met are shown in Figure 2, 3a and 3b. The final wash temperatures for each screening are shown in Table 2. The efficiency of mutagenesis is about 3% to 5%. For checking the resistance phenotypes of these mutants against beta-lactam antibiotics, the mutagenized genes were cloned back into pBR322. HB101 was used for growth of the plasmids containing the mutagenized beta-lactamase genes. For production of the mutant proteins, the mutagenized genes were cloned into the pKK plasmid containing a tac promoter. The tac promoter can be induced by IPTG; the host used for growth of these plasmids is *E. coli* D1210, which is a lac I^q derivative of HB101.

The conditions for the induction of the tac promoter

for the production of beta-galactosidase by IPTG have previously been studied (33). The induction becomes optimal after 2 hours by the use of 1 mM IPTG. However, this condition can't be used for inducing the production of beta-lactamases since overproduction of beta-lactamase may cause the cells to become very unhealthy and the resulting purification of beta-lactamase may become difficult. Figure 4 shows that by the use of 100 mM to 200 mM IPTG, the induction will reach the optimal in 5 hours.

For the unstable mutant proteins, the induction was done at 4 ^{0}C for 6 hours by 1mM IPTG. The conditions for inducing the tac promoter for the production of the unstable mutant beta-lactamase have not been optimized.

The T71M and T71L mutants of beta-lactamase were too unstable to be further characterized. The purity of the isolated mutant proteins was checked by 12% SDS polyacrylamide gel electrophoresis. Some catalytic properties of purified T71I mutant of beta-lactamase has been characterized. Table 3 shows the kcat and Km values of T71I mutant of beta-lactamase against benzylpenicillin, ampicillin, and cephalothin. Table 4 shows the differences between the thermal stability of the T71I and T71L mutants of beta-lactamase as well as wild-type beta-lactamase.

DISCUSSION

IN VITRO MUTAGENESIS

I have generated three single mutations at site 71 of RTEM-1 beta-lactamase using oligonucleotide-directed mutagenesis. Two primers were used; one was the mutagenic oligonucleotide and the other was an oligonucleotide that primes about 50 base pairs away. Each primer was extended on the same single-stranded template by the Klenow fragment of *E. coli* DNA polymerase. The reaction mixture was used directly for transfection without the isolation of covalently closed double-stranded DNA. Screening for mutant phages was accomplished by the use of the ^{32}P labeled mutagenic oligonucleotides as hybridization probes. The efficiency of mutagenesis is approximately 3% to 5%.

The features of the mutagenic oligonucleotides used in this work are basically the same as described by Zoller and Smith (34). Generally, in making small changes of one or two bases, 17mer or 18mer can be used. Another thing that should be taken into consideration is the prevention of the self-hybridization of the mutagenic oligonucleotide. For example, 5'- G ATG AGC ATG TTT AAA is not suitable for directing mutagenesis because it tends to hybridize to itself; in the presence of the large fragment (Klenow) of DNA polymerase, double-strand oligonucleotides may be

formed. In such uses, the efficency of mutagenesis can be expected to be very low.

Another factor that may affect the efficiency of mutagenesis is the position of the upstream primer. In this work, the upstream primer is a sequencing primer that extends upstream from a position 50bp of the mutagenic oligomer. The efficiency of mutagenesis is about 3-5%. When the M13 sequencing primer is used as the upstream primer that extends from a position 2.9kb upstream of the mutagenic oligomer, the efficiency can go up to 50% (34). In addition, another successful experiment has been completed in which the upstream primer is only 200 bases away; and the efficiency is around 1% (34). These differences reflect the proximity of the two oligonucleotides (34).

Two procedures have been used in screening for the mutants. One is adapted from the procedure developed by Gergen et al. (35), with some modifications. This procedure is originally designed for screening colonies containing recombinant plasmids. In this method, Whatman 540 filter papers are used to make replicas. With the modified hybridization conditions, this method can be successfully applied to screening for plaques containing mutagenized M13 phages. The results obtained by the use of the method described above are as good as those obtained by another method, the dot-blot hybridization (34). The

major advantages of the first method are that Whatman 540 filter papers are cheaper, easier to handle and to store than nitrocellulose filter papers, which are used in the dot blot hybridization procedure.

After the putative mutants are identified, the plaque purification procedure is essential because these original plaques may contain a mixture of wild-type and mutant plaques. Some investigators think that, theoretically, each original suspected plaque should contain a 50 : 50 mixture of wild-type and mutant phage (34). When checking the life-cycle of M13, we can find that this is not true (36). Theoretically, in each original suspected plaque, the ratio of the mutant phage to the wild type should be larger than 60%. In practice, we found that most suspected plaques contained almost 100% mutant by analysis of plaque-purified clones. Similar results have also been observed by other investigators (34). These observations indicate that the replaced parental (+) strand of wild-type M13 phage can't be used as a template as efficiently as the (-) strand of the mutagenized M13 phage.

Currently, a number of relatively simple and reliable procedures for oligonucleotide-directed mutagenesis have been developed (37, 38, 39, 40). The choice of which method to use may depend simply on the familarity of an individual with the particular vector system and enzymes utilized with the method.

ROLES OF RESIDUE 71 OF RTEM-1 BETA-LACTAMASE

All the cells containing these mutant beta-lactamases show significant resistance to benzylpenicillin and ampicillin, indicating that the residue Thr71 may not be directly involved in either binding or catalysis. The T71I mutant of beta-lactamase shows dramatic changes in the kcat and Km values against benzylpenicillin and ampicillin. Moreover, this single mutation has a stronger effect on the kcat/Km value against cephems than on the kcat/Km value against penams. Another mutant beta-lactamase with the replacement of Thr71 by Ser has also been purified and partially characterized at the same time (27). The differences in the thermal stability of these mutant beta-lactamases are shown in Table 4. When comparing the differences in the structures of Thr, Ile, Ser and Leu, we can find that there are a methyl group and a hydroxyl group on the beta-carbon of Thr; there is a corresponding methyl group, but there is no such hydroxyl group on the beta-carbon of Ile; in contrast, there is a corresponding hydroxyl group, but there is no such methyl group on the beta-C of Ser; finally, there is neither a corresponding methyl group nor a corresponding hydroxyl group on the beta-C of Leu. Combining the differences in the thermal stability of these mutant beta-lactamases with those in the structures between Thr, Ile, Ser and Leu, we conclude that both the methyl and the hydroxyl group on the beta-carbon

of Thr play a very important role in stabilizing the conformation of the wild-type RTEM-1 beta-lactamase; in this regard the methyl group seems more important than the hydroxyl group.

The role of this residue has also been investigated by site-saturation (21). The residue Thr71 of RTEM-1 beta-lactamase has been changed into all 19 possible amino acid residues. The results obtained by that study indicate again that the residue 71 is not directly involved in either binding or catalysis, and the same conclusion has been reached that both the methyl and the hydroxyl groups of Thr71 of RTEM-1 beta-lactamase play a very important role in stabilizing the conformation of the protein.

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TABLE I

MUTATIONS AT THE SITE 71 OF RTEM-1 BETA-LACTAMASE

mutation	a.a. sequence	DNA sequence
None (ie. wild-type sequences)	Met Ser Thr Phe Lys	G ATG AGC ACT TTT AAA GT
Thr71→ Met	Met Ser Met Phe Lys	*G ATG AGC ATG TTT AAA G
The71→ Leu	Met Ser Leu Phe Lys	*G ATG AGC CTG TTT AAA GT
Thr71→ Ile	Met Ser Ile Phe Lys	*G ATG AGC ATT TTT AAA G

* These are the exact DNA sequences of the mutagenic primers used for directing each mutations in the beta-lactamase gene.

FIGURE I

THE SCHEME FOR OLIGONUCLEOTIDE-DIRECTED MUTAGENESIS

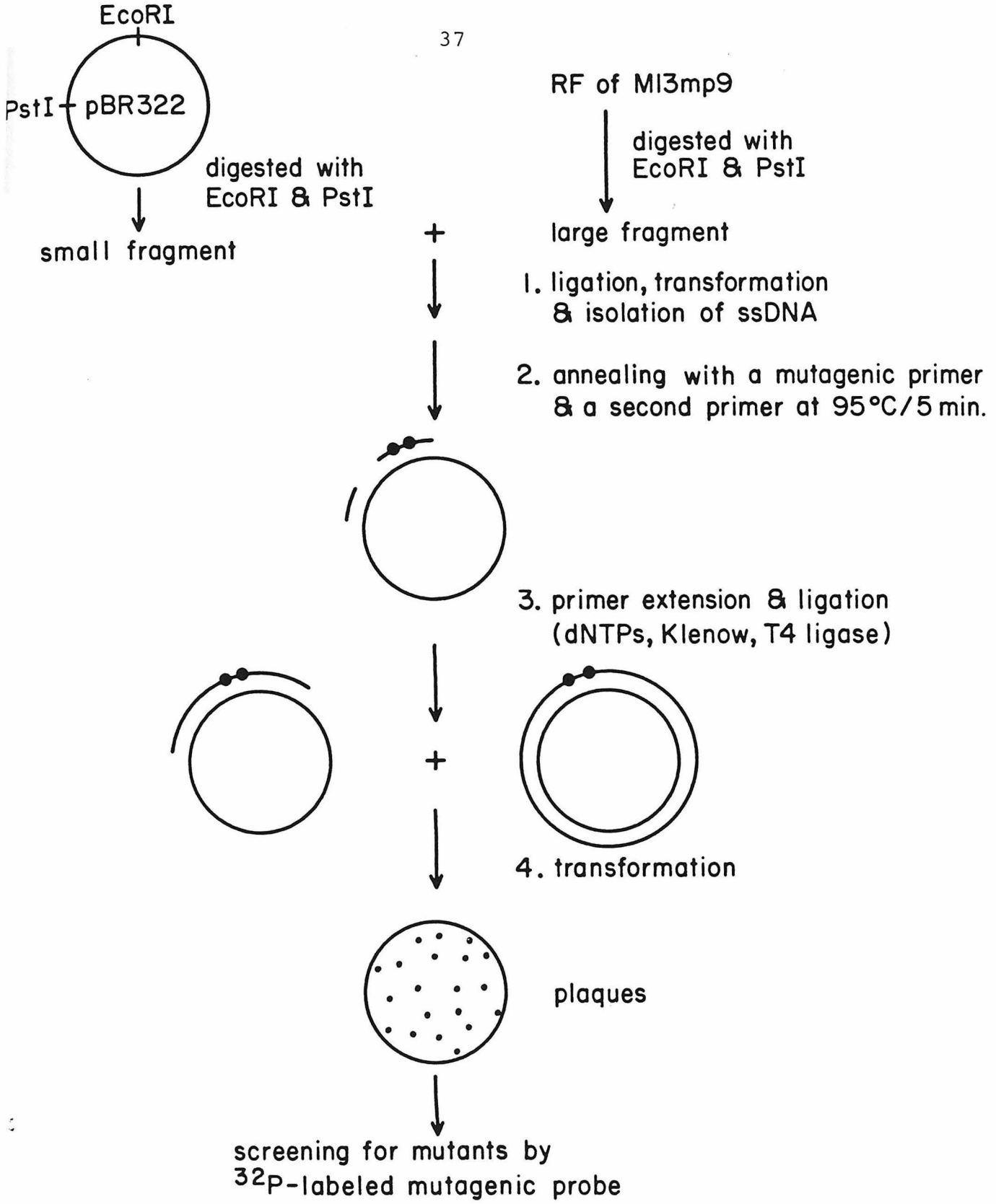

EcoRI
PstI
pBR322
digested with
EcoRI & PstI
small fragment
RF of M13mp9
digested with
EcoRI & PstI
large fragment
+
1. ligation, transformation
& isolation of ssDNA
2. annealing with a mutagenic primer
& a second primer at 95°C/5 min.
3. primer extension & ligation
(dNTPs, Klenow, T4 ligase)
+
4. transformation
plaques
screening for mutants by
32P-labeled mutagenic probe

FIGURE II

AUTORADIOGRAM OF A DOT BLOT

The dot blot procedure used for screening mutants has been described in the Methods section of this chapter. The ^{32}P labeled mutagenic probe used here is 5' - G ATG AGC ATG TTT AAA G - 3'. The nitrocellulose membranes were washed at room temperature (A), and at 43 °C (B) in 6XSSC.

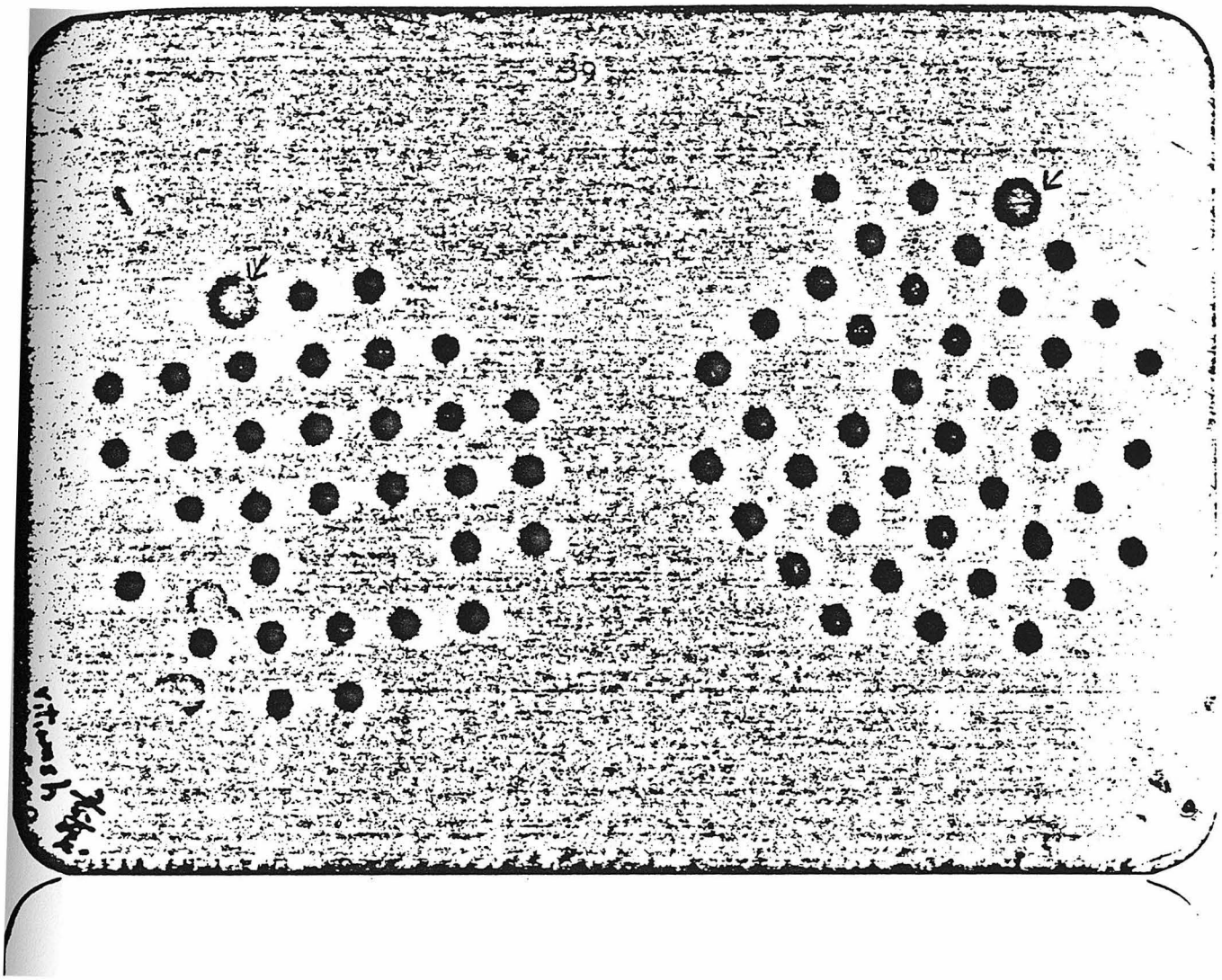
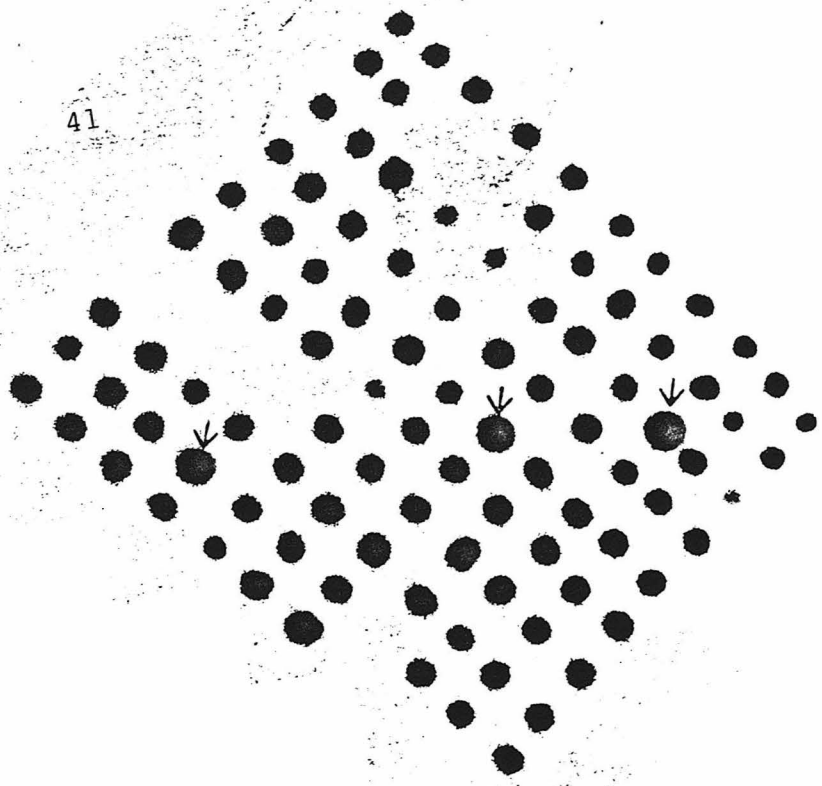

39

FIGURE III-a

Autoradiograms of Whatman 541 filter replicas of phage-infected colonies which have been treated according to the hybridization procedure described in the methods section and washed at room temperature in 6XSSC. The ^{32}P labeled mutagenic primer used here is 5' - G ATG AGC ACT TTT AAA G - 3'. The arrows indicate the colonies that may contain the mutagenized genes.



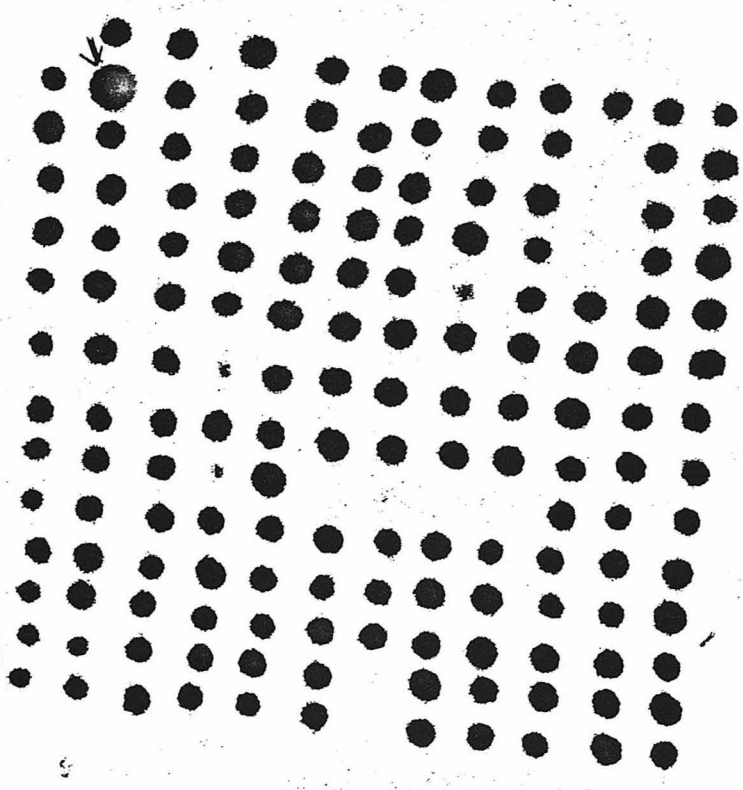


FIGURE III-b

AUTORADIOGRAMS OF WHATMAN 541 FILTER REPLICAS OF PHAGE-INFECTED COLONIES WHICH HAVE BEEN TREATED ACCORDING TO THE HYBRIDIZATION PROCEDURE AND WASHED AT 44 °C.

The arrows indicate the colonies that may contain the mutagenized genes.

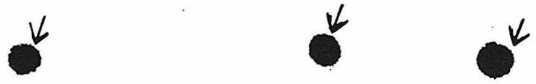


TABLE II

THE DNA SEQUENCES OF THE MUTAGENIC PRIMERS

The DNA sequences of the mutagenic primers used to introduce mutations in the RTEM-1 beta-lactamase gene are shown as above; mismatched bases are indicated in bold-face characters, and the corresponding wild-type bases are shown below in parentheses. Td is the melting temperature estimated from the empirical relationship Td = 4X (G-C base pairs) + 2X (A-T base pairs) (41). The discriminating temperature is the wash temperature (in 6XSSC) at which a difference in hybridization was observed between a perfectly matched DNA duplex (oligomer/mutant) and the corresponding heteroduplex (oligomer/wild type).

TABLE II

DNA sequence of mutagenic primers	Td	observed discriminating temperature
G ATG AGC ATG TTT AAA G (GC)	46°C	43°C
G ATG AGC CTG TTT AAA GT (A)	48°C	46°C
G ATG AGC ATT TTT AAA G (G)	44°C	43°C

FIGURE IV

INDUCTION OF TAC PROMOTOR FOR PRODUCTION
OF WILD-TYPE BETA-LACTAMASE BY IPTG.

Aliguots of culture solutions containing different concentrations of IPTG were taken out and centrifuged. The pallets were resuspended in 10XTE buffer and centrifuged. Pallets were resuspended in cold water and centrifuged again. The specific activity against benzylpenicillin of each supernatant was then assayed.

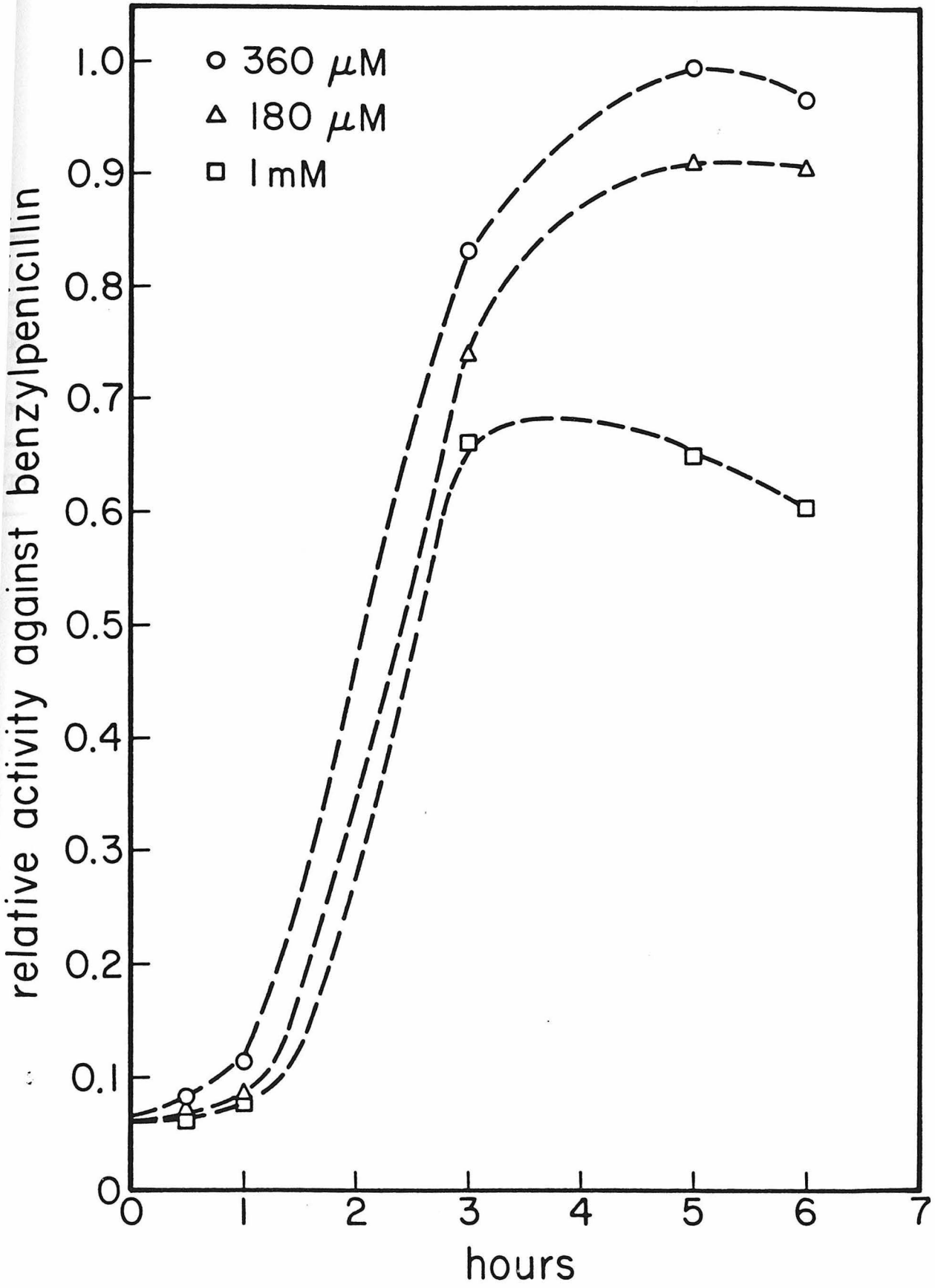

360 μM
180 μM
1 mM
relative activity against benzylpenicillin
hours
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0
1
2
3
4
5
6
7

TABLE III

COMPARISON OF CATALYTIC PARAMETERS OF WILD-TYPE AND THR71 -> ILE MUTANT BETA-LACTAMASE

	Km(μM)	kcat(sec^{-1})	kcat/Km($sec^{-1}\mu M^{-1}$)
Ampicillin	50(50)	145(2,000)	2.9(40)
Benzylpenicillin	350(50)	1,530(1,700)	4.4(34)
Cephalothin	1,250(250)	4.59(230)	3.7×10^{-3}(0.92)

* : wild-type value in brackets.

	Km	kcat	kcat/Km
Ser70Thr71(wild type) (benzylpenicillin/cephalothin)	0.2	7.39	36.9
Ser70Ile71 (benzylpenicillin/cephalothin)	0.28	333.3	1,189
Ser70Thr71(wild type) (ampicillin/cephalothin)	0.2	8.70	43.5
Ser70Ile71 (ampicillin/cephalothin)	0.04	31.6	784

TABLE IV

THERMAL STABILITY OF ACTIVE BETA-LACTAMASES

Temperature	$t_{\frac{1}{2}}$			
	*S70T71 (wild type)	S70, T71S	S70, T71I	S70, T71L
40°C	------	9 min	> 30 min	3 min
50°C	> 30 min	< 1 min	5 min	< 1 min
55°C	13 min	------	< 1 min	------
60°C	1-2 min	------	------	------

* From Jim Neitzel's data.

CHAPTER II

STUDIES OF THE ROLES OF RESIDUES 71 AND 72 OF RTEM-1 BETA-LACTAMASE BY CASSETTE MUTAGENESIS

INTRODUCTION

There are two groups of bacterial enzymes that interact strongly with beta-lactam antibiotics, the beta-lactamases and the penicillin-binding-proteins. Both groups of proteins can be acylated by beta-lactam antibiotics, although only for the beta-lactamases is deacylation a rapid process (1). Beta-lactamases have not been found to hydrolyze acyclic peptides, involving D-Ala-D-Ala, which are model substrates for D,D-carboxypeptidases (2, 3). However, certain acyclic depsipeptides, analogous in structure to the terminal D-Ala-D-Ala moiety of the peptidoglycan cross-link, are substrates for both beta-lactamases and D,D-carboxypeptidase (4). It has been proposed by Tipper and Strominger (5) that beta-lactam antibiotics are structural mimics of the C-terminal, D-Ala-D-Ala, residues of the peptide chain of uncross-linked peptidoglycan. They have also suggested that beta-lactamases may be evolutionary descendants of the penicillin-binding-proteins involved in peptidoglycan synthesis (5). Subsequent structural and mechanistic investigations have in general tended to support these ideas (6, 7, 8, 9).

Recently, structural data became available for comparing a penicillin-binding-protein (or D,D-

carboxypeptidase) from Streptomyces R61 with class A beta-lactamases from B. licheniformis 749/C (10) and B. cereus 569 (11). The significant similarity found by X-ray crystallography in the spatial arrangement of the elements of secondary structure in these proteins strongly supports the hypothesis described above. These results led us to study the possibility of creating a substantial D,D-carboxypeptidase activity within the structural background of a beta-lactamase by mutagenesis.

There is homology between the amino acid sequence around the penicillin-binding sites of the D,D-carboxypeptidases and the sequence around the active site serine of the class A beta-lactamases (12). The specific amino acid residues involved in catalysis for both enzymes, in addition to the active site serine, are still unclear. Interestingly, both enzymes contain a conserved triad. The one for class A beta-lactamases is Ser-Thr-Xaa-Lys; [residue 70 to 73 in the numbering system of Ambler (13)], however, the one for D,D-carboxypeptidases is Ser-Xaa-Thr-Lys. The serine at residue 70 is the active site for both enzymes (3, 8, 14, 15). The lysine at residue 73 is conserved in class A beta-lactamases (13), class C beta-lactamases (16) and all membrane-associated D,D-carboxypeptidases (17). The role of this residue is under thorough investigation (18). The role of residue Thr71 of RTEM-1 beta-lactamase has been studied in Chapter I. It is

not directly involved in catalysis or binding; however, it may play an important role in stabilizing the conformation of class A beta-lactamases.

Here, to study the possibility of creating substantial D,D-carboxypeptidase activity within a beta-lactamase background and to further study the roles of residue 71 and 72 in the catalytic activity, substrate specificity and thermal stability of class A beta-lactamases, I have changed the diad Thr71Phe72 of RTEM-1 beta-lactamase into Thr71Thr72 and D,D-carboxypeptidase homology sequences, Leu71Thr72 as well as Ile71Thr72, by cassette mutagenesis. The mutant protein containing the diad, Leu71Thr72, is too unstable to be purified and characterized. The mutant proteins containing the diads, Thr71Thr72 and Ile71Thr72, were purified and partially characterized. The results indicate that the residue 72 of RTEM-1 beta-lactamase is not directly involved in catalysis; however, residues 71 and 72 may play a very important role in keeping Ser70 and Lys73 in the correct orientation in beta-lactamases for efficiently catalyzing the hydrolysis of beta-lactam antibiotics. None of these mutant beta-lactamases shows detectable D,D-carboxypeptidase activity. This implies that some of the amino acid residues involved in binding or catalysis by D,D-carboxypeptidases may be different from those involved in binding or catalysis by beta-lactamases.

MATERIALS AND METHODS

ENZYMES AND CHEMICALS

Restriction enzymes and the large fragment of DNA polymerase I (Klenow) were purchased from Boehringer Mannheim. The T4 DNA ligase and T4 polynucleotide kinase were obtained from Bethesda Research Laboratories. All antibiotics were obtained from Sigma Chemical Co. The [Alpha-^{32}P] dTTP, 3,000 uCi/mmle (1Ci = 37 GBq), was purchased from Amersham.

BACTERIAL CELL LINES

E. coli HB101 cells containing wild-type or mutant beta-lactamase genes in pBR 322 were grown in LB media. For growth of plasmid containing the tac promoter, *E. coli* strain D1210 was used as a host. *E. coli* D1210 is a lac I^q derivative of HB101.

DNA

Plasmid DNA was prepared according to standard procedures (20). Numbering of the pBR322 is the one commonly used (20).

DNA fragments were purified from 1.2% low-melting-point agarose gels according to standard methods (20).

Oligonucleotides were synthesized by using the phosphoramidite chemistry (21) on the Applied Biosystems (Foster City,CA) DNA synthesizer, model 380A and purified

by preparative polyacrylamide gel electrophoresis.

REACTIONS FOR CASSETTE MUTAGENESIS

Individual oligonucleotide strands were phosphorylated using standard procedures (20).

Oligonucleotide strands were annealed by mixing 0.5 pmole /µl of each strand and incubating at 95 °C for 5 min in 50 mM Tris-HCl buffer, pH 8, containing 10 mM $MgCl_2$, followed by gradual cooling to room temperature over a 45 minute period.

For ligations, approximately 0.05 pmole of each restriction fragment and 0.5 pmole of the annealed synthetic fragment, were mixed in 50 mM Tris-HCl buffer, pH8, containing 10 mM $MgCl_2$, 0.5 mM ATP, 5 mM dithiothreitol and 10 units of T4 DNA ligase and incubated at 15 °C for 16 hours. The reaction mixture was used directly to transform competent E. coli HB101 cells. Cell transformations were done by standard methods (22).

CASSETTE MUTAGENESIS

To introduce the double mutations at site 71 and site 72 of RTEM-1 beta-lactamase, the following mixture of oligonucleotides,

```
                                                  [AT ]
5' - CC GAG GAA CGT TTT CCA ATG ATG AGC           [CCT]  ACT AAA GT
       C CTT GCA AAA GGT TAC TAC TCG              [TAA]  TGA TTT CA-5'
                                                  [GG ]
```

was ligated into the plasmid as shown in Figure 1.

After ligation and transformation as described above

the cells were spread on L-agar plates containing 25 µg/ml tetracycline.

DNA SEQUENCING

The plasmids were digested with Ava I and the resulting 2,962 base-pair fragments were isolated as described above. The fragment was labeled at nucleotide T-3973 using [alpha-^{32}P] dTT, and the Klenow fragment of DNA polymerase I. The labeled fragment was sequenced by the method of Maxam and Gilbert (23).

AMINO ACID DERIVATIVES

The benzyl ester of D-alanine was prepared by the method described by Guttmann and Boissonnas (24). Alpha-Boc-ε-benzyloxycarbonyl-L-lysine was purchased from Sigma. Boc-D-alanine was prepared by the procedure as described (25). The N-hydroxy-succinimide ester of alpha-Boc-ε-benzyloxycarbonyl was prepared as described (26).

SYNTHESIS OF ALPHA-BOC-ε(Z)-L-LYS-D-ALA-D-ALA(BZL)

This compound was synthesized by the method of Nieto and Perkins as shown in Figure 1 (27). The benzyl ester (free base) of D-alanine was coupled to the Boc-D-alanine by means of dicyclohexylcarbodiimide in dichloromethane at 0 °C overnight. Dicyclohexylurea was removed by filtration and the protected dipeptide was purified by recrystallization from ethylacetate-n-hexane. The yield was about 86% of the theoretical. The tert-butyloxycarbonyl group was removed by treatment with anhydrous

trifluoroacetic acid at 0°C for 15 minutes. The trifluoroacetic acid was evaporated under vacuum, and the excess was removed by repeated dissolution in 80% acetic acid and concentration *in vacuo* at 40°C. Finally, the trifluoroacetate salt was dried *in vacuo* over KOH and P_2O_5. Coupling of the dipeptide benzyl ester trifluoroacetate with the N-hydroxysuccinimide ester of alpha-Boc- -benzyloxycarbonyl-L-lys was carried out as described by Anderson et al. (26). The protected tripeptide was recrystallized from ethylacetate-n-hexane. The yield was about 82% of the theoretical value. The melting point of the protected tripeptide is 127° - 128°C, which is very close to the published one, 126° - 127°C.

SYNTHESIS OF DI-ACETYL-L-LYS-D-ALA-D-ALA

The benzyl group was first removed by hydrogenation in 80% acetic acid, with 10% Pd/C as catalyst, to give the N-acetylated peptide. The tert- butyloxycarbonyl group was then removed by the method as described above. The free peptide L-lys-D-ala-D-ala (1 mmole) was dissolved in dioxane-water (1:1, v/v) (10 ml), cooled at 0°C (ice bath), and triethylamine (5 mmole) was added followed by acetic anhydride (2.5 mmole). The mixture was kept on ice for 2 hours, boiled for 3 minutes to destroy any excess of acetic anhydride, evaporated to dryness, redissolved in water and evaporated again. The yield of crude product was about 60% of the theoretical value. The crude product was

redissolved in water and further purified on a column of Dowex 50 (H^+ form) and recrystallized from ethanol-hexane. The melting point is 200 -201 °C.

SYNTHESIS OF [^{3}H]-DIACETYL-L-LYS-D-ALA-D-ALA

This substrate, which was radioactively labeled in the acetyl group, was prepared as described by Perkins and Nieto (28). [1-^{3}H] - acetic anhydride (50 mCi; 26.6 mCi/µmole) was condensed at the bottom of the ampoule provided by the supplier (The Radiochemical Centre, Amersham, Bucks., U.K.) by cooling it to -80 °C and warming slightly the rest of the ampoule. The top of the ampoule was broken and L-lysyl-D-alanyl-D-alanine (3 µmole) and triethylamine (1 µl) in water-dioxane (1:1,v/v) (4 µl) were added quickly. The frozen solution was melted, mixed thoroughly and kept in an ice bath for 1 hour. Then 1 µl of acetic anhydride (non-radioactive) was added and the reaction was allowed to proceed for one more hour in the ice bath, The reaction mixture was heated for 1 minute at 100 °C, concentrated to dryness, redissolved in water and finally purified by paper electrophoresis in pyridine-acetate buffer (acetic acid : pyridine : water = 1 : 25 : 475), pH 6.5 (60 v/cm, 1 hour). The purified product gave a single radioactive spot after high-voltage electrophoresis under conditions that would detect > 0.1% radioactive impurity.

ASSAY FOR D,D - CARBOXYPEPTIDASE ACTIVITY

Assays of the mutant beta-lactamases and RTEM-1 beta-lactamase were performed at 37 °C in mixtures containing 0.1 M Tris-HCl (pH 7.5), 160 nmole of [^{3}H] diacetyl-L-Lys-D-Ala-D-Ala (specific activity, 67.1mCi/mmole) and 20 ug of enzyme in a total volume of 60 µl. The extent of conversion of diacetyl-L-Lys-D-Ala-D-Ala to diacetyl-L-Lys-D-Ala was determined by seperating the compounds by high-voltage paper electrophoresis in water / acetic acid / pyridine, 1,000 : 10 : 1 (v/v), buffer pH 3.5, locating each by cutting Whatman 3MM paper in pieces and assaying the radioactivity (relative mobilities: diacetyl-L-Lys-D-Ala-D-Ala, 20cm; diacetyl-L-Lys-D-Ala, 29cm).

RESULTS

The diad Thr71Phe72 of RTEM-1 beta-lactamase has been simultaneously changed into Thr71Thr72, Leu71Thr72, Ile71Thr72 and Pro71Thr72 by cassette mutagenesis. The scheme for cassette mutagenesis shown in Figure 1 is adapted from Schultz and Richards (19). A mixture of synthetic oligonucleotides was used in directing mutagenesis. After three-piece ligation and transformation, about 120 colonies were obtained on the tetracyline plate. These colonies were separated into four groups according to their resistant phenotypes to benzylpenicillin and ampicillin. Several colonies from each group were selected and their DNA sequences were confirmed by the method of Maxam and Gilbert (23). The mutagenized genes were subcloned into pJN plasmid for expression. The pJN plasmid was constructed by Jim Neitzl and contains a tac promoter as well as a gene for kanamycine resistance. This plasmid can be used in production of a beta-lactamase mutant that shows no beta-lactamase activity.

Cells producing either T71L, F72T or T71P, F72T mutant of beta-lactamase display no resistant phenotype to ampicillin and cephalothin. These two mutant proteins have not been further characterized. However, the T71T, F72T

and T71I, F72T mutants of beta-lactamases have been purified and partially characterized. The kcat and Km values of these mutant beta-lactamases are shown in Table 1. The T71T, F72T mutant of beta-lactamase shows 52.7% kcat/Km value of the wild-type beta-lactamase. The T71I, F72T mutant of beta-lactamase shows only 0.11% kcat/Km value of the wild-type beta-lactamase. Table 2 shows that the thermal stability of the T71T, F72T mutant of beta-lactamase is worse than that of the wild-type beta-lactamase; the thermal stability of T71I, F72T mutant of beta-lactamase is worse than that of the T71I mutant of beta-lactamase.

DISCUSSION

When suitable restriction sites are available in the original vector plasmid or suitable new restriction sites have been created, cassette mutagenesis becomes a much easier way to generate specific mutations at desired sites than the oligonucleotide-directed mutagenesis. It also becomes possible to generate many mutations at the same time by cassette mutagenesis; for example, one amino acid can be replaced by the 19 possible substitutions in one experiment (9).

To continue studying the roles of residue 71 and 72 in the catalytic activity, thermal stability and substrate specificty of RTEM-1 beta-lactamase, and to study the possibility of creating a substantial D,D-carboxypeptidase activity within the structural background of a beta-lactamase, I have changed the diad Thr71Phe72 of RTEM-1 beta-lactamase into Thr71Thr72, Ile71Thr72, Pro71Thr72 and Leu71Thr72 by cassette mutagenesis.

Among those mutant proteins, the T71T, F72T and T71I, F72T mutants of beta-lactamase were purified and partially characterized. The thermal stability of T71T, F72T mutant of beta-lactamase is worse than that of the wild-type beta-lactamase and the thermal stability of T71I, F72T mutant

beta-lactamase is also worse than that of the T71I mutant of beta-lactamase. These results indicate that the Phe72 residue of RTEM-1 beta-lactamase may play an important role in the stability of this enzyme.

The T71T, F72T mutant beta-lactamase shows 52.7% of the kcat/Km value of the wild-type beta-lactamase, indicating that residue 72 may not be involved directly in either binding or catalysis. The T71I mutant of beta-lactamase shows 7.25% of the kcat/Km value of wild-type beta-lactamase; however, the T71I, F72T mutant of beta-lactamase shows only 0.11% of the kcat/Km value of wild-type beta-lactamase. These results suggest that residues 71 and 72, though themselves not being involved directly in catalysis, may play a very important role in keeping the active site Ser70 and the conserved residue Lys73 in the correct orientation for efficiently catalyzing the hydrolysis of the beta-lactam antibiotics. In other words, the orientation between the active site Ser and the conserved Lys residue in mutant beta-lactamases, wild-type beta-lactamase and D,D-carboxypeptidases may be different from one another because each of them contains different diads between the two conserved residues, so that different mutant beta-lactamases and D,D-carboxypeptidases might have different catalytic activities against beta-lactam antibiotics. This may be the reason that the D,D-carboxypeptidases containing the conserved triad, Ser-Xaa-

Thr-Lys, can't catalyze the hydrolysis of the beta-lactam antibiotics very efficiently and that the mutant beta-lactamase of B. aureus PC1 containing the sequence, Ser-Ile-Ser-Lys, shows no beta-lactamase activity (10), but the mutant RTEM-1 beta-lactamase containing the sequence, Ser-Ile-Phe-Lys, still shows significant beta-lactamase activity. It would be interesting to know whether a mutant D,D-carboxypeptidase containing the sequence, Ser-Thr-Phe-Lys, can catalyze the hydrolysis of beta-lactam antibiotics more efficiently than the wild-type D,D-carboxypeptidase and whether a mutant beta-lactamase of B. aureus PC1 containing the sequence, Ser-Ile-Phe-Lys, can regain some beta-lactamase activity. Positive answers for the questions posed above may further support the view that the two residues between the active site Ser and the conserved Lys residues do play an important role in keeping the two conserved residues Ser and Lys in the correct orientation for efficiently catalyzing the hydrolysis of beta-lactam antibiotics.

Another more interesting question is whether the orientation between Ser71 and Lys73 and the penicillin-binding site in these mutant beta-lactamases have been changed in such a way that these mutants can now catalyze reactions characteristic of D,D-carboxypeptidases. This possibility was tested by the use of the model substrate [^{3}H]-diacetyl-L-Lys-D-Ala-D-Ala. Neither of the mutant

proteins described in Chapter 1 and this chapter show detectable D,D-carboxypeptidase activity. It is possible that either the amino acid residues used in binding or catalysis by D,D-carboxypeptidases are different from those used by beta-lactamases or that, in these two groups of enzymes, the spatial arrangement of the same amino acid residues involved in binding or catalysis is different.

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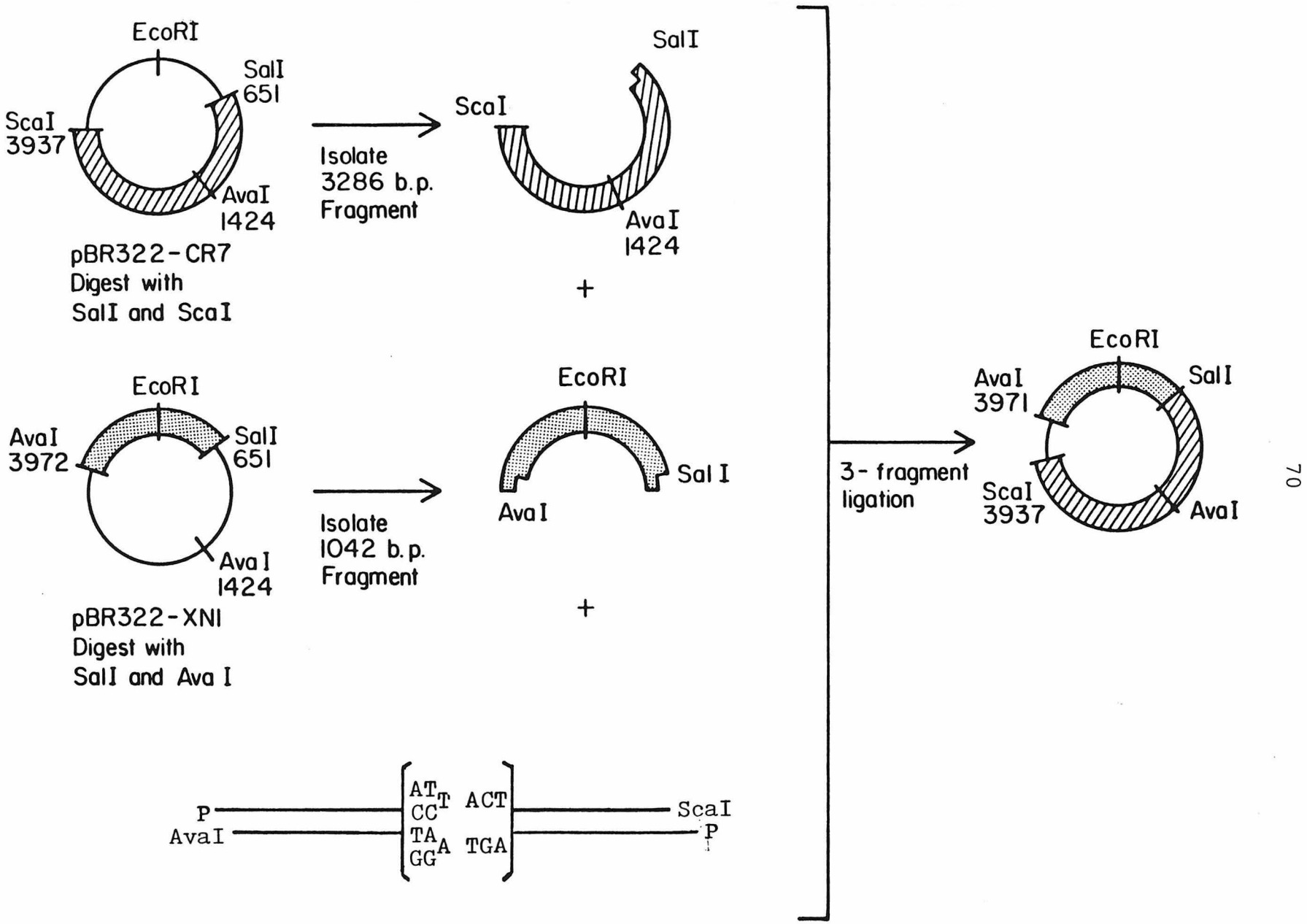
FIGURE I

THE SCHEME FOR CASSETTE MUTAGENESIS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SalI
651
ScaI
3937
AvaI
1424
pBR322-CR7
Digest with
SalI and ScaI
Isolate
3286 b.p.
Fragment
SalI
ScaI
AvaI
1424
+
EcoRI
AvaI
3972
SalI
651
Ava I
1424
pBR322-XNI
Digest with
SalI and Ava I
Isolate
1042 b.p.
Fragment
EcoRI
Sal I
Ava I
+
P
AvaI
AT
CCT
ACT
TA
GG
A
TGA
ScaI
P
3- fragment
ligation
EcoRI
AvaI
3971
SalI
ScaI
3937
AvaI

TABLE I

COMPARISON OF CATALYTIC PARAMETERS OF WILD-TYPE AND MUTANT BETA-LACTAMASES AGAINST AMPICILLIN

	Km(μM)	kcat(sec^{-1})	kcat/Km($\mu M^{-1} sec^{-1}$)
T71F72 (wild type)	50	2,000	40
T71I, F72	50	145	2.9
T71I, F72T	80	3.6	0.045
T71T, F72T	16	338	21.1

kcat/Km of mutant : kcat/Km of wild type		
T71I, F72	T71I, F72T	T71, F72T
0.073	0.0011	0.53

TABLE II

THERMAL STABILITY OF ACTIVE BETA-LACTAMASES

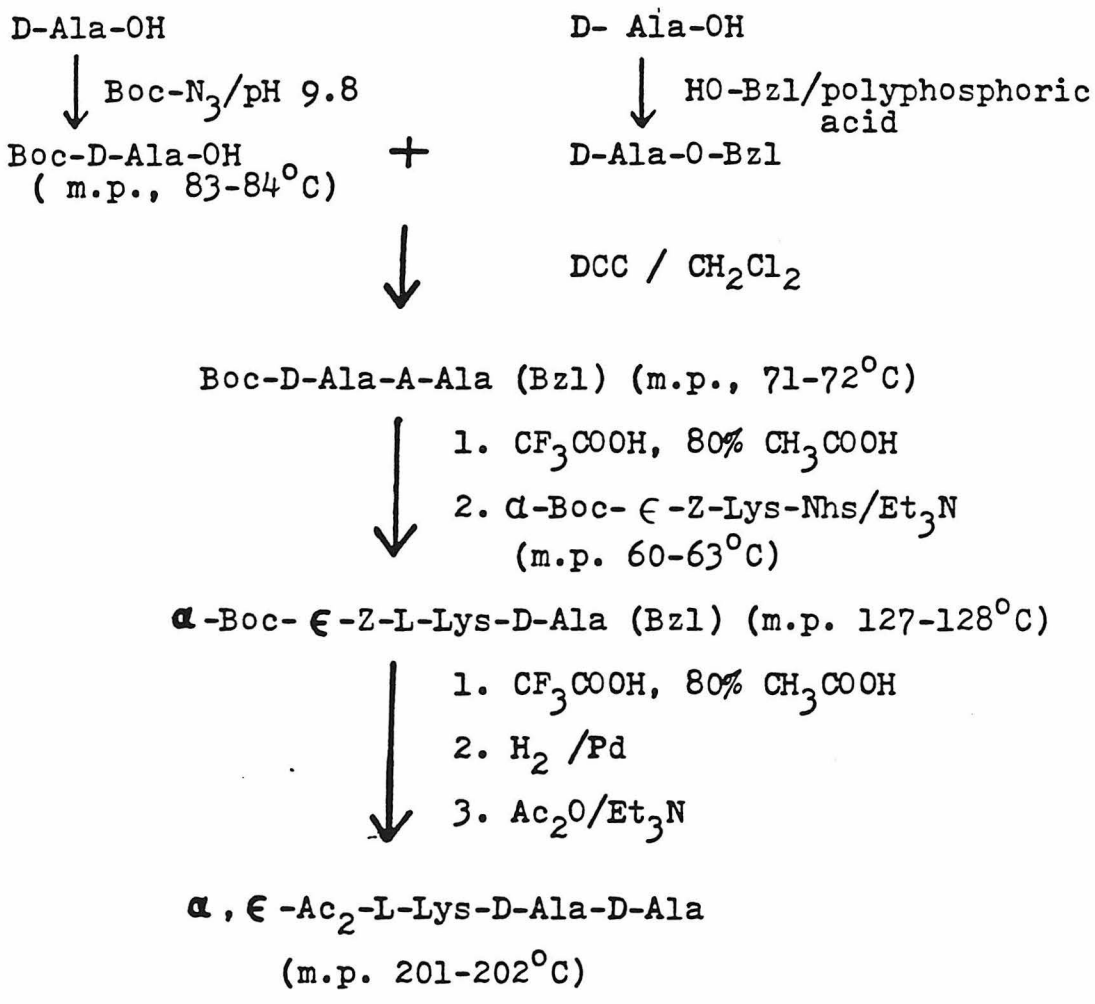
Temperature	$t_{\frac{1}{2}}$			
	*T71, F72	T71, F72T	T71I, F72	T71I, F72T
40°C	------	> 30 min	> 30 min	> 30 min
50°C	> 30 min	15 min	5 min	7 min
55°C	1-3 min	8 min	< 1 min	< 1 min
60°C	1-2 min	< 1 min	------	------

* From Jim Neitzel's data for wild-type beta-lactamase.

FIGURE II

SYNTHESIS OF DIACETYL-L-LYS-D-ALA-D-ALA

Abbreviations : Boc, t-butyloxycarbonyl; Bzl, benzyl; DCC, dicyclohexylcarbodiimide; Nhs, N-hydroxysuccinimide; Z, benzyloxycarbonyl.


D-Ala-OH
Boc-N3/pH 9.8
Boc-D-Ala-OH
(m.p., 83-84°C)
+
D- Ala-OH
HO-Bzl/polyphosphoric acid
D-Ala-O-Bzl
DCC / CH2Cl2
Boc-D-Ala-A-Ala (Bzl) (m.p., 71-72°C)
1. CF3COOH, 80% CH3COOH
2. α-Boc- ε -Z-Lys-Nhs/Et3N
(m.p. 60-63°C)
α -Boc- ε -Z-L-Lys-D-Ala (Bzl) (m.p. 127-128°C)
1. CF3COOH, 80% CH3COOH
2. H2 /Pd
3. Ac2O/Et3N
α , ε -Ac2-L-Lys-D-Ala-D-Ala
(m.p. 201-202°C)

CHAPTER III

CONSTRUCTION OF A HYBRID PROTEIN TO STUDY THE STRUCTURE-FUNCTION RELATIONSHIPS BETWEEN BETA-LACTAMASES AND D,D-CARBOXYPEPTIDASES

INTRODUCTION

The amino acid sequences around the active site serine of beta-lactamases show significant homology to those around the penicillin binding sites of the D,D-carboxypeptidases, leading to the proposal that these beta-lactamases may have evolved from D,D-carboxypeptidases (1, 2). Both groups of enzymes are acylated by beta-lactam antibiotics (3) and are able to catalyze the hydrolysis of certain acyclic depsipeptides (4). However, only D,D-carboxypeptidases can catalyze the hydrolysis of the acyclic peptides, involving the terminal D-Ala-D-Ala moiety of the peptidoglycan cross-link (1). It has been demonstrated that there is a significant similarity in the spatial arrangement of the elements of secondary structure in these two groups of enzymes (5, 6).

In Chapter II, to study the possibility of creating a substantial D,D-carboxypeptidase activity within the background of beta-lactamase structure, I have generated several mutant beta-lactamases with D,D-carboxypeptidase-like sequences in the immediate vicinity of the active site serine 70. None of these mutants shows detectable D,D-carboxypeptidase activity. Possibly, the amino acid residues used in binding or catalysis by D,D-carboxypeptidases are different from those used by beta-

lactamases. It is also possible that the spatial arrangement of the same amino acid residues involved in binding or catalysis in D,D-carboxypeptidases is different from the spatial arrangement of those corresponding amino acid residues in beta-lactamases. In other words, to be able to generate a mutant beta-lactamase that can catalyze appreciable D,D-carboxypeptidase activity, we may have to replace more than two amino acid residues around the active site serine of beta-lactamases with those corresponding amino acid residues of D,D-carboxypeptidases.

In both enzymes, the active site serine is near the NH_2-terminus. The spatial location of the beta-lactam binding site in the D,D-carboxypeptidase is shown in Figure 2a (7). It is flanked on one side by the beginning of the alpha-A helix, at the rear by the beta-A strand of the beta-sheet and at the bottom by the alpha G helix. Amino acid side chains potentially able to contact a beta-lactam are in a 20-residue coil of polypeptide in the NH_2-terminal portion of the molecule as shown in Figure 2b (7). Although the spatial location of the beta-lactam binding site in the beta-lactamase has not been worked out, one comparable to that of the D,D-carboxypeptidase might be expected.

Among the D,D-carboxypeptidases whose amino acid sequences are known, PBP5 of *E. coli* is the one that contains the most homologous amino acid sequence around the active site serine when compared with the class A beta-

lactamases (8). PBP5 can also catalyze a weak beta-lactamase activity, indicating that it may be more closely related to beta-lactamases than other D,D-carboxypeptidases (9). Combining the reasons described above with the consideration of suitable restriction endonuclease sites existing in the gene of RTEM-1 beta-lactamase, I decided to construct a hybrid protein with the replacement of a polypeptide of 29 amino acid residues of RTEM-1 beta-lactamase by the corresponding polypeptide of PBP5 of E. coli, which contains the amino acid residues potentially able to contact the binding substrate (7); this peptide consists of 30 amino acids.

The gene encoding the hybrid protein was constructed by the use of cassette mutagenesis as shown in Figure 3. For checking the in vivo stability of the hybrid protein, the mutagenized gene was cloned into pBR322. For production of hybrid protein, the mutagenized gene was cloned into pJN plasmid containing a tac promoter. Cells producing the hybrid protein do not display a resistance phenotype against any beta-lactam antibiotics. The result obtained by the use of western blot indicates that the in vivo stability of the hybrid protein is reasonably good. Interestingly, given the objective, the purified hybrid protein shows 1.8% D,D-carboxypeptidase activity of the wild-type PBP5 of E. coli. This result further supports the hypothesis that beta-lactamases are evolutionarily

related to D,D-carboxypeptidases(1). Moreover, this hybrid protein shows no transpeptidase activity, suggesting that the alpha-G helix in D,D-carboxypeptidase may play an important role in the transpeptidation reactions.

MATERIALS AND METHODS

ENZYMES AND CHEMICALS

Restriction enzymes and the large fragment of DNA polymerase I (Klenow) were purchased from Boehringer Mannheim. The T4 DNA ligase and T4 polynucleotide kinase were obtained from Bethesda Research Laboratories. All antibiotics were obtained from Sigma Chemical Co. The [alpha-^{32}P]dCTP, 3,000 µCi/mmole (1 Ci =37 GBq) was purchased from Amersham.

CELLS

E. coli HB101 cells containing wild-type or hybrid gene in pBR322 were grown in LB media. For growth of plasmid containing the tac promoter, *E. coli* strain D1210 was used as a host. *E. coli* D1210 is a lac I^q derivative of HB101.

DNA

Plasmid DNA was prepared according to standard procedures (10). Numbering of the pBR322 is the one commonly used (10).

DNA fragments were purified from 1.2% low melting point agarose gels according to standard methods (10).

Oligonucleotides were synthesized by the use of phosphoramidite chemistry (11) on the Applied Biosystems (Foster City, CA.) DNA synthesizer, model 380A and purified by preparative polyacrylamide gel electrophoresis.

REACTIONS

Individual oligonucleotide strands were phosphorylated using standard procedures (10).

Oligonucleotide strands were annealed by mixing 0.5 pmole/µl of each strand and incubating at 90 °C for 5 min in 50 mM Tris-HCl buffer, pH 8, containing 10 mM $MgCl_2$, followed by gradual cooling to room temperature over a 45 minute period.

For ligations, approximately 0.05 pmole of each restriction fragment and 0.5 pmole of the annealed synthetic fragment were mixed in 50 mM Tris-HCl buffer, pH8, containing 10 mM $MgCl_2$, 0.5 mMATP, 5 mM dithiothreitol and 10 units of T4 DNA ligase and incubated at 15 °C for 16 hours. The reaction mixture was used directly to transform competent *E. coli* HB101 cells. Cell transformations were done by standand methods (12).

CONSTRUCTION OF THE HYBRID GENE

The EcoRI-PstI fragment containing part of the beta-lactamase gene was first cloned into M13mp8. The newly constructed vector DNA was designated as M13mp8-DC9. The annealed four-piece synthetic oligonucleotide, which encodes the exact amino acid sequence around the active site serine of PBP-5 of *E. coli*, was then cloned into M13mp8-DC9 as shown in Figure 2.

After ligation and transformation as described above, the plaques were formed on YT-agar plates. The plaques

containing the mutant gene were identified by the restriction maps of the RFs digested with Ava I and Bgl I.

DNA SEQUENCING

The plasmids were digested with Xho II and Pst I, and the resulting 314 base pair fragment was isolated. This fragment was labeled at nucleotide G-3923 using [alpha-^{32}P]dCTP and the Klenow fragment of DNA polymerase I. The labeled fragment was sequenced by the method of Maxam and Gilbert (13).

PROTEIN GELS AND IMMUNO BLOT

Whole cell extracts were prepared from E. coli harboring plasmids encoding either mutant or wild-type beta-lactamase. The cells were grown to late log-phase (O.D. at 600 nm^{-1}), and the absorbance at 600 nm^{-1} was measured. The cells were pelleted by centrifugation and resuspended in loading buffer [10% (vol/vol) glycerol / 5% (vol/vol) 2-mercaptoethanol /3% (wt/vol) sodium dodecyl sulfate/ 62.5 mM Tris-HCl, pH 6.8 /1.0 mM EDTA] and lysed after being incubated 10 minutes at 95^{o}C. Cells from cultures with densities of 1 O.D. were resuspended in 100 µl of loading buffer; cells from cultures with slightly higher or lower densities were resuspended in a proportionally greater or lesser volume of loading buffer. Aliquots of 20 µl of these mixtures were loaded directly onto a 15 cm 12% polyacrylamide gel with a 2 cm 4% stacking gel and electrophoresed at 5 mA for approximately 12 hours.

The protein was transferred from the polyacrylamide gel to nitrocellulose by electrophoresis (electrophoretic blot or western blot) according to the Bio-Rad procedure (14) : the polyacrylamide gel was washed for 30 minutes in 25 mM Tris base / 192 mM glycine (pH 8.3) / 20% (vol/vol) methanol; the gel was then sandwiched between blotting paper and pure nitrocellulose (Schleicher and Scheull) and electrophoresed in a Bio-Rad Trans-Blot cell at 50 V for 5 hours.

The nitrocellulose membrane was first treated with a solution of 1% (wt/vol) BSA and 0.1% (vol/vol) normal goat serum for 1.5 hours at room temperature. The membrane was stained according to the procedure from the Vectastain ABC kit (15) using antibodies raised against beta-lactamase in rabbits. The initial injection used to raise the antibodies contained denatured beta-lactamase purified by SDS/polyacrylamide gel electrophoresis (16); booster shots contained protein purified as described elsewhere (16). Two weeks after the booster shot, the rabbit was bled and the serum was collected and used without further purification. For binding of the primary antibody, a 1 : 1,000 dilution of rabbit antiserum in TPBS was incubated with the nitrocellulose membrane for 1 hour at room temperature.

PRODUCTION and PURIFICATION OF THE HYBRID PROTEIN

The mutagenized gene was cloned into pJN plasmid for expression. The induction condition was the same as that

described in Chapter 2. The purification procedure was basically the same as that described in Chapter 1 except that ampicillin was added to a concentration of 40 µg/ml in all the buffers used in the procedure. Before purifying the hybrid protein on the Ultragel column, I had previously purified the hybrid protein twice on a DE-52 column. Those fractions that showed single bands on 12% SDS-polyacrylamide gel were pooled and used for further tests.

ASSAY FOR D,D-CARBOXYPEPTIDASE & TRANSPEPTIDASE ACTIVITY

Assays of the mutant beta-lactamases and RTEM-1 beta-lactamase were performed at 37 ^{o}C in mixtures containing 0.1 M Tris-HCl (pH 7.5), 160 nmole of [^{3}H] diacetly-L-Lys-D-Ala-D-Ala (specific activity, 67.1 µCi/umole) and 20ug of enzyme in a total volume of 60µl. The starting material, diacetyl-L-Lys-D-Ala-D-Ala, and the reaction products were separated by high-voltage electrophoresis in water / acetic acid / pyridine, 1,000 : 10 : 1 (v/v) buffer pH 3.5. The Whatman 3 MM paper was then cut into pieces (0.25 inch/piece). Each piece of the 3 MM paper was soaked in 1 ml of ddH_2O at room temperature with constant shaking; after 30 minutes, an aliquot of 100 from each vial was transferred into a scintillation vial containing 10 ml of scintillation fluid. The starting material and the reaction products were located by assaying the radioactivity of each vial and comparing their relative mobilities (18). The plot was shown in Figure VIII

(relative mobilities : diacetyl-L-Lys-D-Ala-D-Ala, 20 cm; diacetyl-L-Lys-D-Ala, 29 cm). The extent of conversion of diacetyl-L-Lys-D-Ala-D-Ala was derived from the ratio of the radioactivity of diacetyl-L-Lys-D-Ala to diacetyl-L-Lys-D-Ala-D-Ala.

The pH profile of the D,D-carboxypeptidase activity of the hybrid protein was obtained by the use of Tris-HCl or KOAc buffer at different pH values. Carbonate and phosphate buffers at different pH values have also been used to test their effects on the D,D-carboxypeptidase activity of the hybrid protein.

For assaying the transpeptidase acitvity of the hybrid protein, the same conditions described above were used except that the reaction mixture contained 50 mM or 100 mM glycine.

RESULTS

Table 1 shows the amino acid sequence of RTEM-1 beta-lactamase, which has been replaced, and the amino acid sequence of PBP5 of E. coli, which has been substituted in its place into RTEM-1 beta-lactamase. The gene encoding the hybrid protein was constructed by cassette mutagenesis as shown in Figure 3. After three-piece ligation and transformation, the plaques containing the hybrid gene were identified by comparing the restriction maps of the RF DNAs digested with Ava I and Bgl I. After the digestion, the wild-type RF shows three bands; however, the mutant RF shows only two bands. The results are shown in Figure 4. The DNA sequence was further confirmed by the method of Maxam and Gilbert (17). The mutagenized gene was cloned back into pBR322 for checking its resistance to beta-lactam antibiotics and into pJN for expression. Cells containing the mutagenized gene do not display a resistant phenotype against any beta-lactam antibiotics. The in vivo stability of the hybrid protein was checked by western blot. The results are shown in Figure 5. The hybrid protein produced in D1210 cells was purified twice on a DE-52 column and then once on an Ultagel column. This hybrid protein elutes off the DE-52 column more slowly than the wild-type RTEM-1 beta-lactamases as shown in Figure 6. To stabilize the

hybrid protein, ampicillin was added to 40 ug/ml in all the buffer used in the purification procedure. The hybrid protein purified under this modified condition shows about 30 times increase in its beta-lactamase activity and about 15 times increase in its D,D-carboxypeptidase activity over the protein purified in the absence of ampicillin. The purity of the isolated hybrid protein was checked on 12% SDS-polyacrylamide gel. The results are as shown in Figure 7.

The purified hybrid protein has been partially characterized. The specific activity of this hybrid protein is 1.1 sec^{-1} on ampicillin and 1.32 sec^{-1} on benzylpenicillin. The D,D-carboxypeptidase activity of this hybrid protein is about 3.5×10^{-3} min^{-1} as shown in Figure 9 and Table 2. Figure 10 shows a comparison between the pH profile of the D,D-carboxypeptidase activity of the hybrid protein and that of PBP-5 of E. coli (9). This figure also shows the differences in the effects of phosphate and carbonate buffers on the D,D-carboxypeptidase activity of these two proteins.

The transpeptidase activity of the purified hybrid protein has also been tested. The substrates used were [^{3}H]-diacetyl-L-Lys-D-Ala-D-Ala and glycine. When 100 mM glycine was added to the reaction mixture, the hybrid protein showed only 80% of its D,D-carboxypeptidase activity obtained under the condition without glycine.

After the reaction mixture containing 100mM glycine had been incubated at 37°C for 24 hrs, [^{3}H]-diacetyl-L-Lys-D-Ala was the only reaction product detected. In other words, this hybrid protein shows no detectable transpeptidase activity. However, under the same reaction conditions for the wild-type PBP-5 of E. coli, in 30 minutes, 80% of the reaction product is diacetyl-L-Lys-D-Ala-Gly, and only 20% of the reaction product is diacetyl-L-Lys-D-Ala (9).

DISCUSSION

Since the introduction of site-directed mutagenesis as an approach for studying structure-function relationships in proteins in 1982 (19, 20), many such studies have been reported (21-32). This work strengthens the view that the site-directed mutagenesis technique, when combined with classical enzymology and structural analysis, can become a very powerful tool to gain insights into the molecular basis of enzymatic catalysis. Recently, construction of hybrid proteins has been demonstrated to be a successful approach for creating new proteins with desired functional properties (33, 34); it has also been used as another approach for studying the structure-function relationships between evolutionarily and structurally related proteins.

Using cassette mutagenesis, when suitable endonuclease restriction sites exist in a cloned gene, one can easily replace a segment of the cloned gene with another designed gene fragment. Hybrid protein can thus be obtained by the expression of the hybrid gene.

The mutant 434 repressor with the replacement of its putative recognition alpha-helix with the putative recognition alpha-helix of 434 cro protein shows a change in its DNA-binding specificity to that of 434 cro protein (33). The construction of a hybrid protein of Lex A, an E.

coli repressor protein, and GAL4, a Saccharomyces cerevisiae transcriptional activator, successfully demonstrated that the DNA binding function of GAL4 can be replaced with that of a prokaryotic repressor without loss of the transcriptional activation function (34). Moreover, chimeric antibodies, which represent unique combinations of heavy and light chains, have been created by means of recombinant DNA technology (35, 36). Here, we present the first example of creating a new enzyme with desired catalytic function by the construction of a hybrid protein.

This hybrid protein was constructed by the replacement of a polypeptide chain containing 29 amino acid residues [from residue 47 to 75, in the number system of Ambler (37)] of RTEM-1 beta-lactamase with the corresponding sequence containing 30 amino acid residues of PBP-5 of E. coli. When comparing the amino acid sequence of this hybrid protein with that of RTEM-1 beta-lactamase, we find that there is an insertion between residue 56 and 57 in the hybrid protein and that there are 18 substitutions of amino acid residues between these two proteins.

Although the in vivo stability of this hybrid protein is reasonably good, the thermal stability of the purified hybrid protein is poor. When assaying the catalytic activity of the hybrid protein purified in the absence of ampicillin, I found that there was a lag of about 15 minutes. A likely explanation for this finding is that the

hybrid protein undergoes a conformational change after it binds to the beta-lactam antibiotics. Interestingly, although the thermal stability of the hybrid protein is poor, it can catalyze the hydrolysis of beta-lactam antibiotics with a constant rate for a period of 30 minutes when assayed at 55 °C. This result suggests that the conformation of the hybrid protein can be stabilized by its binding to beta-lactam antibiotics. This finding led me to add excess ampicillin in all the buffers used in the purification procedure as described in the Methods section. Indeed, the hybrid protein purified by this modified condition shows about 30 times increase in its beta-lactamase activity and about 15 times increase in its D,D-carboxypeptidase activity over the protein purified in the absence of ampicillin. All the data shown in this chapter, unless specifically indicated, were obtained by the use of the hybrid protein purified by the improved purification procedure.

The purified hybrid protein shows a specific activity of $1.32\ sec^{-1}$ against benzylpenicillin; interestingly, this protein also shows a specific D,D-carboxypeptidase activity of $3.5 \times 10^{-3}\ min^{-1}$. The wild-type RTEM-1 beta-lactamase has a kcat value of $1,700\ sec^{-1}$ against benzylpenicillin and shows no detectable D,D-carboxypeptidase activity (38). The wild-type PBP5 of *E. coli* has a half-life of about 10 minutes for releasing the

bound benzylpenicillin and shows a specific D,D-carboxypeptidase of 0.19 min^{-1} (9). In other words, this hybrid protein shows about 1.8% of the D,D-carboxypeptidase activity of the wild-type PBP5 of E. coli and about 10^{-3} of the specific activity of the wild-type beta-lactamase against benzylpenicillin. These results further support Tipper and Strominger's hypothesis that beta-lactamase may be evolutionarily related to D,D-carboxypeptidases (1).

The pH profile of the D,D-carboxypeptidase activity of the hybrid protein is different from that of the wild-type PBP5 as shown in Figure 10. The optimal pH for the D,D-carboxypeptidase activity of the hybrid protein is about 8.5; at pH 9, the hybrid protein shows only 40% of its optimal D,D-carboxypeptidase activity. However, the optimal pH for the wild-type PBP5 in Tris-HCl buffer is about 10 as shown in Figure 10. Moreover, both phosphate and carbonate buffer have been shown to be inhibitory for the activity of the wild-type PBP5 of E. coli (9), but phosphate buffer shows no such effect on the D,D-carboxypeptidase activity of the hybrid protein, and carbonate buffer shows less effect on the D,D-carboxypeptidase activity of the hybrid protein than on the activity of wild-type PBP5 of E. coli.

Another interesting finding is that the wild-type PBP5 (mol. wt. 42,000) of E. coli shows transpeptidase activity (9), but the hybrid protein (mol. wt. 28,500) does not. It

has been postulated that the portion of the COOH-terminal domain of helices E, F, G and H in R-61 D,D-carboxypeptidase could be involved in peptidoglycan binding during cell well biosynthesis (7). The major structural difference between the R61 D,D-carboxypeptidase and the class A beta-lactamase of B. licheniformis 749/C and B. cereus 569 is that both class A beta-lactamases lack the correponding alpha-G helix in their chystallographic structures. It is likely that there is no such alpha-G helix in the structure of hybrid protein either; this may be the reason that the hybrid protein shows no transpeptidase activity. Further understanding in the structure-function relationships between beta-lactamases and D,D-carboxypeptidases will require higher resolution of three-dimensional structures of these two groups of proteins.

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TABLE I

A. - 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 -

B. - D L N S G K v L a e e q n a d v R r d p a S* e t K m m t s G -

A. The amino acid sequence of RTEM-1 beta-lactamase which has been replaced.
B. The amino acid sequence of PBP-5 of E. coli which has been substituted into RTEM-1 beta-lactamase.
* The active site Ser.

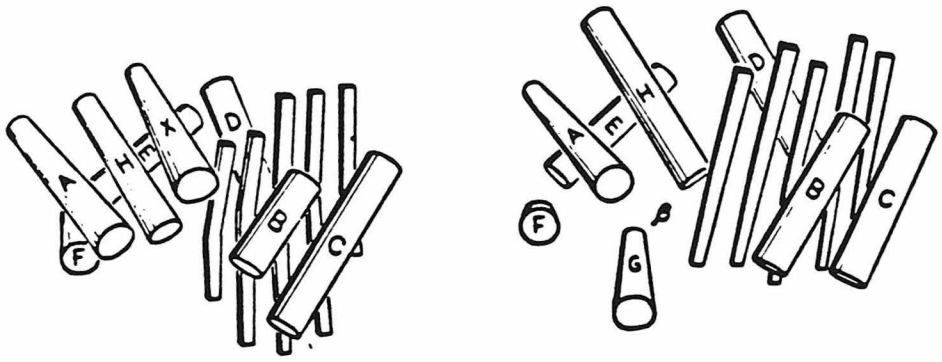
FIGURE I

SECONDARY-STRUCTURE ELEMENTS IN B. LICHENIFORMIS 749/C, STREPTOMYCES R61 AND BETA-LACTAMASE I.

A : Secondary-structure elements in B. licheniformis 749/C beta-lactamase (left) and in Streptomyces R61 D,D-carboxypeptidase (right). This is taken from Ref.5.

B : Secondary-structure elements of beta-lactamase I derived from analysis at 2.5 Å resolution. This is taken from Ref.6.

(A)



(B)

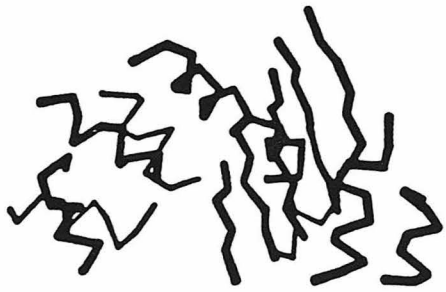
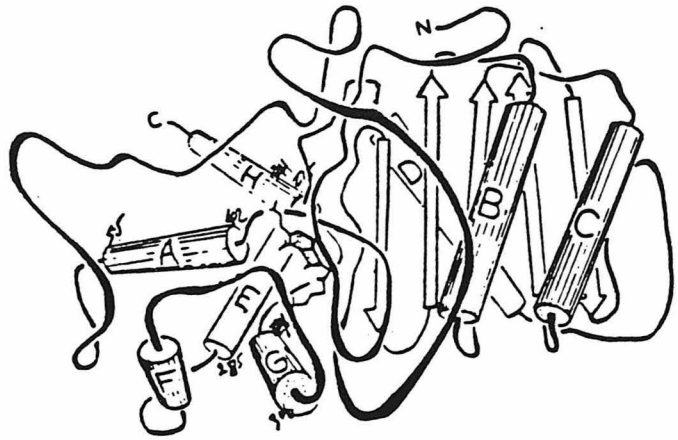


FIGURE II

A : Main chain folding schematic of Streptomyces R61 D,D-carboxypeptidase.

B : A yz projection of alpha-carbon atoms 25-42 (dots) showing folding of active site polypeptide around the beta-lactam position. Both A and B are taken from Ref.7.

(A)



(B)

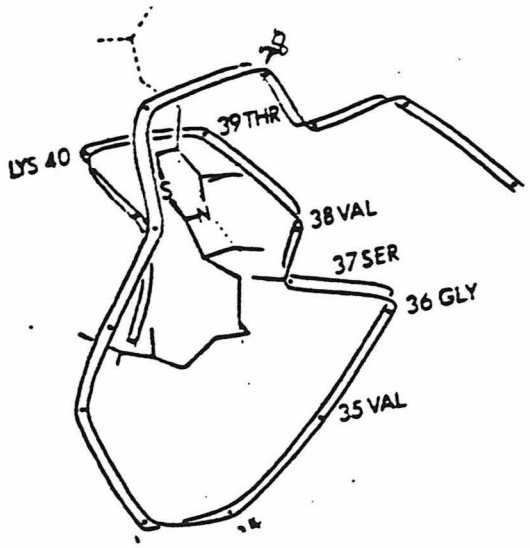


FIGURE III

THE SCHEME FOR THE CONSTRUCTION OF THE HYBRID GENE

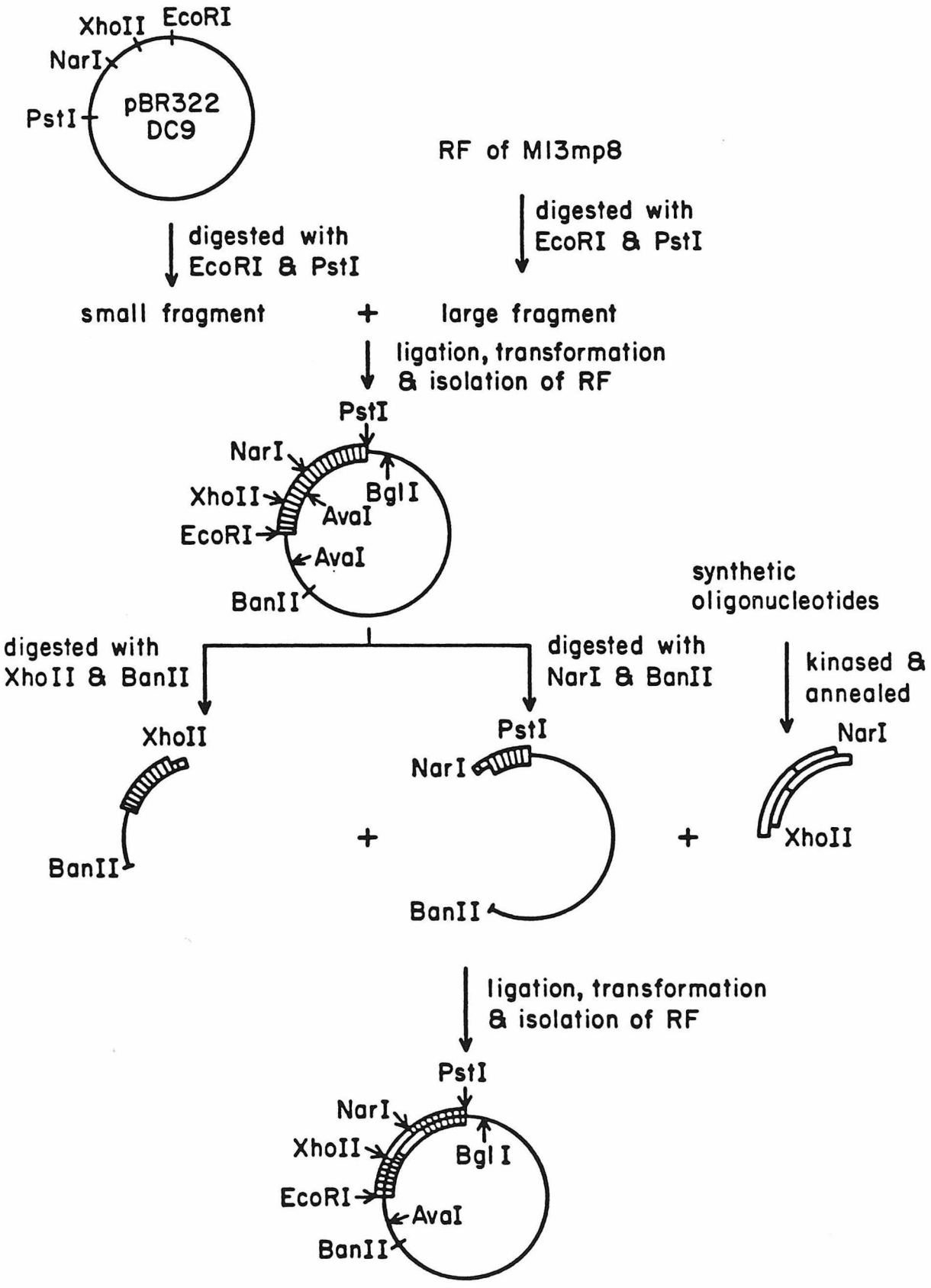

XhoII
EcoRI
NarI
PstI
pBR322
DC9
RF of M13mp8
digested with
EcoRI & PstI
digested with
EcoRI & PstI
small fragment
+
large fragment
ligation, transformation
& isolation of RF
PstI
NarI
XhoII
EcoRI
BglI
AvaI
AvaI
BanII
synthetic
oligonucleotides
digested with
XhoII & BanII
digested with
NarI & BanII
kinased &
annealed
XhoII
BanII
PstI
NarI
+
BanII
+
NarI
XhoII
ligation, transformation
& isolation of RF
PstI
NarI
XhoII
BglI
EcoRI
AvaI
BanII

FIGURE IV

RESTRICTION MAPS OF RFs DIGESTED WITH Ava I AND Bgl I

After the digestion of RFs with Ava I and Bgl I,the RF of the wild type will be cut into three pieces, but the RF of the putative mutant will be cut into only two pieces. The plaque that gave the result shown in b lane contained the mutagenized gene.

c ba

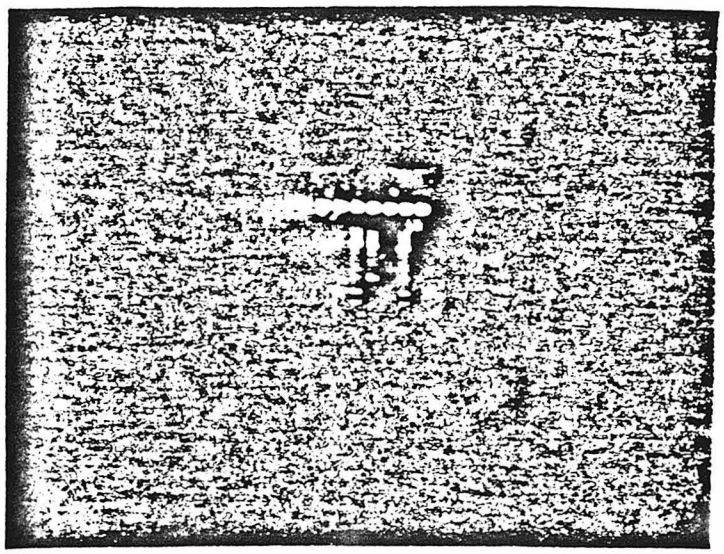


FIGURE V

WESTERN BLOT OF THE MUTANT BETA-LACTAMASES

37 °C
30°C
E. coli HB101
pBR322
hybrid
thr 71 thr 72
leu 71 thr 72
ile 71 thr 72
leu 71 phe 72
ile 71 phe 72
E. coli HB101
pBR322
hybrid
thr 71 thr 72
leu 71 thr 72
ile 71 thr 72
leu 71 phe 72
ile 71 phe 72
E. coli protein
precusor β-lactamase
β-lactamase
E. coli protein

FIGURE VI

THE ELUTION PROFILE OF THE HYBRID PROTEIN ON DE-52 COLUMN

The arrows indicate the fractions pooled for further purification. When the wild-type beta-lactamase was purified on the same column under the same condition, the fractions pooled were from about 350 ml to 380 ml.

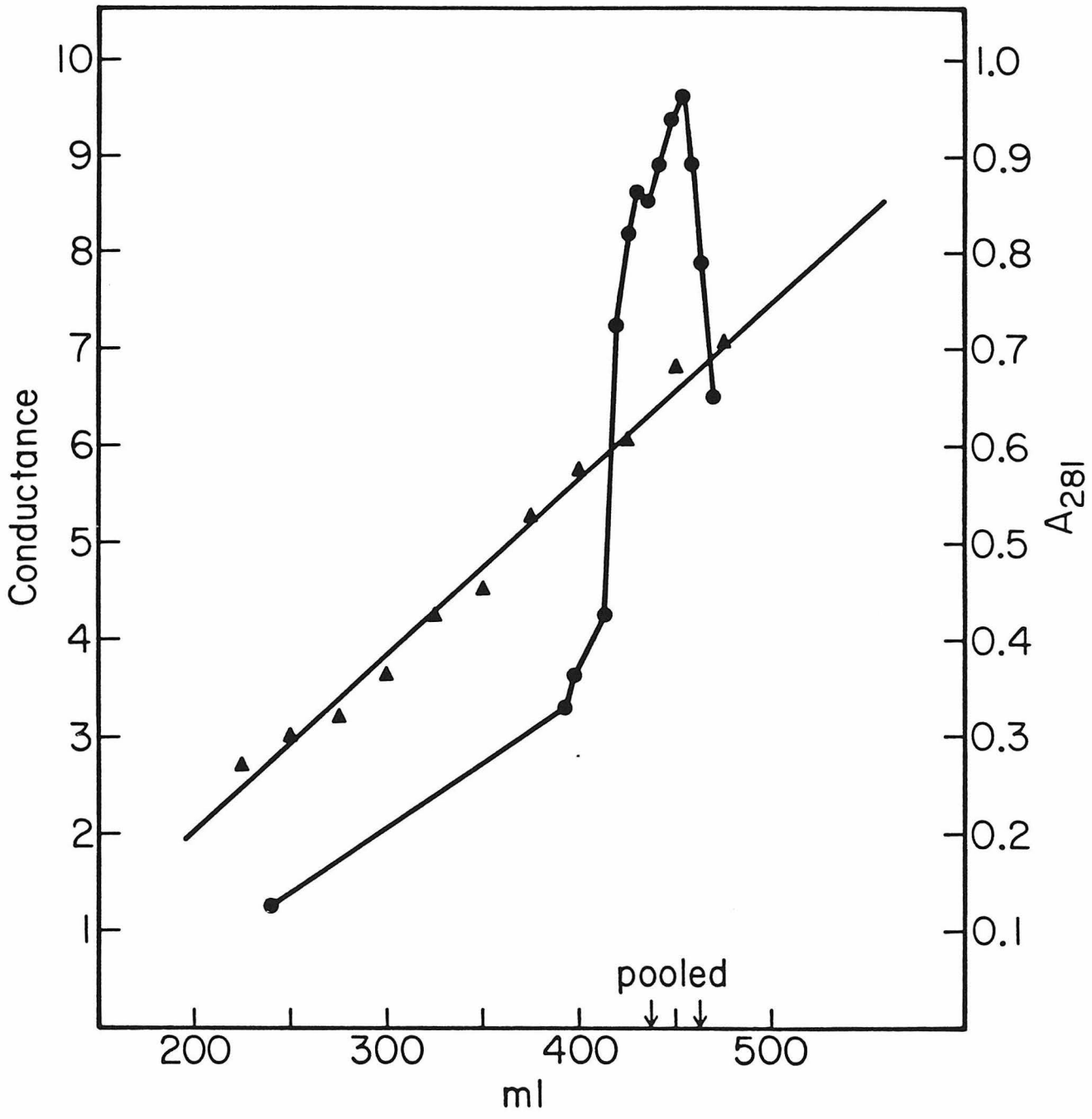
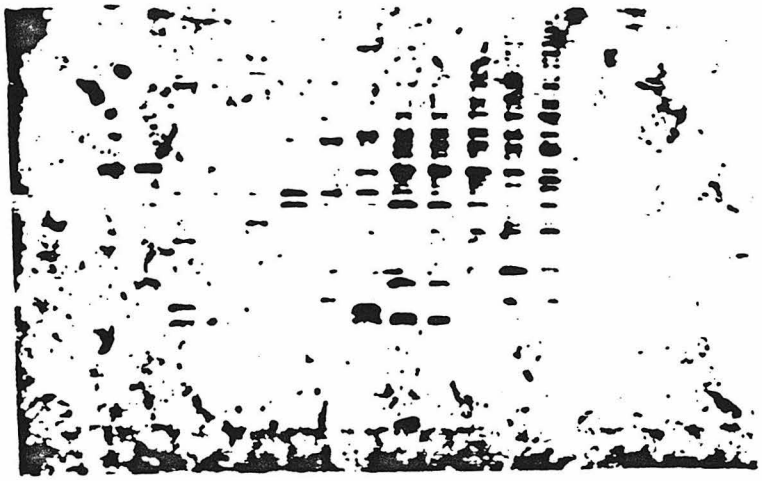

Conductance
A_{281}
ml
pooled
10
9
8
7
6
5
4
3
2
1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
200
300
400
500

FIGURE VII

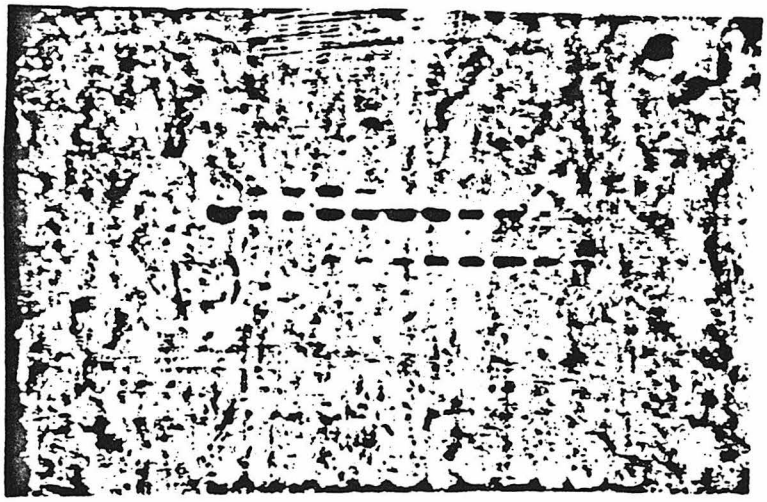
12% SDS/POLYACRYLAMIDE GEL ELECTROPHORESIS
OF THE HYBRID PROTEIN

This hybrid protein was purified once on a DE-52 column (A), twice on a DE-52 column (B) and finally on an Ultragel column (C). In picture C, lane a shows the purity of the wild-type beta-lactamase; lane b shows the purity of the hybrid protein; lane c shows the purity of the partially purified hybrid protein.

(A)



(B)



(C)

a b c

FIGURE VIII

SPECTRUM OF THE RESULT OF THE HYDROLYSIS OF [^{3}H]-DIACETYL-L-LYS-D-ALA-D-ALA BY THE HYBRID PROTEIN

[^{3}H]-diacetyl-L-Lys-D-Ala-D-Ala (specific activity, 67.1 mCi/mmole) was used as the substrate. The D,D-carboxypeptidase activity of the hybrid protein was assayed under the condition as described in the Methods section. After the reaction mixture had been incubated at 37°C for 25 hours, the starting material and the reaction products were separated by high voltage paper electrophoresis. The spectrum was obtained by locating these materials and measuring their radioactivity as described in the Methods section.

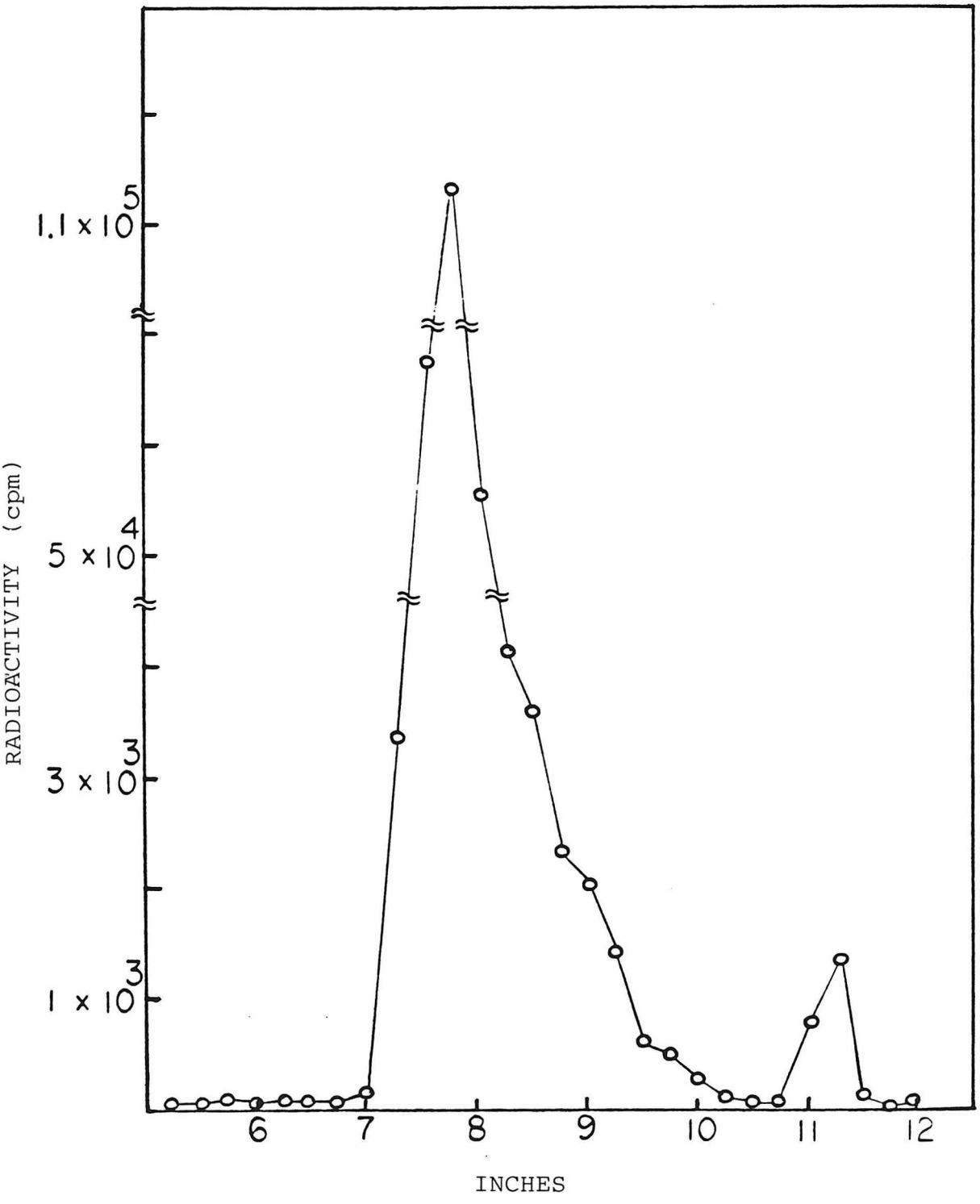

RADIOACTIVITY (cpm)
1.1 x 10^5
5 x 10^4
3 x 10^3
1 x 10^3
6
7
8
9
10
11
12
INCHES

FIGURE IX

D,D-CARBOXYPEPTIDASE ACTIVITY OF THE HYBRID PROTEIN

Assays were carried out as described in the Methods section. [^{3}H]-L-Lys-D-Ala-D-Ala (specific activity, 67.1 µCi/µmole was used as the substrate. Release of [^{3}H]-L-Lys-D-Ala was followed with time by high-voltage paper electrophoresis. The following proteins have been assayed : the hybrid protein in 50 mM Tris buffer (1µg/µl), pH 7.0, containing 150 µg/ml of ampicillin (□-□); the hybrid protein in 50 mM Tris buffer, pH 7.0, in which the ampicillin has been dialyzed away during a 5-hour period (△-△); the wild-type beta-lactamase and the T71I, F72T mutant of beta-lactamase (○-○).

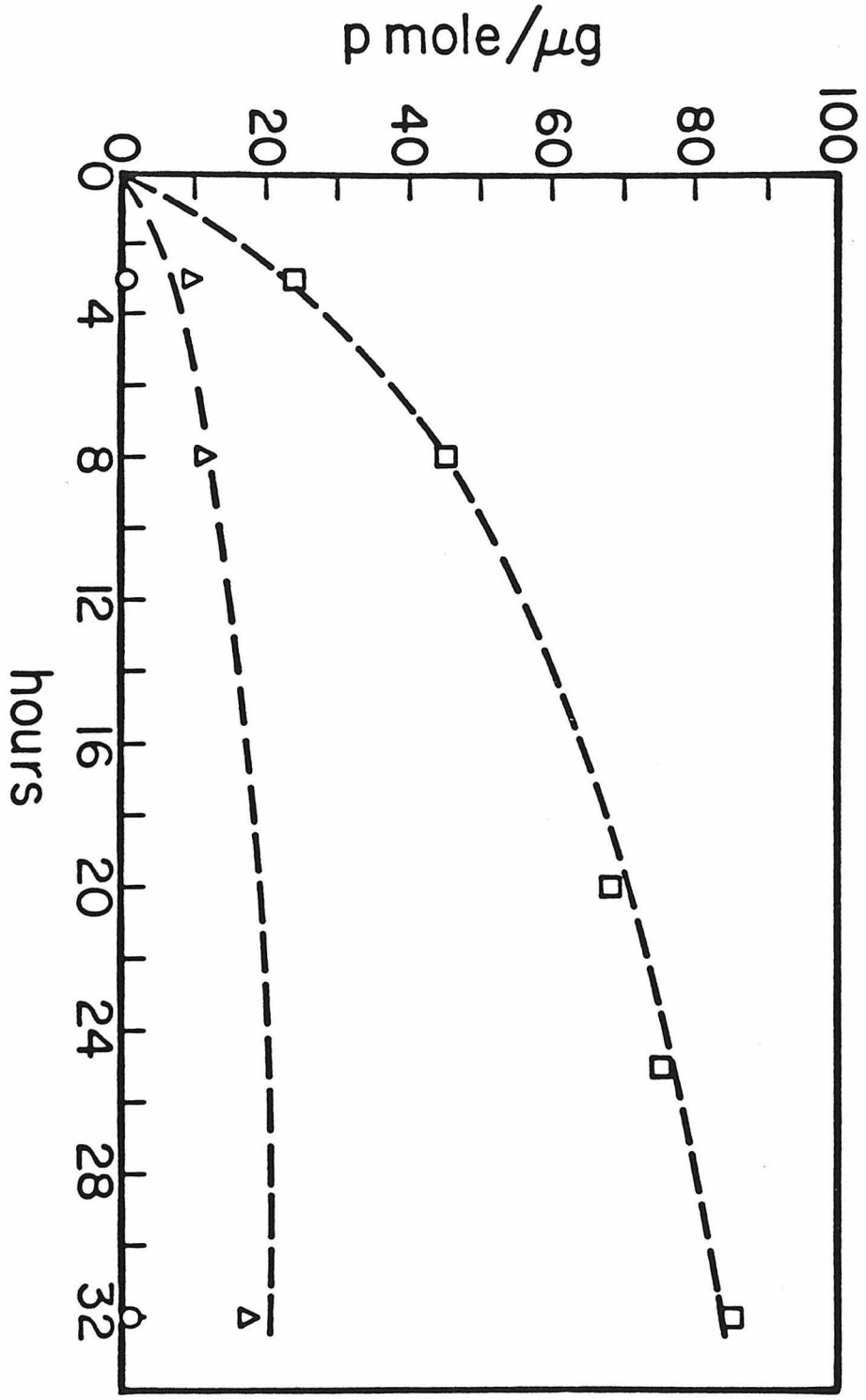

p mole/μg
0
20
40
60
80
100
hours
0
4
8
12
16
20
24
28
32

TABLE II

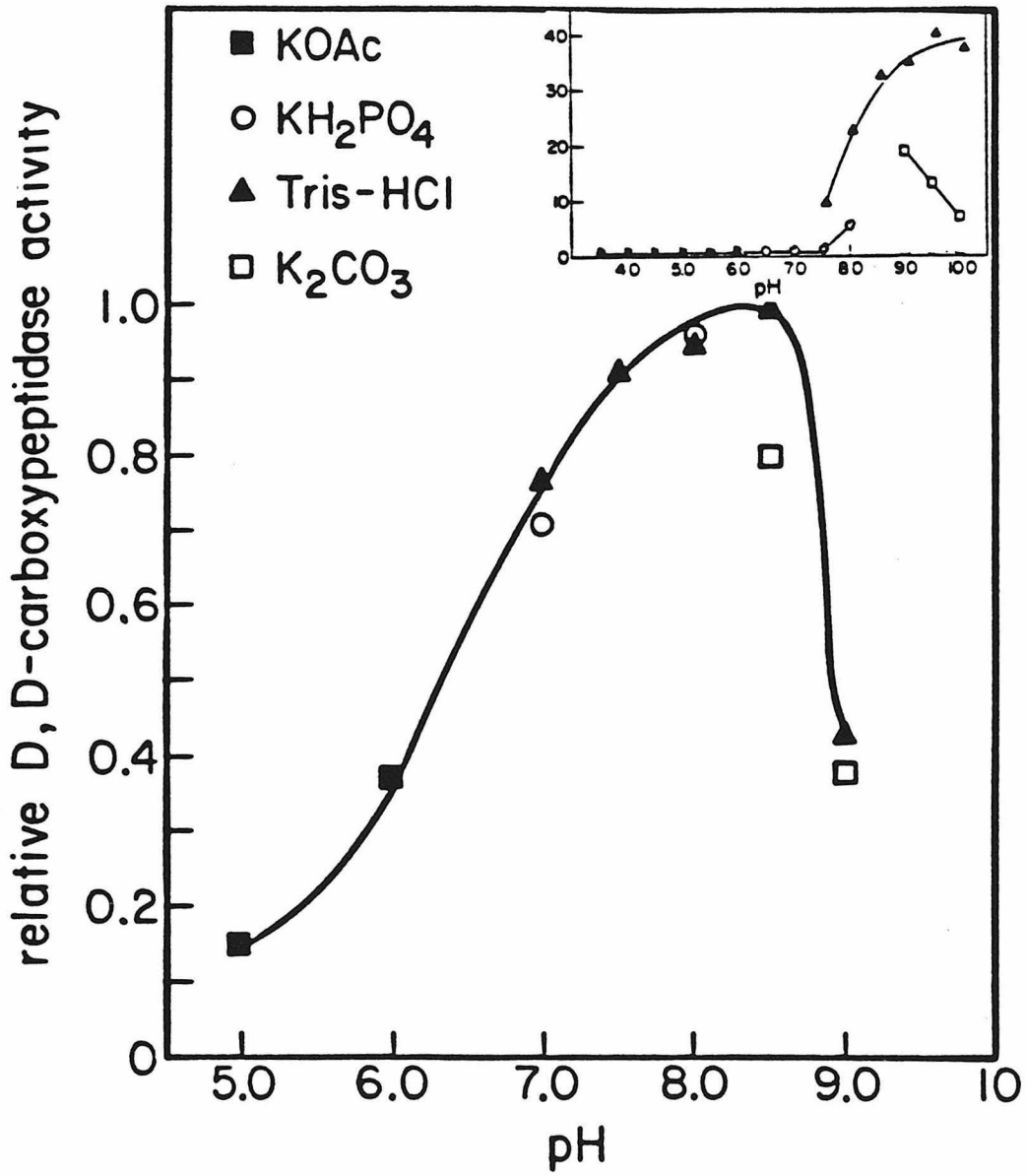
SPECIFIC ACTIVITY OF THE HYBRID PROTEIN, RTEM-1 BETA-LACTAMASE AND PBP5 OF *E. coli*.

substrate	hybrid protein	*PBP5	RTEM-1 beta-lactamase
^{3}H -L-Lys-D-Ala-D-Ala	$3.5 \times 10^{-3} min^{-1}$	$0.19 min^{-1}$	-------
benzylpencillin	$1.32 sec^{-1}$	$1.5 \times 10^{-3} sec^{-1}$	$1700 sec^{-1}$

*Data derived from Ref. 9.

FIGURE X

THE PH PROFILES OF THE D,D-CARBOXYPEPTIDASE ACTIVITY OF THE HYBRID PROTEIN AND PBP5 OF E. COLI (upper right corner)


KOAc
KH2PO4
Tris-HCl
K2CO3
relative D, D-carboxypeptidase activity
pH
0
0.2
0.4
0.6
0.8
1.0
5.0
6.0
7.0
8.0
9.0
10
40
30
20
10
0
40
50
60
70
80
90
100
pH

APPENDIX

^{13}C-NMR OF MONOCLONAL ANTIBODIES CONTAINING ^{13}C-ENRICHED TRYPTOPHAN

INTRODUCTION

One of the most characterized effector functions mediated by immunoglobulins is the triggering of the complement cascade (1). Complement consists of a group of nine proteins. These proteins, when triggered by antibody-antigen complexes, can produce a variety of results including the elaboration of chemotactic factors, cell lysis and viral inactivation (2). The precise mechanism by which the binding of antigen triggers this system is controversial, and two models have been advanced. One of these postulates that antigens act by aggregating antibodies. Such aggregation can have two effects. It can cause pre-existing sites on individual molecules to be brought together. Alternatively, new sites may result (or pre-existing sites may disappear), as a consequence of the polymerization of the antibodies (3, 4). The other model postulates that antibodies are allosteric proteins and that antigens function as allosteric regulators in addition to aggregating antibody molecules into complexes or clusters that function allosterically (4, 5, 6, 7).

Much evidence has been accumulated to support the contention that binding of antigens has an effect on an antibody at some distance from the binding site. Using neutron and an X-ray small-angle scattering technique, Cser

et al. have demonstrated that after binding to the haptens, pig antibody shows a conformational change (8). The results obtained by Liberti et al. (9) also indicate that a ligand can induce a conformational effect at some distance from the combining site. Additionally, it has been found that reduction of the inter-heavy chain disulfide bonds, which is known to eliminate complement fixation (10), also abolishes circular dichroism changes known to originate in the Fc region, which take place when antigen is bound (11). A study utilizing the technique of circular polarized luminescence (CPL) found changes in the CPL spectrum of rabbit antibodies to pneumococcal type III polysaccharide when large fragments of the antigen were bound, but not with smaller fragments (12).

Although Wright et al.(13) have prepared dimeric, trimeric and tetrameric complexes of IgG and have demonstrated that these complexes of IgG alone can activate complement fixation by the classical pathway, further studies by Sand (14) indicate that under certain experimental conditions components C5 to C9 are still not activated. Furthermore, it has been confirmed by Hardy that monovalent antigen can enhance the complement fixation of a monoclonal dansyl-specific IgM (15). This result led us to study the possibility of detecting directly the conformational change in antibodies by nuclear magnetic resonance.

The highly sensitive technique of ^{13}C nuclear magnetic resonance (16) provides a very useful probe of protein microenvironments (17). Using myeloma cells growing in culture (18), one can incorporate a ^{13}C-enriched amino acid into antibodies (19). This approach produces a minimal perturbation of the antibody and greatly increases the chances of observing the signals. The natural abundance of ^{13}C is so low that, coupled with the inherent low intensity of the signals, the observation of a few carbons at a concentration of 0.1 millimolar would be extremely difficult (20). In addition, the background signal from all the other protein carbons would tend to obscure any specific resonance (21). Enrichment serves both to improve the signal-to-noise ratio and to diminish background noise relative to the enriched signal.

Tryptophan enriched in ^{13}C at the gamma carbon was chosen to be incorporated into antibodies for the following reasons. First, to minimized spectral complexity, it is preferred to label an amino acid which is one of the less frequently occurring ones in proteins; tryptophan is the least common amino acid residue in most proteins (22). Secondly, it is important to consider that the probe we used in detecting the conformational changes in antibodies should be in a position likely to sense any changes that take place; the evidence described below indicates that both the gamma carbon in tryptophan and the

tryptophan residue itself in antibodies are very sensitive to their enviromental changes. Previous studies (23) have shown that the solvent exposure of tryptophan in IgG changes depending on whether antigen is bound or not. It has also been shown that labeling of tryptophan differs for an antibody and for an antibody-antigen complex (14). Moreover, the gamma carbon in tryptophan is a quaternary carbon and it is known to shift over five parts per million depending on its environment (24). Also, because this carbon has no directly bonded hydrogens, one may accumulate a spectrum without proton-decoupling and so diminish the background resonances relative to the quaternary carbons (25). An additional advantage of the gamma carbon is that its resonance is outside both the aliphatic and aromatic envelopes of resonances, further reducing interference by either antibody or antigen background resonances.

In this work, I optimized the tissue culture conditions for incorporating ^{13}C-enriched tryptophan into antibodies. Using ^{14}C-tryptophan, I demonstrated that tryptophan will not convert to other amino acid residues under these culture conditions. The activities of immunoglobulins purified by two different procedures have been compared. Finally, preliminary NMR spectra of IgG containing ^{13}C-enriched tryptophan have been obtained.[2a]

MATERIALS AND METHODS

CULTURE CONDITIONS

Myeloma cells were cultured either in Dubecco's modifed Eagle's medium (DMEM) or in RPMI1640 medium supplemented with either 10% fetal calf serum or 10% horse serum (Flow Labs, Inglewood, CA.). The medium was made up with double glass distilled water using a commercial powder formulation (Grand Islang Biological Co., Santa Clara, CA.) and then sterilized by pressure filtration through a 0.2 micron pore filter (Bio-Rad, Richmond, CA.). Labeling medium was made up from individual components without L-tryptophan according to either modified RPMI1640 or DMEM formulation. Just prior to use, 20 milligrams of enriched L-tryptophan were dissolved in 5 milliliters of saline solution and pressure-filtered into one liter of labeling medium. Prior to labeling, cells were grown in 250 milliliter screwcap glass erlenmeyer flasks at 37 ^{0}C in a humidified, 10% carbon dioxide-gassed incubator (Lab line 715). Large suspension cultures were grown in 2.8 liter flasks containing 1.5 liter of labeling medium supplemented with 10% fetal calf serum and stirred with a "floating" stir bar (Nalge, Ace Scientific, Linden, N.J.).

ISOLATION OF ENRICHED IgG ANTIBODIES

(1) From Affinity Column

The spent culture medium was centrifuged to remove cells and then applied directly to a DNP-Sepharose 4B column. Prior to each use, the column was washed with 20% acetic acid and then equilibrated with PBS. After the effluent was protein-free (as determined by the absorbance at 280 nm) the antibody was specifically eluted with 0.05 M DNP glycine. The protein fractions were pooled and a ten-fold molar excess of sodium dithionite was added with stirring to the solution buffered at pH 8.0 with 50 millimolar Tris. The dithionite reduced the bound DNP as observed by the disappearance of the yellow color and was immediately removed by pressure dialysis using PBS as the replacement buffer. The solution was concentrated to 20 milligrams per milliliter in deuterated PBS and then centrifuged to remove precipitated protein (5000 xg for 10 minutes) just prior to NMR study.

(2) From Ammonium Sulfate Precipitation Method

Tris buffer (0.05 M) was obtained by diluting 0.5 M Tris buffer, pH 8.3. Two liters of culture supernatant were concentrated to 200 ml (original serum volumes). Aliquots of one hundred milliliter saturated ammonium sulfate, pH 7.8, were added drop by drop into concentrated medium with stirring at 4 ^{o}C. The precipitate was collected after two hours by centrifuging the solution at 10 Krpm (RC2B rotor) for 10 minutes and then resuspended in a minimum amount of 50 mM Tris and eluted with a linear salt

gradient to 0.6 M NaCl. That protein eluted in the first peak was pooled; its purity was examined by 12% SDS gel electrophoresis; the concentration of the protein was determined by absorbance measurement assuming an extinction coefficient of 1.4 for 1 mg/ml IgG[2a] (280 nm, 1 cm path).

COMPLEMENT ASSAY

A standard microcomplement ^{51}Cr release assay was employed in the complement fixation studies with a few modifications (15). The buffer used in all studies was hepes-buffered saline (0.01 M HEPES, 0.15 M NaCl, 0.15 mM $CaCl_2$, 0.50 mM $MgCl_2$, 0.1% gelatin, pH 7.40). Hemolysin and sheep red blood cells were purchased from Flow Labs (Inglewood, Calif.). Hemolysin incubation was for 1/2 hour at 37°C followed by 1/2 hour at 0°C. Cells were then washed three times with Hepes-buffered saline and standardized as in the original reference. As has been found with some other IgM preparations above a certain concentration, large amounts of complement were spontaneously fixed, which limited concentrations in these assays to 10 µg/well (or 80 µg/ml).

Briefly, the antibody in 25 µl was added to the various dilutions of antigen in 25 µl, complement (2 CH_{50} units) was added in 25 µl, and the 96 well plate was incubated at 37°C for 45 minutes. Controls included wells containing just the antigen dilutions plus complement, just the

antibody plus complement, just complement, just the buffer and a 100 percent lysis standard of water. After the 45 minute incubation, 50 µl of 2% activated ^{51}Cr loaded-erythrocytes were added to each well and the plate was again incubated for 45 minutes at 37°C. At the end of this time the plates were centrifuged using plate holders (Flow Labs, Inglewood, Calif.) in a GLC-2 centrifuge (Sorvall) for 10 minutes at 2,200 rpm at 4°C. Aliquots of 50 µl per well were withdrawn and counted in gamma counter vials on a Beckman Gamma 4,000 Counter using the standard ^{51}Cr isoset.

All complement assays and titrations were carried out in Microtiter 96 well plates (Flow Labs, Inglewood, Calif.). Pipetting was carried out with Gilson automatic pipettors of 20, 200 and 1,000 µl maximum capacity and a Pipetteman pipettor of 5,000 µl maximum capacity. Incubations were carried out in a Lab Line 715 CO_2-100% humidity incubator.

ACID HYDROLYSIS OF IgG

Purified IgG[2a] was hydrolyzed by a previously published procedure[2a] with slight modifications. Hydrolysis was performed in a small culture tube (Kimble, 6x50 mm), which was put into a thick-walled sealed tube (Pyrex, 80x11 mm). Two milligrams of protein were hydrolyzed with 40 µl of 3N mercaptoethane sulfonic acid in the small culture tube and 200 µl of 3N mercaptoethane sulfonic acid in the large

sealed tube at 110°C for 24 hours. After hydrolysis, the tube was cooled, opened and 78 µl of 1N NaOH were added to the solution to bring the pH value up to 3.6. Aliquots of 50 ul were used for the amino acid analysis.

AMINO ACID ANALYSIS

A Beckman 120B amino acid analyzer was used. Fifty-microliter aliquots obtained from the procedure were applied to a 0.6x40 cm Aminex A5 column and eluted with three different buffers sequentailly at 58°C. They are 2N sodium citrate (pH 3.2), 2N sodium citrate (pH 4.2) and 1.2N sodium citrate, 1M NaCl (pH 8.9). (This experiment was kindly done by Mr. John Racs.)

PAPAIN DIGESTION OF IgG_{2a}

The protein was digested according to the method of Porter (26). Papain (0.2 mg) was added in 10 ml of buffer (0.05 M sodium acetate, pH 5.5, 0.0 M cystein, 2mM ethylenediamine tetraacetate) containing 20 mg of IgG_{2a}. This solution was incubated at 37°C for 15 minutes, 30 minutes, 1 hour and 1.5 hours, respectively. P-hydroxymercuribenzoate was added to stop the digestion after incubation and dialyzed against water overnight. the results were examined by SDS-polyacrylamide gel electrophoresis.

ISOLATION OF Fab AND Fc FRAGMENTS

The protein was digested as described above for 30 minutes. After dialysis against water overnight, the

solution was applied to the DNP-affinity column equilibrated with PBS buffer, pH 7.4, and eluted with PBS buffer. The first peak was pooled and concentrated to 5 mL. The concentrated solution was then applied to a G-75 Sephacel column (2.5x50 cm) equilibrated with PBS, pH 7.4, and eluted with the same buffer. The fractions of the first peak containing Fc fragment were pooled and examined by SDS/polyacrylamide gel electrophoresis. The Fab fragment was isolated by the same procedure as described for the purification of the intact antibody.

CELL STORAGE

Aliquots of cells were frozen in fetal calf serum containing 10% DMSO and stored in a liquid nitrogen refrigerator. Cells were pelleted and resuspended at 5×10^6 to 1×10^7 cells per milliliter in the freezing medium. One-milliliter aliquots were dispensed into 2 ml polypropylene freezer vials and the vials placed in a styrofoam block (approximately one centimeter of insulation around each vial). The block was placed in a -70^{o}C refrigerator and after 24 hours the vials were transferred to the liquid nitrogen refrigerator. Warming the vials in a water bath to 38^{o}C and washing the cells to remove the DMSO medium restored the cells to a viable state. The average recovery is on the order of 70 to 90% depending on storage time.

NMR CONDITIONS

A Bruker WM-500 (117 KG) and a Varian IX-299 (47 KG) were used to observe ^{13}C spectra. Ten millimeter sample tubes with 2.5 milliliter volume were used and were locked to an internal D_2O signal. Protein concentrations were 0.1 to 0.3 millimolar. At 117 KG, the flip angle we used was 18^{o} and the acquisition time was 0.557 milliseconds; at 47 KG the flip angle we used was 12^{o} and the acquisition time was 0.2 milliseconds. No decoupling was used. Spectra were accumulated for 10- to 12-hour periods.

RESULTS

Using ^{14}C-tryptophan, we have found that RPMI1640 medium supplemented with 10% fetal calf serum is the best culture medium for incorporating this labeled amino acid into antibodies. The results are shown in Table 1. Figure 1 indicates that there is less than 10% conversion of tryptophan to other amino acids. The optimal conditons for the papain digestion of IgG$_{2a}$ were established; the results are shown in Figure 2. After the papain digestion of IgG$_{2a}$, the Fc and other small fragments were separated from the Fab fragment by the use of an affinity column. The Fc fragment was separated from the other small fragments on a G-75 column. The results are shown in Figure 3. Figure 4 shows the elution profile of IgG$_{2a}$ on a DEAE Sephacel column. Figure 5 shows the comparison of the activity of antibodies purified by the ammonium precipitation method and by the affinity column. Figures 6 and 8 show the spectra of intact IgG$_{2a}$ and its Fab fragment. Figure 7 shows the difference spectrum obtained from the subtraction of the spectrum of IgG$_{2a}$ by the spectrum of its Fab fragment.

DISCUSSION

CULTURE CONDITIONS

Using ^{14}C-tryptophan, I have found that modified RPMI1640 supplemented with 10% fetal calf serum is the best culture medium for incorporating enriched tryptophan into antibodies. The differences between the formulation of DMEM and modified RPMI1640 are discussed below. In modified RPMI1640, HEPES is the major buffer material. It contains more vitamins than DMEM. They are p-amino benzoic acid, biotin and vitamin B12. It also contains hypoxanthin and thymidine. In DMEM, however, $NaH_2PO_4 \cdot H_2O$ is the major buffer material.

P-Amino benzoic acid can be used by plants and procaryotes, but not by mammalian cells. Biotin participates in the conversion of pyruvate to oxaloacetate and serves as a carrier for the "active" CO_2. The detailed mechanism of the action of vitamin B12 is still unknown (27). Hypoxanthin is a purine source; thymidine is a pyrimidine source. HEPES is known to be a better buffer material for tissue culture. With these poor understandings, it is difficult to explain why modified RPMI1640 is a better medium than DMEM for incorporating ^{13}C-enriched tryptophan into antibodies. It is also very difficult to explain why fetal calf serum is better than

dialyzed horse serum. However, the optimal growth conditions for incorporation have been found. Modified RPMI1640 suplemented with 10% fetal calf serum was always used as the culture medium after this finding. Furthermore, amino acid analysis gave recoveries of more than 90% of the ^{14}C-tryptophan, indicating that little conversion of it to other amino acids occurs.

PURIFICATION OF LABELED IgG$_{2a}$

To obtain free DNP-specific antibodies purified by the affinity column, we have to use sodium dithionite to remove the DNP-glycine which bound tightly to the antibodies. Although there is only a small difference between the activities of the antibodies purified by the affinity column and those purified by the precipitation method, we prefer using a method with the least danger of perturbing the conformation of the intact antibody. At the same time, five new cell lines have been obtained from Dr. Herzenberg's Laboratory. All of these new cell lines produce dansyl-specific immunoglobulins. Since we may face the same problem when purifying these immunoglobulins by an affinity column, a more general method of purification of antibodies is desirable.

A DEAE-Sephacel column has been found to be a better ion exchange column for purifying the antibody than DEAE-cellulose column (14). The exact procedure has been described in the Methods section. Antibody was precipitated

from the supernatant by ammonium sulfate; the crude antibody was then purified on a G-150 column followed by a DEAE-Sephacel column.

NMR CONDITIONS

All of the ^{13}C spectra were accumulated with a very small flip angle because the T_1 for tryptophan resonance at high fields is very long. The value of T_1 can be calculated under the assumption that $1/T_1 = 1/T_1^D + 1/T_1^{CSA}$. $1/T_1^{CSA}$ can be calculated from the following equation :

$$1/T_1^{CSA} = (2/15)H_o\gamma^2(\Delta\sigma)^2\tau_R(1+\gamma^2H_o\tau_R^2) \qquad (25).$$

Here, is the gyromagnetic ratio; Ho is the magnetic field strength. $1/T_1^D$ can be calculated from the following equation : $1/T_1^D = \sum_j 1/T_{1j} = (2/15)\hbar\gamma_C^2\sum_j S_j(S_j+1)\gamma_j^{2-6}r_{cj}\chi_j$ (24). Here, 1/Tij is the contribution to $1/T_1$ from a dipolar interaction between the pertinent ^{13}C nucleus and a nucleus j, γ_c and γ_j are the gyromagnetic ratios of ^{13}C and of nucleus j, respectively, Sj is the spin quantum number of nucleus j, each cj represents a nonbonded C-j distance, and

$$\chi_j = \frac{\tau_R}{1+(\omega_j-\omega_c)^2\tau_R^2} + \frac{3\tau_R}{1+\omega_c^2\tau_R^2} + \frac{6\tau_R}{1+(\omega_j+\omega_c)^2\tau_R^2}$$

Here, Wc and Wj are the resonance frequences, in radians/sec., of ^{13}C and of nucleus j, respectively; τ_R is the correlation time for rotational reorientation of the molecule. Using the estimate value of of about 150 nanoseconds (15), we can estimate values of T_1. At 47 KG (Varian XL-200), the T_1 of the gamma carbon of tryptophan

in IgG$_{2a}$ is about 14 seconds. At 117 KG (Brucker 500), the chemical shift anisotropy dominates the relaxation mechanism of the nonprotonated unsaturated carbons in a native protein (25), and the T_1 is about 20 seconds. According to these values of T_1, the best flip angle can be calculated at a fixed acquisition time. After several tests, the optimal conditions for accumulating the spectrum of intact IgG$_{2a}$ were found. At 47 KG, the flip angle we used was 12^{o} and the acquisition time was 0.2 milliseconds; at 117 KG, the flip angle we used was 18^{o}, and the acquisition time was 0.557 milliseconds.

The preliminary spectra of IgG$_{2a}$ and its Fab fragment obtained under these conditions are shown in Figures 6, 7 and 8. Unfortunately, although I have used the optimal conditions for incorporating the ^{13}C-enriched tryptophan into IgG$_{2a}$ and for accumulating the spectra of the ^{13}C-enriched IgG$_{2a}$, the signal-to-noise ratio of the spectra I obtained are still not very good. This is because the T_1 for tryptophan resonance is too long and, to avoid saturating the resonance, we have to use a very small flip angle. This problem won't be solved even when we use an NMR with higher magnetic field because, at a higher magnetic field, the chemical shift anisotropy dominates the relaxation mechanism of the nonprotonated unsaturated carbons in a native protein causing T_1 to become yet longer and thereby requiring the use of a still smaller flip

angle.

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TABLE I

NUMBER OF MOLECULES OF ^{14}C-TRP INCORPORATED INTO ONE MOLECULE OF IgG_{2a}

Modified RPMI 1640 & 10% Fetal Calf Serum	10.96
Modified RPMI 1640 & 10% Dialyzed Horse Serum	6.36
DMEM & 10% Fetal Calf Serum	5.37
DMEM & 10% Dialyzed Horse Serum	3.18

FIGURE I

SPECTRUM OF THE RESULT OF ACIDIC HYDROLYSIS

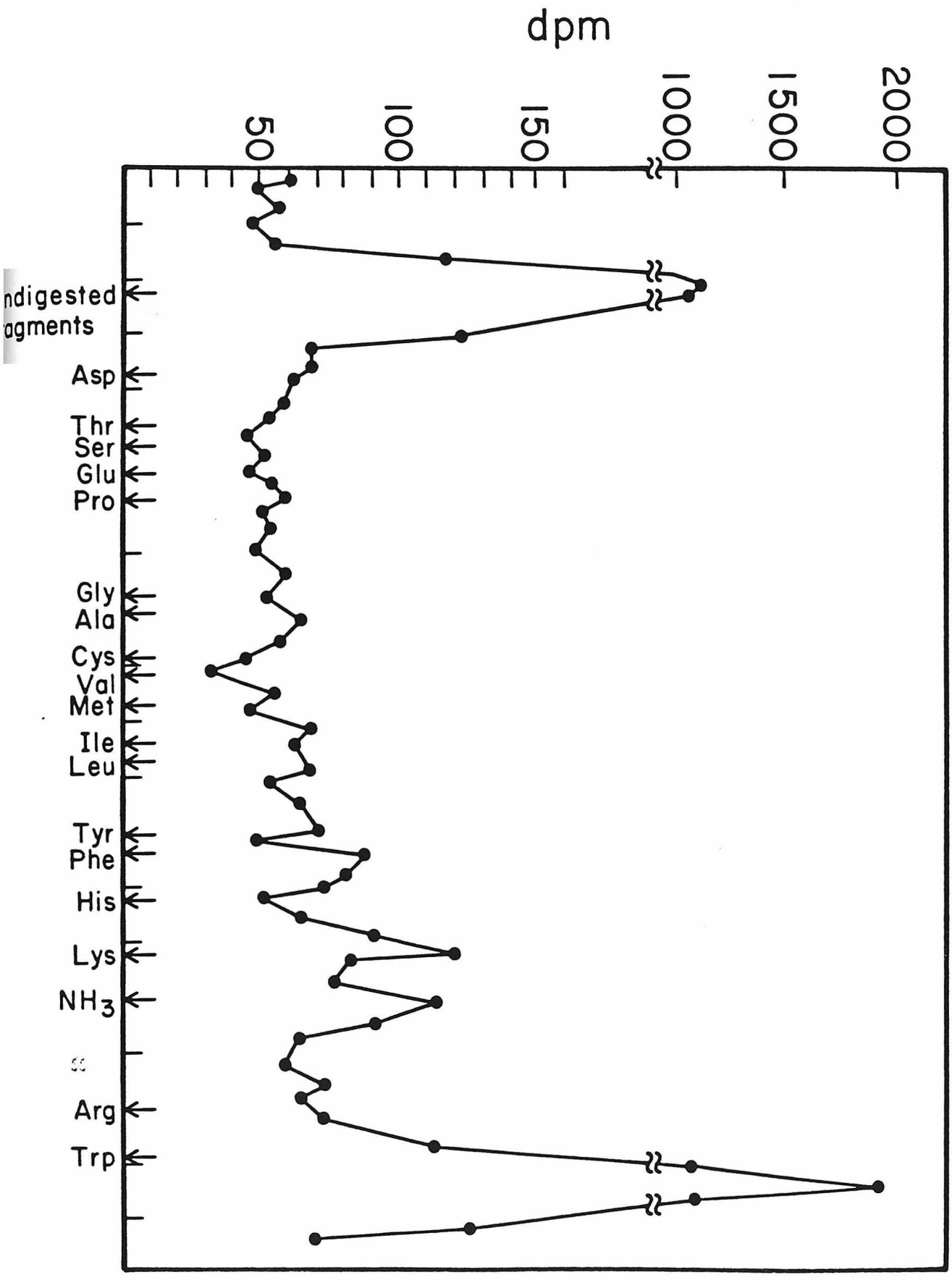

dpm
50
100
150
1000
1500
2000
ndigested
agments
Asp
Thr
Ser
Glu
Pro
Gly
Ala
Cys
Val
Met
Ile
Leu
Tyr
Phe
His
Lys
NH3
Arg
Trp

FIGURE II

12% SDS/PAGE OF IgG$_{2a}$ DIGESTED BY PAPAIN AND PURIFIED Fab AND Fc FRAGMENTS OF IgG$_{2a}$

Upper spectrum : 12% SDS/polyacrylamide gel electrophoresis of IgG$_{2a}$ digested by papain for 15, 30, 60 and 90 minutes, respectively.

Lower spectrum : 12% SDS/polyacrylamide gel electrophoresis of purified Fab and Fc fragments of IgG$_{2a}$.

90 60 30 15 min

Fab Fc

FIGURE III

THE ELUTION PROFILE OF Fc AND OTHER SMALL FRAGMENTS, OBTAINED BY DIGESTION OF IgG^{2a} ON G-75 SEPHADEX COLUMN

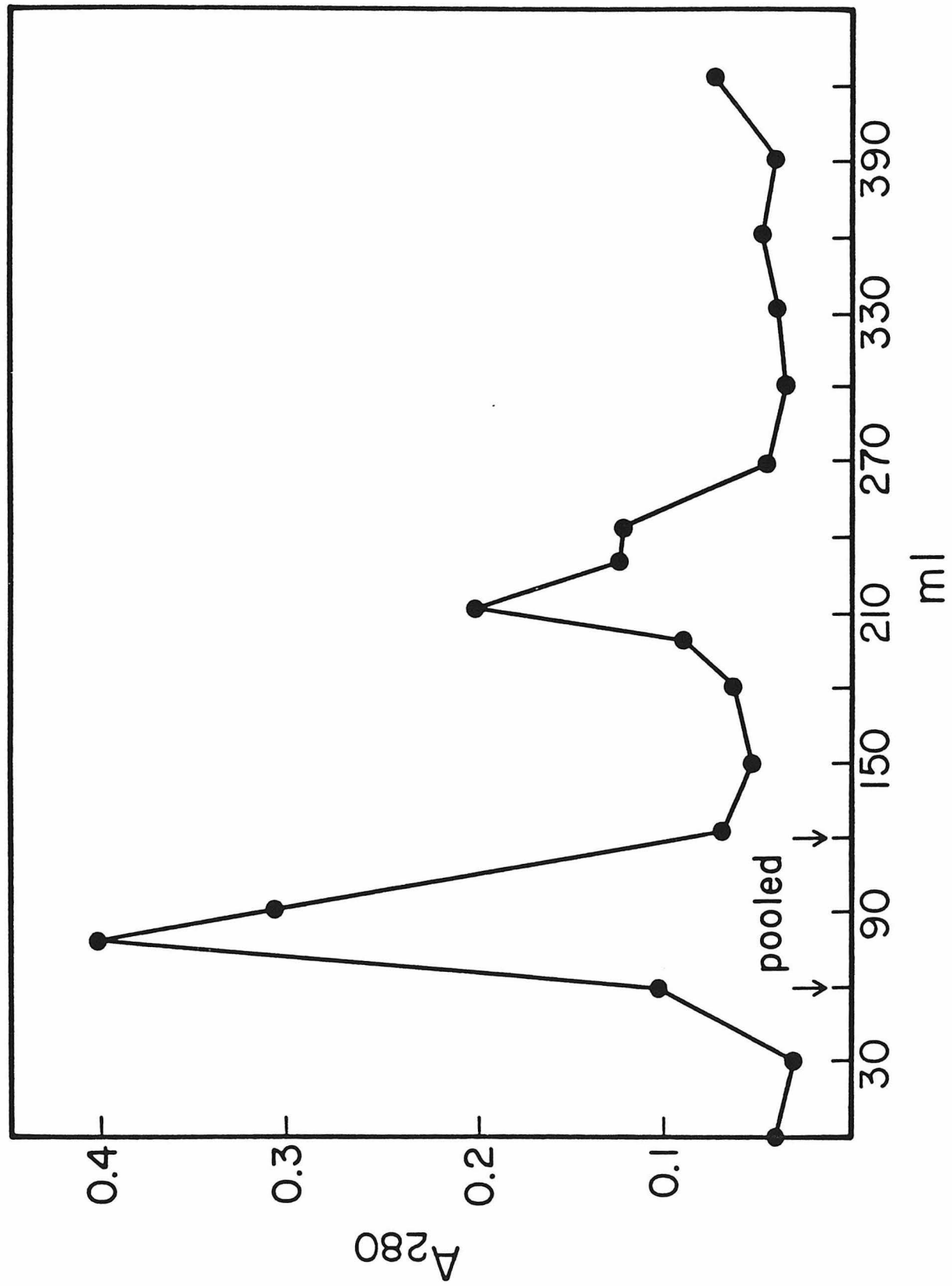

0.4
0.3
0.2
0.1
A280
30
90
150
210
270
330
390
ml
pooled

FIGURE IV

THE ELUTION OF PROFILE OF IMPURE IgG $_{2a}$
ON A DEAE SEPHACEL COLUMN

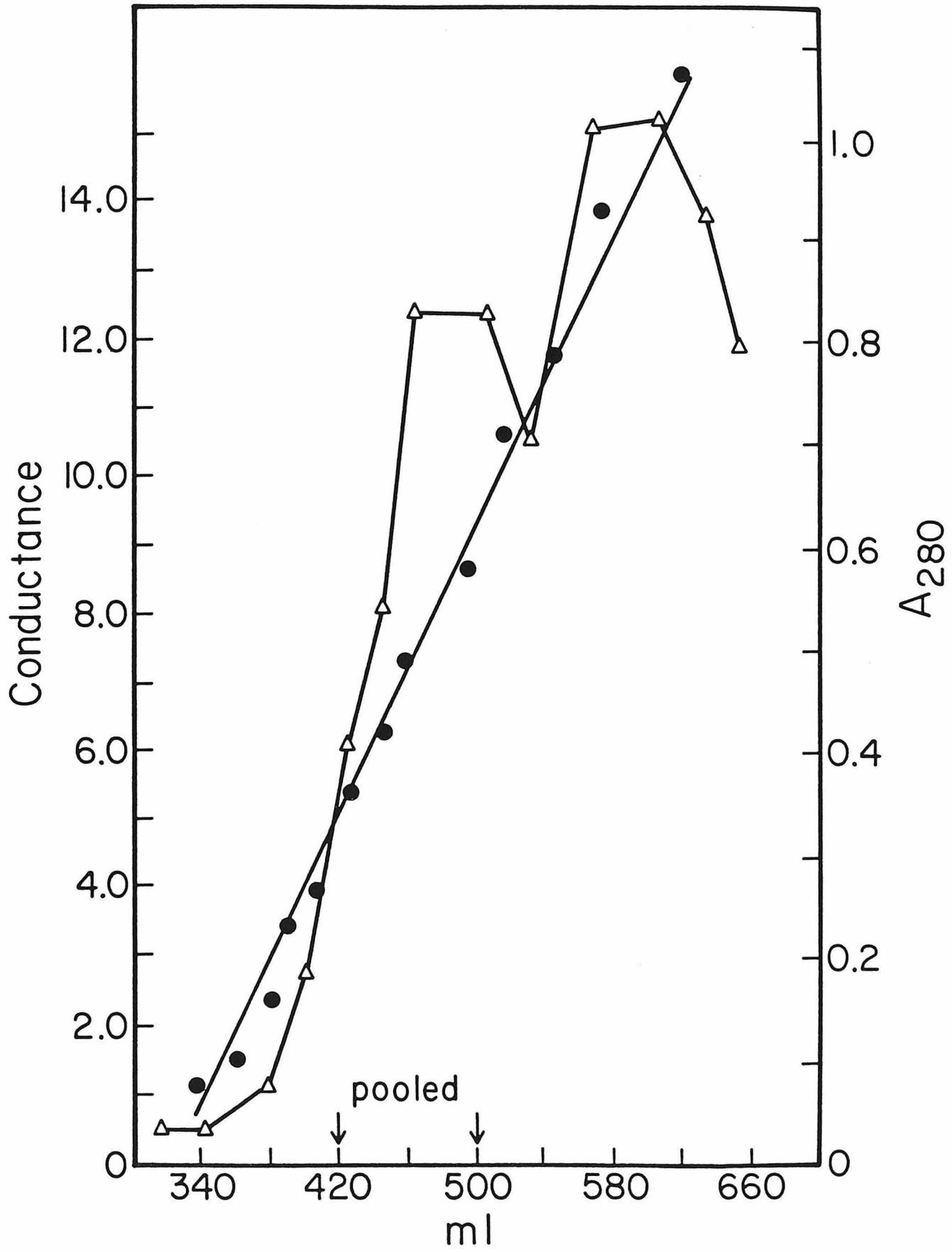

Conductance
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
1.0
0.8
0.6
0.4
0.2
0
A_{280}
pooled
340
420
500
580
660
ml

FIGURE V

COMPLEMENT FIXATION OF IgG_{2a}

□: purified by $(NH_4)_2SO_4$ precipitation method

●: purified by the affinity column

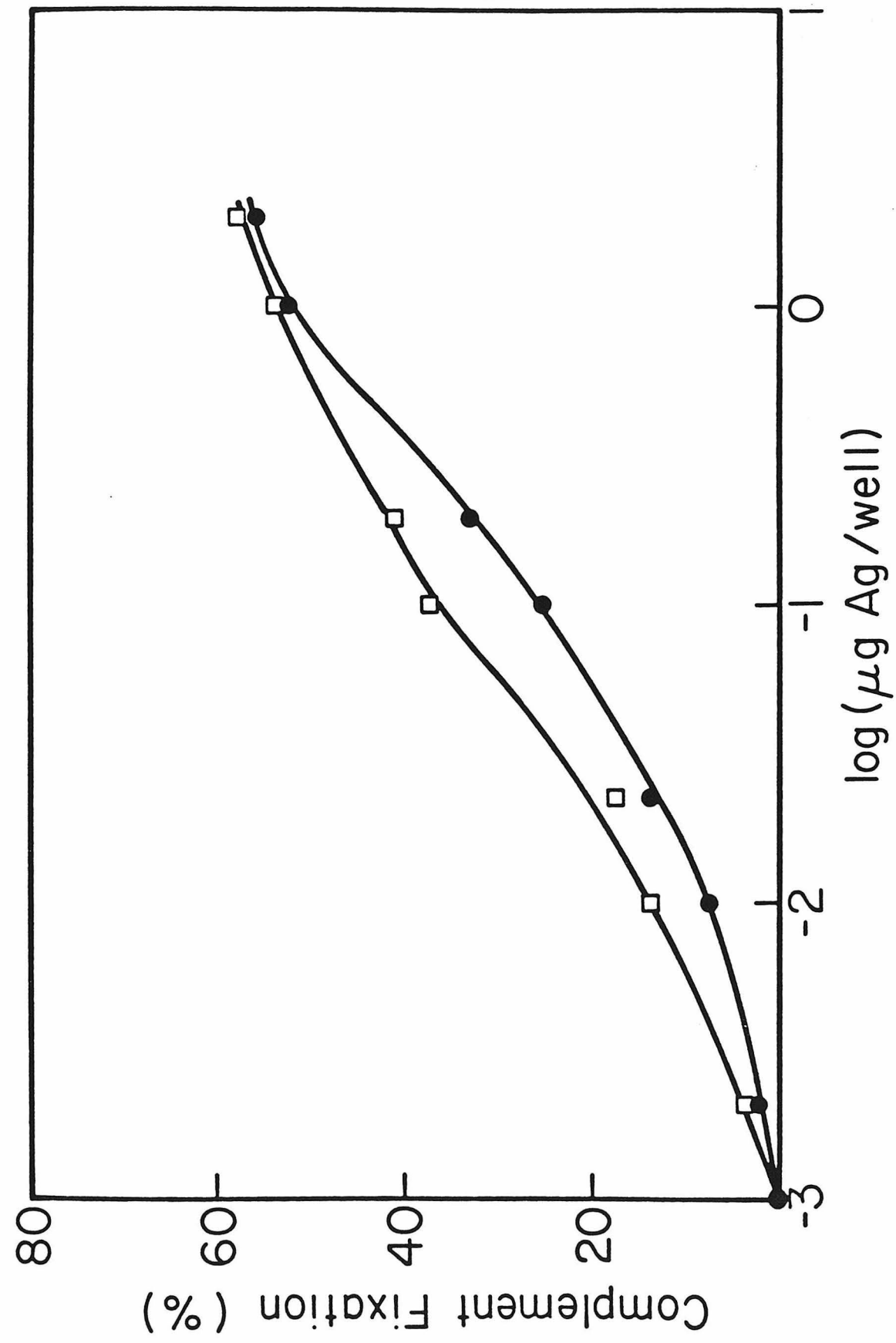

Complement Fixation (%)
80
60
40
20
-3
-2
-1
0
log (μg Ag/well)

FIGURE VI

THE SPECTRA OF γ-^{13}C-Trp ENRICHED IgG$_{2a}$ AND ITS Fab FRAGMENT AT 125 M Hz

Upper spectrum : γ-^{13}C-Trp enriched IgG$_{2a}$ (0.8 mM) 55,129 transients at 125 M Hz.

Lower spectrum : Fab fragment of γ-^{13}C-Trp enriched IgG$_{2a}$ (0.65 mM) 85,090 transients at 125 M Hz.

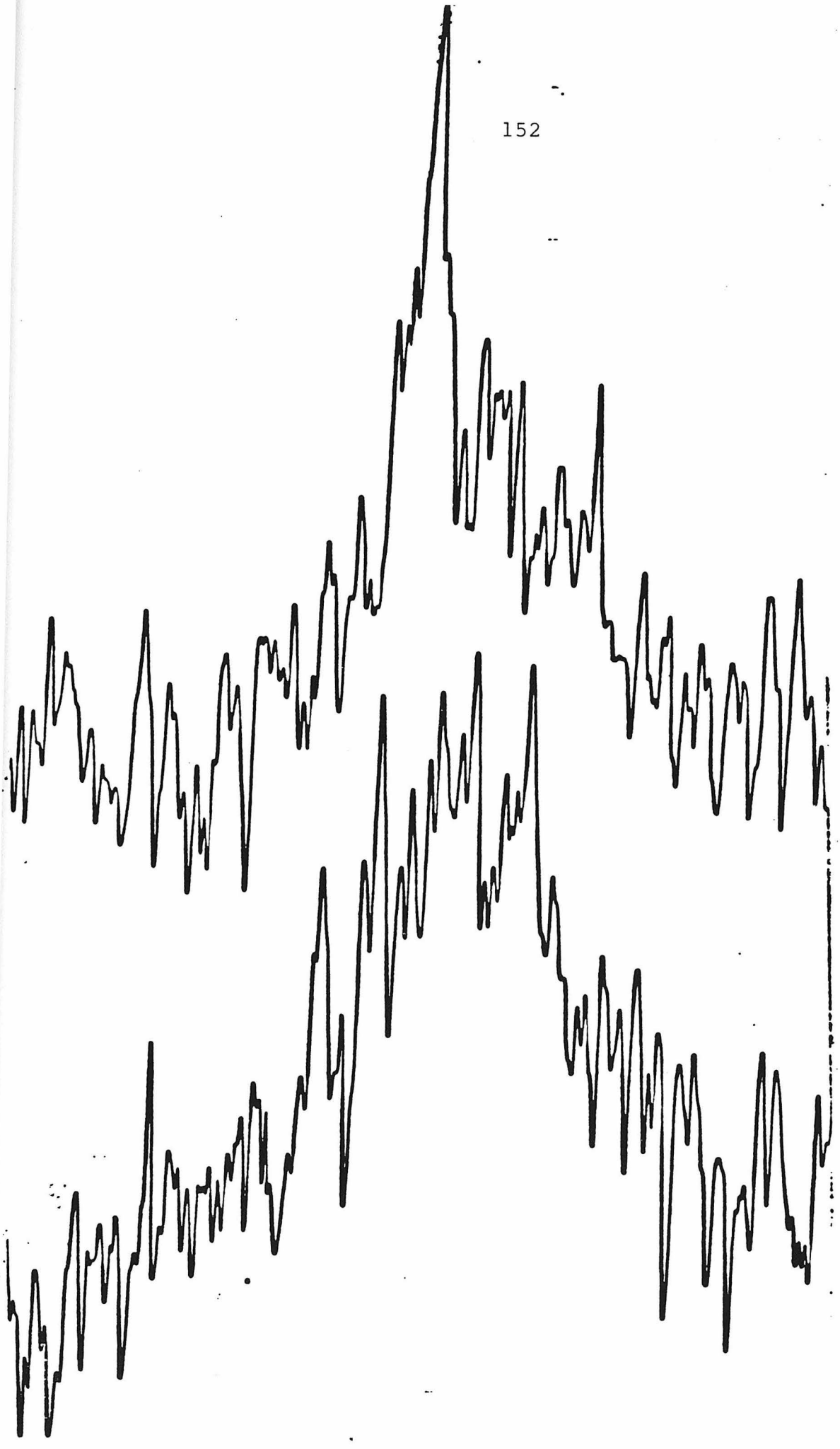


FIGURE VII

THE DIFFERENCE SPECTRUM OF INTACT IgG_{2a}
AND ITS Fab FRAGMENT

Upper spectrum : The difference spectrum of those two spectra shown in Figure VI.

Lower spectrum : γ- ^{13}C-Trp enriched IgG_{2a} 55,129 transients at 125 MHZ.

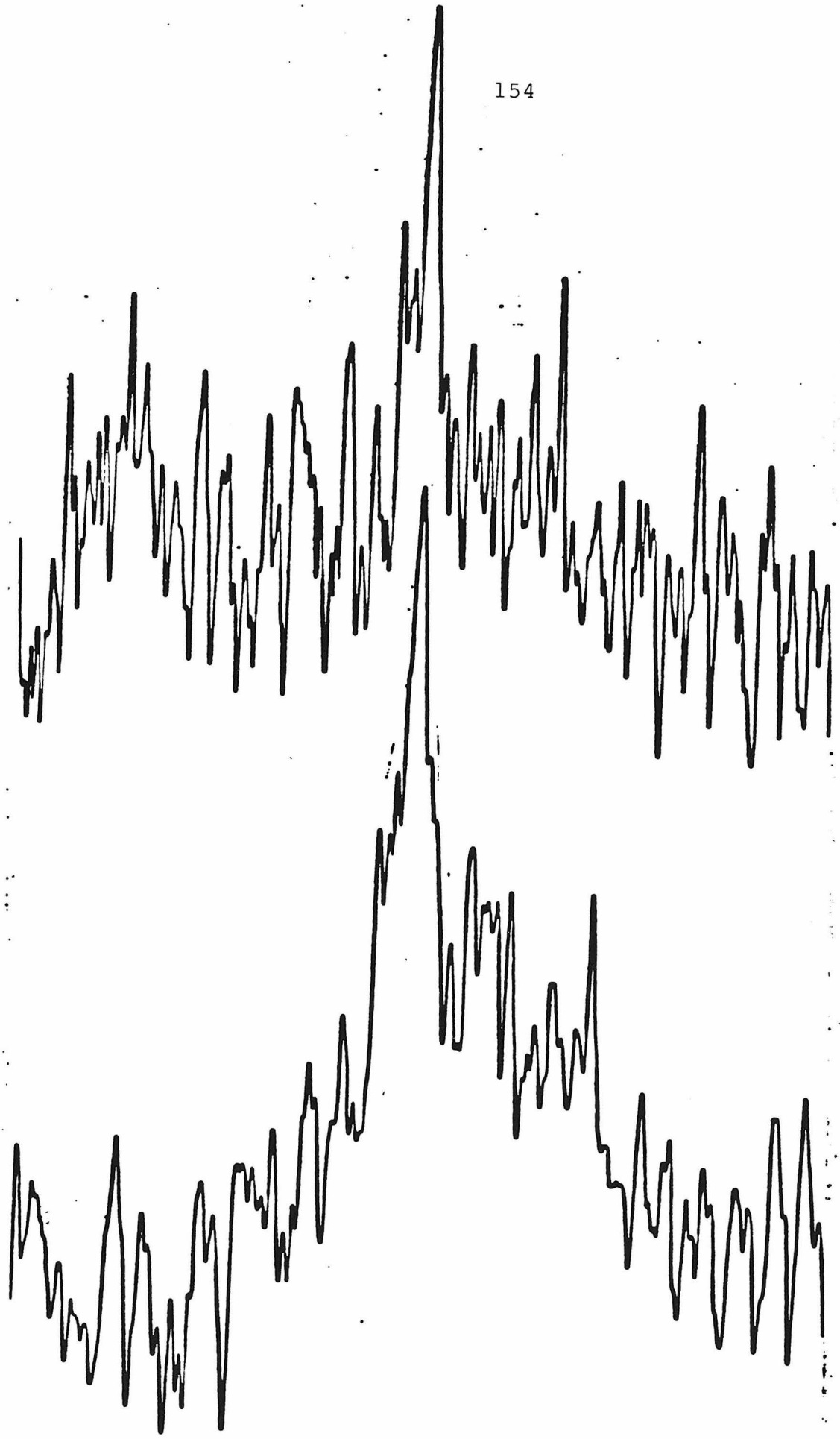
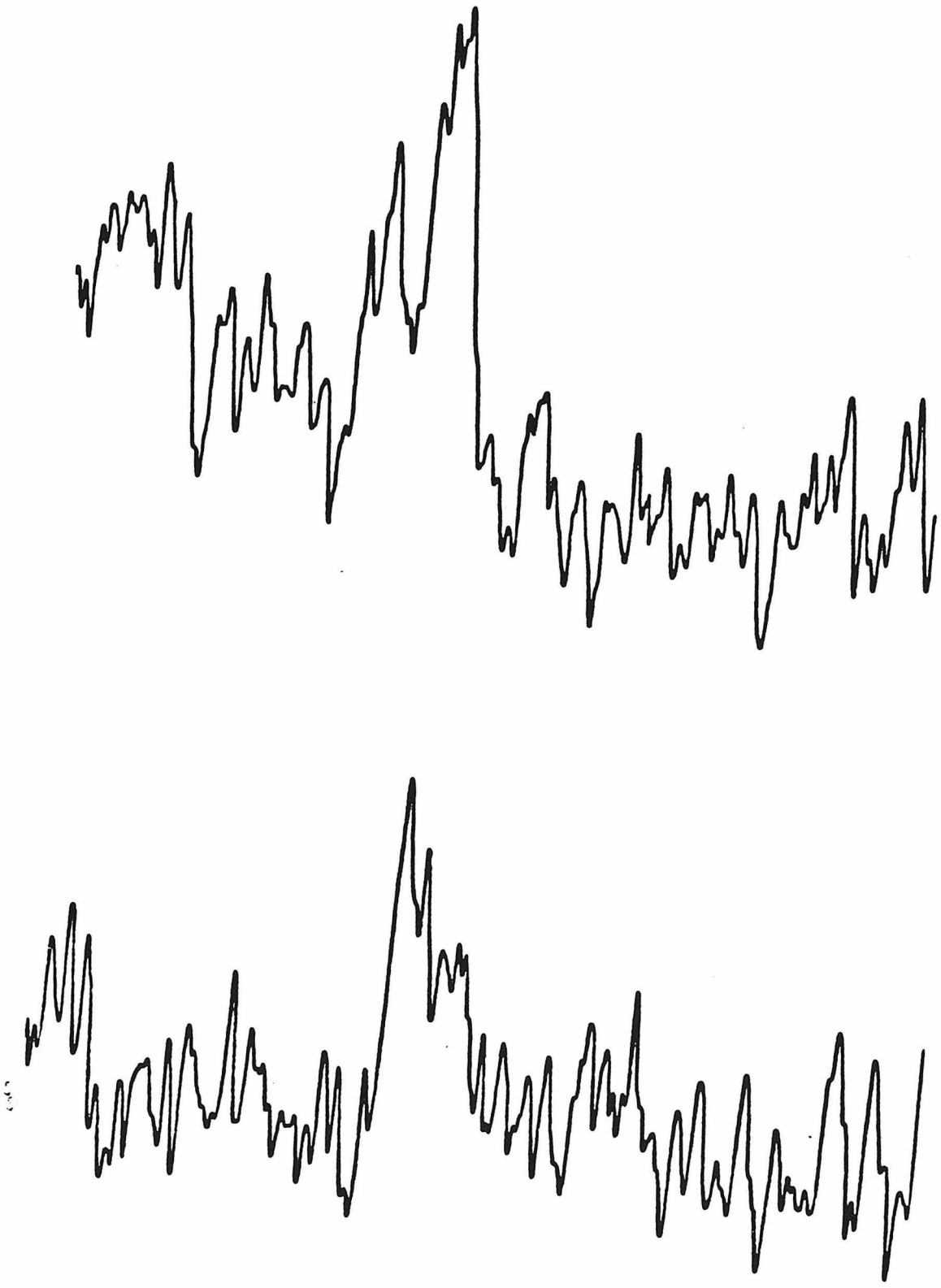


FIGURE VIII

THE SPECTRA OF γ_{2a}-^{13}C-Trp ENRICHED IgG AND ITS Fab FRAGMENT AT 50 M Hz

Upper spectrum : γ_{2a}-^{13}C-Trp enriched IgG (0.8 mM) 222,885 transients at 50 M Hz.

Lower spectrum : Fab fragment of γ_{2a}-^{13}C-Trp enriched IgG (0.65 mM) 630,531 transients at 50 M Hz.



PROPOSITION I

STUDIES OF THE MECHANISM OF NUCLEAR ACCUMULATION OF PROTEINS

The nuclear and cytoplasmic compartments of eukaryotic cells contain large distinct sets of proteins (1, 2). Studies of the fate of endogenously synthesized and exogenously introduced proteins suggest that all nuclear proteins are synthesized in the cytoplasm, from which the mature polypeptides migrate rapidly to the nucleus (3, 4, 5, 6). Thus the steady-state segregation of the proteins between nucleus and cytoplasm appears to be governed by an intrinsic property of mature polypeptides. It is not clear how the structures of proteins specify their distribution between the two compartments.

Several models have been commonly used to explain the nuclear accumulation of certain proteins (7, 8, 9, 10). The first envisages the free diffusion of all proteins into the nucleus with the subsequent retention of nuclear proteins by their binding to nondiffusible nuclear elements. The second model suggests that the accumulation of nuclear proteins is due to their selective transport across the nuclear envelope. The third model suggests that both selective transport and selective binding play a role in nuclear accumulation. This model also suggests that it is possible that these two activities are controlled by different regions of the protein.

Since nuclear accumulation is selective and occurs posttranslationally, it has been proposed that nuclear proteins contain within their mature molecular structure a

signal sequence that controls their accumulation (2, 3, 7). In the first model, this signal would be responsible for the nuclear retention of the protein, while in the second model its interaction with the nuclear envelope would allow selective transport of the protein into the nucleus. Experimental evidence, however, suggests that neither the first model nor the second model is wholly correct. If all proteins entered nuclei by free diffusion, then assuming a functional nuclear pore radius of 45 Å for the Xenopus oocyte nucleus (11), one would predict that, for these nuclei, proteins with mol. wt. larger than 70,000 daltons would be excluded and proteins smaller than this would enter freely. However, many nuclear proteins that are considerably larger than 70,000 daltons can enter nuclei at a rate faster than predicted (1, 3). Evidence against protein accumulation by selective transport across the nuclear envelope is based on experiments which show that the puncture or removal of the nuclear envelope has little effect on the ability of the nucleus to accumulate nuclear proteins (8, 11).

Recent studies favor the third model (10, 12). The sequence responsible for the nuclear accumulation of the influenza virus nucleoprotein in Xenopus oocytes has been identified (10). When the globin cDNA is fused to a portion of cDNA of NP including the region encoding amino acids 327-345, the resulting fusion protien enters and

accumulates in the nucleus. Fusion proteins lacking this region of the NP enter but do not accumulate in the nucleus. Despite these findings, they have not identified another signal peptide that is responsible for nuclear entry of the NP.

Furthermore, a short sequence of amino acids including Lys-128 has been demonstrated to be required for nuclear accumulation of SV40 large T antigen (12). The implication that the sequence element around Lys-128 acts as an autonomous signal capable of specifying nuclear location has been tested directly by transferring it to the amino termini of beta-galactosidase and of pyruvate kinase. They are normally cytoplasmic proteins. Sequences that include the putative signal can induce each of the fusion proteins to accumulate completely in the nucleus. However, due to the absence of the kinetic data of nuclear entry of these fusion proteins, it is still unclear whether this sequence is responsible for either nuclear entry or specific binding to nondiffusible nuclear elements, or it is responsible for both.

To further study whether both selective transport and selective binding play a role in nuclear accumulation and whether these two activities are controlled by different regions of the proteins, the following experiments are proposed :

It has been demonstrated that Pro-Lys-Lys-Lys-Arg-Lys-

Val can act as a nuclear location signal (12). However, it is still unclear whether this sequence is responsible only for nuclear entry. This can be studied by first isolating the fusion protein of beta-galactosidase and this signal sequence that has been produced by E. coli, then microinjecting the isolated fusion protein into the cytoplasm of Xenopus oocytes and finally detecting the rate of nuclear accumulation of the injected fusion protein.

If this fusion protein accumulates in the nucleus of Xenopus oocytes much faster than the intact beta-galactosidase, we can conclude that this signal sequence is responsible for nuclear entry. However, this result still can't tell us whether this signal sequence is also responsible for selective binding in the nucleus of Xenopus oocytes because of the large size of the fusion proteins that could not diffuse out of the nucleus once they entered. To further study this question, we can make a series of deletions of the gene encoding the fusion protein. We can then obtain a series of fusion proteins that contain different parts of beta-galactosidase but the same signal peptide.

With these fusion proteins, we can study the rate of their entry into the nucleus of Xenopus oocytes as described above. The results obtained from this study may provide evidence for the effects of the conformation and the size of the fusion proteins on the rate of their nuclei

entry. With these fusion proteins we can also study the effects of the size of the fusion proteins on the amount of their nuclear accumulation. The results obtained from this study may provide evidence for the role of the single sequence in specific binding in the nucleus of Xenopus oocytes.

As I described at the beginning, it has also been demonstrated that a short amino acid sequence from 327 to 345 is responsible for nuclear accumulation of the influenza virus nucleoprotein (NP) in Xenopus oocytes (10). It has been suggested that this sequence is responsible for the specific binding to nondiffusionable elements in the nucleus of Xenopus oocytes. We can use the same approach I proposed above for studying whether this sequence is responsible for specific binding alone. First, we can fuse the gene of beta-galactosidase with that encoding the amino acid sequence from 327 to 345 of the influenza virus nucleoprotein at either the 5'-end or the 3'-end. The fusion proteins can be produced by E. coli, then be isolated and microinjected into the cytoplasm of Xenupos oocytes. If this sequence is responsible for specific binding to the nucleus elements alone, these fusion proteins will not be able to enter the nucleus because of their large size. We can then make a series of deletion of the genes encoding the fusion proteins. After these fusion proteins of different sizes were isolated and microinjected

into the cytoplasm of the Xenopus oocytes, the rate of their nuclear entry and the amounts of their nuclear accumulation can then be studied. The results obtained from this study may provide evidence for the role of this specific sequence in nuclear entry and nuclear accumulation.

Finally, we can construct a series fusion proteins that contain parts of beta-galactosidase, the signal sequence, Pro-Lys-Lys-Lys-Arg-Lys-Val, and the amino acid sequence from 327 to 345 of the NP. Again, we can study the rate of nuclear entry and the amount of nuclear accumulation of these fusion proteins using the same approach as I decribed above. Combining all the results obtained from these studies, we may gain further insights into the ideas that both selective transport and selective binding play a role in nuclear accumulation and that these two activities may be controlled by different regions of nucleoproteins.

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PROPOSITION II

THE RELATIONSHIPS BETWEEN THE HINGE REGION AND THE BIOLOGICAL FUNCTIONS OF ANTIBODIES

IgG is a divalent molecule of mol. wt. 150,000. Antigen is recognized by sites on the Fab domain of the molecule. A number of molecules involved in triggering effector functions, however, interact with sites on the Fc domain of the molecule. These include complement subcomponent Clq, Cls, complement component C4 and Fc receptor molecules (1). It is generally agreed that Clq interacts with the C2 domain of IgG, but the precise location of the Clq-binding site is still a matter of some controversy (2). Complement subcomponent Cls is thought to occupy the extremities of a very extended $Cl\gamma_2$ Cls molecule, and its interaction with a site on IgG has still to be definitively demonstrated (3). Activated complement component C4b is thought to form a covalent linkage to a lysine on the Fab arms of IgG, possibly in the V_γ domain (2). Activated C3b forms a covalent linkage to a residue in the V_γ or $C_\gamma 1$ domain (4). The involvement of both $C_\gamma 2$ and $C_\gamma 3$ domains in the interaction with Fc receptor molecules have been claimed (5). The extent to which the receptor molecules from different cell types are overlapping or identical sites on IgG is unkown.

The molecular structure of IgG is now known in some detail. High-resolution crystal structures are available for pooled human Fc (6), rabbit Fc (7), guinea pig pFc (8), human Fab New (9) and Kol (10). Due to the flexibility of

the hinge region (1), complete structures are available only for the hinge-deleted IgG, proteins Dob (11) and Mcg (12). The lack of information on the relative dispositions of Fab and Fc in an intact IgG molecule, which may be very important in revealing or masking binding sites, is probably the most serious gap in our understanding of IgG structure.

Although there are probably no functional binding sites in the hinge region, this structure unit seems to be very important in modulating such sites existing in other structural domains of antibodies. Because of the lack of information on the complete three-dimensional arrangement of the heavy-chain residues between Fab and Fc for even a single hinge conformation, we do not know with any certainty the pivotal area about which Fab-Fc relative movement occurs or the degree of such movement allowed in a given case. These factors may be crucial in modulation of functional sites (1).

It is well known that the isotypes shown in Table 1 can express different biological activities (14, 15). The human IgG1 and IgG3 are more effective at activating complement and binding to cell receptors than IgG2 and IgG4; mouse IgG1 and guinea pig IgG1 are much less effective in activating complement than mouse IgG_{2a}, mouse IgG_{2b} or rabbit IgG [reviewed in (13) and (14)]. The idea that the hinge can modulate biological activity comes from

a combination of observations. First, sequence differences in the $C_\gamma 2$ or $C_\gamma 3$ domains have not been found to explain the subclass specificities described above. Second, the greatest differences between the subclasses are located in the hinge. Third, hinge-deleted proteins tend to show diminished biological activities [reviewed in (5)]. Here, to obtain convincing evidence for the view that the hinge region can modulate the biological functions of antibodies, I propose to generate a series of systematically designed mutant IgGs that contain exactly the same Fab and Fc domains but different hinge structures.

With the advent of the development of hybridoma and recombinant DNA technology, we can now manipulate immunoglobin genes in vitro and then transfect them into lymphoid cells (18). The transfected genes can be faithfully expressed, and assembly can occur between the transfected and endogenous chains or between two transfected chains (5). Once the immunoglobulin genes have been cloned in a suitable vector, one can replace any specific fragment of the cloned gene with another designed synthetic oligonucleotide duplex when suitable endonuclease restriction sites are available. Thus, we can generate a series of chimeric genes encoding chimeric antibodies that contain exactly the same Fab and Fc domains but different hinge structures.

The most commonly used vectors have been the pSV2

vectors (15), in which the selectable marker is placed under the control of the SV40 early promoter; SV40 sequences provide slice signals and a polyadenylation site. Two selectable bacterial genes have been used : (i) the xanthine-guanine phosphoribosyltransferase gene (gpt) (16), and (ii) the phosphotransferase gene from Tn5 (designated neo) (17). The recipient cell type chosen for the production of Ig molecules would appear to be myeloma cells. Once these designed chimeric genes have been successfully transfected into the myeloma cells, these designed chimeric antibodies can then be isolated either from tissue culture or from the ascites of tumor-bearing mice.

The gene encoding the γ_{2b} heavy chain of the myeloma MPC-11 has been successfully transfected into the myeloma J558 (19, 20). This γ_{2b} heavy chain has been expressed, assembled with the endogenous chain, and secreted as $H_2 L_2$ molecules (19, 20). By the use of cassette mutagenesis, the gene segment encoding the hinge sequence of the 2b heavy chain can be replaced by different gene segments encoding the hinge sequences of human γ_1, γ_2, γ_4, mouse γ_1, γ_{2a} as shown in Table 1. After these designed chimeric genes in the pSV40 vector have been transfected into the myeloma J558 cells and the myeloma J558 cells containing the pSV40 vector can be selected as described (16, 17), the chimeric antibodies can then be isolated either from the

tissue culture of the selected myeloma J558 cells or from the mice bearing the tumor of the selected myeloma J558. Finally, the effectiveness of these chimeric antibodies at activating complement can be assayed.

This study may provide evidence for or against the view that the hinge structure can modulate the biological functions of antibodies. Furthermore, the flexibility of these chimeric antibodies can be measured by the use of nanosecond fluorescence (21, 22). This further study may provide us insights into the relationships between the flexibility and the biological functions of antibodies.

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TABLE I

A COMPARISON OF HINGE SEQUENCES

		216 238
Human	γ_1	E P K S C D K T H T . C P P . . . . . C P A P E L L G G P
Human	γ_2	E R K . . . . . C C V E C P P . . C P A P P V A G . P
Human	γ_4	E S K Y G . . . . P P C P P . . . . . C P A P E F L G G P
Mouse	γ_1	V P R D C G . . . . . C K P C I . . . . C T V P . . E . V S
Mouse	γ_{2a}	E P R G P T I K . . P C C P P K . . . . C P A P N L L G G P
Mouse	γ_{2b}	E P S G P I S T I N P C P P C K E C H K C P A P N L E G G P

↑ First S-S ↑ Last S-S

A dot indicates that the alignment chosen produces no residue corresponding to that position.

PROPOSITION III

E1A TRANSCRIPTION INDUCTION

The adenovirus E1A gene encodes products that function in the modulation of gene transcription initiation and in the oncogenic transformation of mammalian cells (1). At early times following viral infection, the human subgroup-C adenovirus (adenovirus type-2 and -5) E1A gene produces two mRNAs, designated 12S and 13S, which result from alternative RNA splicing of the primary transcript (2). A set of five viral promoters are coordinately regulated by these E1A gene products. In addition, two cellular promoters, hsp70 and beta-tubulin, are also stimulated by the action of these E1A gene products (3, 4, 5). Recently, the mechanism for this transcriptional induction has been investigated with an in vivo exoIII mapping technique to assay for proteins that interact with an E1A-inducible promoter (6). A protein bound to the early E2 promoter has been detected in wild-type infected cells. In the absence of E1A induction, specific interactions at the promoter could not be detected. However, if conditions were established that allowed transcription of the E2 gene in the absence of E1A, the same exoIII protection was observed as was found in the presence of E1A, suggesting a model in which the efficient utilization of the E2 promoter is mediated by a cellular transcription factor. Despite this finding, the precise mechanism by which the complexes of the cellular transcription factor and the E1A gene products activate the transcription of the E2 gene remains

unknown.

Many regulatory proteins have been found. These proteins bind at specific DAN site and influences the transcription of a gene hundreds or even thousands of base pairs away (7). Recent experiments suggest a unified view of these apparently disparate types of gene regulation. First, regulatory proteins recognized specific sequences in DNA using structures that are complementary to the ordinary helix. Second, DNA-bound regulatory proteins influence transcription by excluding binding of other proteins or, more generally, by touching another DNA-bound protein (7). Under the assumption that this view can be applied on the complexes of the cellular transcription factor and the E1A gene products, I postulate that in the complexes, the cellular transcription factor could play a role in DNA binding, and the E1A gene products could play a role in the interaction with another protein,the RNA polymerase. These complexes can thus activate the transcription of certain genes that are under the control of the promoters recognized by the cellular transcription factor.

It has been demonstrated that E. coli expressed E1A proteins will undergo a posttranslational modification and then function normally in microinjected Xenopus oocytes (8). It has also been demonstrated that the DNA binding function of GAL4, a Saccharomyces cerevisial transcriptional activator, can be replaced with that of a

prokaryotic repressor without loss of the transcriptional activation function (9). This is done by the construction of a hybrid protein of GAL4 and Lex A, an E. coli repressor protein. The hybrid protein, synthesized in yeast, activates transcription of a gene if and only if a Lex A operator is present near the transcription start site, leading to the conclusion described above. Similarly, to test the postulation that the E1A gene products could play a role in the interaction with the RNA polymerase, we can construct a gene-encoding hybrid protein of Lex A and an E1A gene product using pBR322 plasmid. The hybrid protein can then be isolated in E. coli and microinjected into the cytoplasm of Xenopus oocytes that have already been microinjected with a chimeric gene (Lex A-CAT) containing the Lex A operator linked to the CAT coding sequence (8). The level of CAT enzyme activity can then be messured (8). If this hybrid protein can activate the transcription of a gene only when a Lex A operator is present near its transcription start site, we can then conclude that the E1A gene product indeed plays a role in the interaction with another protein, most likely to be the RNA polymerase. In other words, these proposed experiments may provide us valuable evidence for the view that the E1A gene products can activate the transcription of specific genes by contacting the RNA polymerase after they bind to the cellular transcription factor.

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PROPOSITION IV

STUDIES OF THE STRUCTURE-FUNCTION RELATIONSHIPS BETWEEN CLASS A BETA-LACTAMASES AND CLASS C BETA-LACTAMASES BY MUTAGENESIS

Beta-lactamases can catalyze the hydrolysis of the amide bond in the beta-lactam ring of penams and cephems. These enzymes have been classified into three groups, class A, B and C. Class A beta-lactamase that has molecular weights of around 29,000 show significant amino acid sequence homology among themselves (1, 2), and preferentially hydrolyze penam antibiotics. Class B beta-lactamase is a metalloenzyme of molecular weight 23,000, which attacks cepham antibiotics and is produced only by Baccilus cereus (1). Class C beta-lactamases, however, are chromosomally determined cephalosporinases of E. coli. They are larger proteins (mol wt. 39,000).

It has been proposed that these beta-lactamases may be evolutionarily related to D,D-carboxypeptidases that are involved in the peptidoglycan cross-linking in cell wall synthesis (3). Recent discoveries of the significant similarity between the three-dimensional structures of R61 D,D-carboxypeptidase and the class A beta-lactamases of B. licheniformis 749/C (4), as well as B. cereus 591 (5), strongly support the hypothesis described above. In Chapter III of this thesis, I further studied the structure-function relationships between beta-lactamases and D,D-caroxypeptidases by the construction of a hybrid protein of RTEM-1 beta-lactamase and PBP-5 of E. coli. This study not only provides an evidence which further supports the hypothesis described above but also

demonstrates that by the construction of a hybrid protein one can create a new enzyme with desired catalytic functions. To further demonstrate the power of this approach, more evidence needs to be accumulated.

As I described above, class A beta-lactamases preferentially hydrolyze penam antibiotics. Class C beta-lactamases, however, preferentially hydrolyze cephem antibiotics. For class A beta-lactamases, both acylation by benzylpenicillin and deacylation are rapid. For both class C beta-lactamase and certain D,D-carboxypeptidases, acylation is more than a million times faster than deacylation, and the acyl enzyme accumulates. Despite these differences, both class A beta-lactamases and class C beta-lactamases possess the important Phe-X-X-X-Ser-X-X-Lys sequence and catalyze cephalosporin via an acyl enzyme intermediate (6). Possibly, these two groups of enzymes may share a common evolutionary origin. It has also been suggested that the class C beta-lactamases may be expected to share some or all of the tertiary structural features that have been found to be common to the class A beta-lactamases and the R61-CPase (5). So, it is worthy of constructing a hybrid protein of class A and class C beta-lactamase to further demonstrate the power of the approach and to study the structure-function relationships between these two proteins.

I propose to replace the polypeptide of 29 amino acids

of RTEM-1 beta-lactamase, including the active site Ser, with the corresponding polypeptide of ampC cephalosporinase of E. coli as shown in Table I. The reason for making this substitution is that this polypeptide contains most of the amino acid residues potentially able to contact the substrates. After the gene encoding the hybrid protein has been successfully constructed as shown in Figure 1, it can then be cloned into pBR322 for checking the resistance of the cells containing the hybrid gene to cephem antibiotics.

The in vivo stability of this hybrid protein can also be checked by western blot. The hybrid proteins constructed in this way are usually not very stable. The instability is possibly due to a bad interaction between their second structures (7 ,8). This problem may be overcome by making further mutations in the hybrid protein. Since it would be difficult to predict what mutations we should make at this point, random mutagenesis followed by selection of desired mutants on agar plates containing different concentrations may be a better approach than site-specific mutagenesis for obtaining more stable or more active mutants of the hybrid protein. These procedures of mutation followed by selection can be done repeatedly until colonies displaying satisfactory resistant phenotypes against cephem antibiotics have been obtained. By comparing the catalytic parameters of these mutants of the

hybrid proteins with the differences in the amino acid sequence of these mutant proteins, we may obtain insights into the structure-function relationships between class A beta-lactamase and class C beta-lactamase. This study may also provide further evidence for the view that construction of hybrid proteins may create new enzymes with desired catalytic functions.

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FIGURE I

THE SCHEME FOR THE CONSTRUCTION OF THE HYBRID GENE

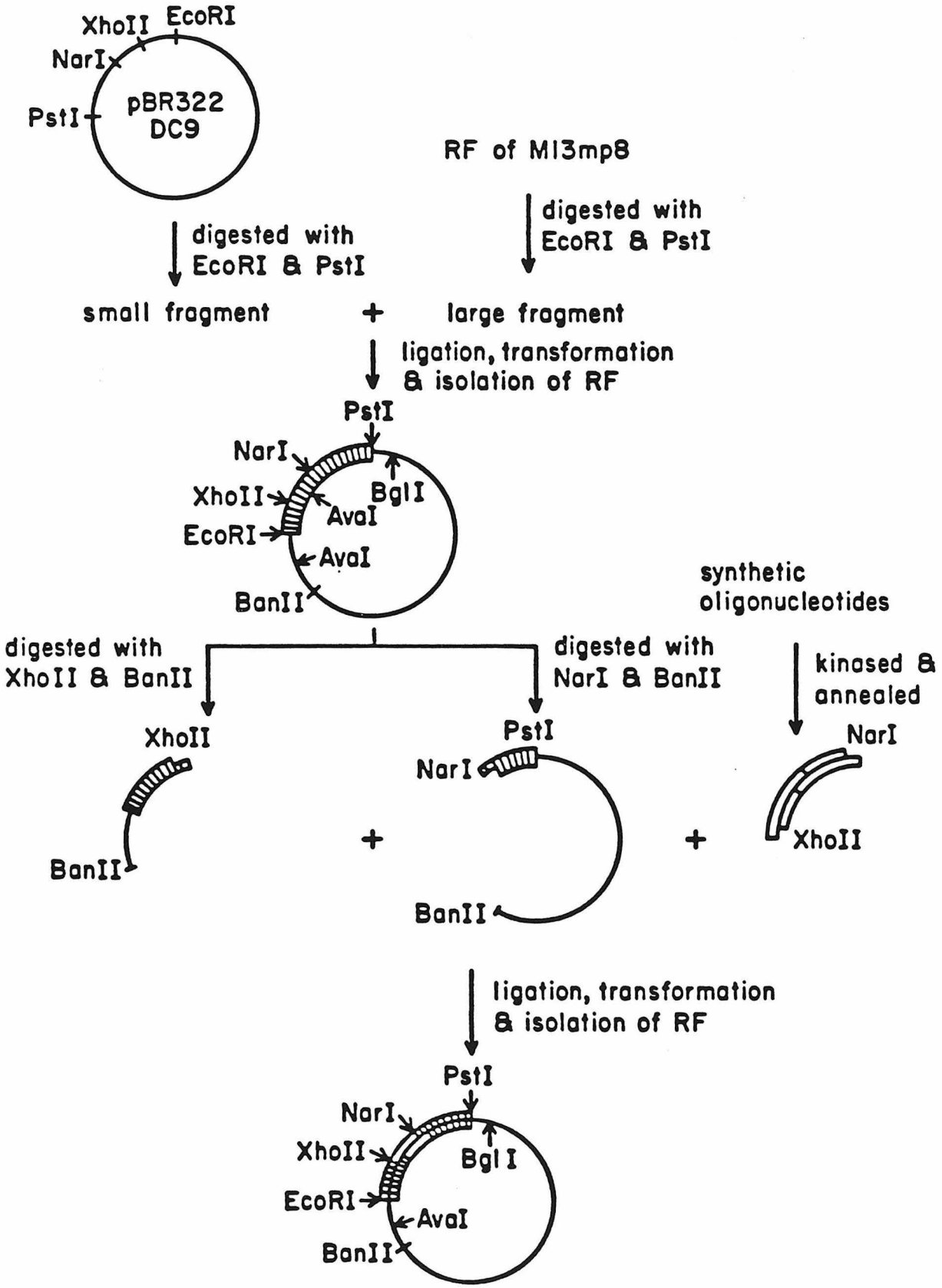
XhoII
EcoRI
NarI
PstI
pBR322
DC9
RF of M13mp8
digested with
EcoRI & PstI
digested with
EcoRI & PstI
small fragment
+
large fragment
ligation, transformation
& isolation of RF
PstI
NarI
XhoII
EcoRI
BglI
AvaI
AvaI
BanII
synthetic
oligonucleotides
digested with
XhoII & BanII
digested with
NarI & BanII
kinased &
annealed
XhoII
BanII
PstI
NarI
BanII
+
+
NarI
XhoII
ligation, transformation
& isolation of RF
PstI
NarI
XhoII
EcoRI
BglI
AvaI
BanII

TABLE I

A. - d l n s q k i l e s f r p e e r F p m m Ś t f K v l l c g -

B. - g y a d i a k k q p v t q q t l F e l g Ś v s K t f t g v -

A. The amino acid sequence of RTEM-1 beta-lactamase which I proposed be replaced.

B. The amino acid sequence of ampC cephalosporinase of E. coli which I proposed be substituted into RTEM-1 beta-lactamase.

*. The active site Ser.

PROPOSITION V

BIOLOGICAL FUNCTIONS OF N-ACETYLTRANSFERASES

N-terminal blocking of proteins is a widespread phenomenon in eukaryotes, prokaryotes and viruses (1, 2, 3). It has been found that about 80% of the soluble proteins from Ehrlich ascites cells are N-acetylated (4) and approximately 90% of the proteins from mouse L cells seem, also, to be N-acetylated (5). Furthermore, in lower eukaryotic organisms, about 50% of the soluble proteins are acetylated (6).

The biological significance of N-acetylation of proteins is still an open question. It has been suggested that a general function for N-acetylation is the protection of proteins from proteolytic degradation by aminopeptidases (7). However, experiments to sustain this hypothesis have not shown significant differences in the turnover rate of acetylated and nonacetylated forms of feline beta-globin and of proteins from mouse L cells (5). The possibility that N-acetylation plays a role in protein secretion has also been suggested (8). The recent result from DNA sequencing has shown that in structural genes, encoding the secretory proteins that are N-acetylated, the codon for the acetylated amino-terminal residue is directly preceded by the initiation codon without the insertion of additional codons for amino acids (9). However, little effort has been made to understand the relationship between N-acetylation and transport of secretory proteins.

The functional role of the N-acetyl group in beta-

endorphin and alpha-melanotropin is more obvious in directly affecting biological activity : in contrast to N-unacetylated native beta-endorphin, the acetylated peptide is nonanalgesic and cannot be bound to opiate receptors in vitro (10). Conversely, native alpha-melanocyte-stimulating hormone (alpha-MSH) is more potent than the N-deacetylated peptide in causing melanocyte dispersion (11).

N-acetylation is an enzyme-catalyzed reaction in which the protein accepts the acetyl group from acetyl-CoA. The N-acetyltransferases have been found in various cells and tissues such as rabbit reticulocytes (12), rat liver (13, 14), rat pituitary (15, 16, 17), calf lens (18), and wheat germs (19). The nature of these N-acetyltransferases is still unknown. This is due to their low concentration and extreme instability after purification.

To facilitate the study of the biological significance of N-acetylation of proteins, it is important to clone the genes encoding these enzymes. After a small part of the amino acid sequences of one N-acetyltransferase is known, we can synthesize a mixed oligonucleotide probe that contains all possible DNA sequences encoding the known amino acid sequence. Using the mixed probe, we can then screen for clones containing a fragment of the gene encoding the N-acetyltransferase from the cDNA library. Combining the sequencing data and the restriction maps of those positive clones, we can obtain the whole DNA sequence

encoding the enzyme. Here, I proposed a series of experiments as a systematic approach for studying the biological functions of the N-acetyltransferase whose gene has been cloned.

The baker's yeast Saccharomyces cerevisiae is one of the most suitable systems for initiating this study because its genome is small, its generation time is short and it can be experimentally manipulated as easily as most prokaryotes.

Once a gene of N-acetyltransferase in yeast is cloned, we will know whether there is only one N-acetyltransferase or whether there is a group of N-acetyltransferases in yeast reponsible for the acetylation reaction of proteins. This can be done easily by screening the whole genome library by the use of ^{32}P-labeled fragments of the cloned gene followed by checking the restriction maps of the positive colonies and finally, by sequencing the DNA fragments.

When there is only one single copy of the gene encoding the N-acetyltransferase in the yeast genome, we can study the essentiality of this gene using the technique of one-step gene disruption as shown in Figure 1 (20). The cloned DNA fragment containing the gene, designated GENE NAT, which encodes the N-acetyltransferase, is digested with a restriction enzyme that cleaves within the DNA sequence of the GENE NAT. Another DNA fragment containing the HIS 3

yeast gene is cloned into the cleaved GENE NAT. The *in vitro* disrupted gene can be liberated from the bacterial plasmid sequences by restriction enzyme digestion. The digested DNA containing the disrupted gene can be transformed into a his 3$^-$, GENE NAT$^+$ yeast cell, and HIS 3$^+$ transformants are selected. Among the transformants are strains that simultaneously become HIS 3$^+$ and gene NAT$^-$. If we can't obtain any transformants that are HIS3$^+$ and NAT$^-$, we may conclude that the disruption of this gene is lethal to yeast cells. The essentiality of this gene can be further studied by first disrupting the gene in a diploid. Subsequent genetic analysis of the disrupted gene will reveal linkage of its lethal function to the selectable genetic marker used to disrupt the gene. If the disruption of this gene is indeed lethal to the yeast cells, the gene product, N-acetylatransferase, must be essential for the viability of the yeast cells. In other words, the N-acetylation of certain proteins is a very important modification process in these cells. If, however, the disruption of this gene is not lethal to the yeast cells, it is possible that the N-acetylation of certain proteins has no significant effect on the biological functions or on the *in vivo* turnover rates of these proteins.

To further study the biological function of N-acetylation of proteins, the following experiments are proposed. First, the GENE NAT can be cloned into a

suitable vector such as YEp13S(21). This vector is a derivative of the yeast plasmid 2-μm circle. In this vector, the LEU2 gene is used as the selectable marker and this GENE NAT can be placed under the control of a promoter such as USA_G (22). The transcription of the GENE NAT can thus be regulated by the protein GAL4 (22). Another gene encoding a protein that is known to be N-acetylated in yeast should also be cloned into the same vector and this gene should be expressed constitutively. The gene encoding alcohol dehydrogenase isozyme-II (23) can be a good candidate for this approach. The vector containing these two cloned genes can then be transformed into either a NAT^- $leu2^-$ $gal4^+$ yeast strain or a NAT^- leu^- gal^+ yeast strain (21).

Comparing the turnover rate of the alcohol dehydrogenase isozyme-II in the $gal4^+$ yeast strain in which the GENE NAT is fully expressed with that in the $gal4^-$ yeast strain in which the GENE NAT is only partially expressed, we may obtain valuable evidence for the effects of the N-acetylation of certain proteins on their <u>in vivo</u> stability in yeast cells.

If, however, there are multiple copies of the gene encoding this enzyme in the yeast genome, the technique of one-step gene disruption could no longer be used for studying the biological functions of N-acetyltransferases. An alternative approach is proposed below. We can

construct a vector that contains the gene that encodes alcohol dehydrogenase isoenzyme-II and the anti-sense gene of the GENE NAT. This anti-sense gene can be placed under the control of a regulatory promoter such as USA_G. Thus, the *in vivo* stability of the alcohol dehydrogenase isozyme II in the yeast cells that normally produce the N-acetyltransferase can be compared with that in the yeast cells that produce reduced amount of the N-acetyltransferase. This study may also provide valuable evidence for or against the view that N-acetylation of protein plays an important role in their *in vivo* stability.

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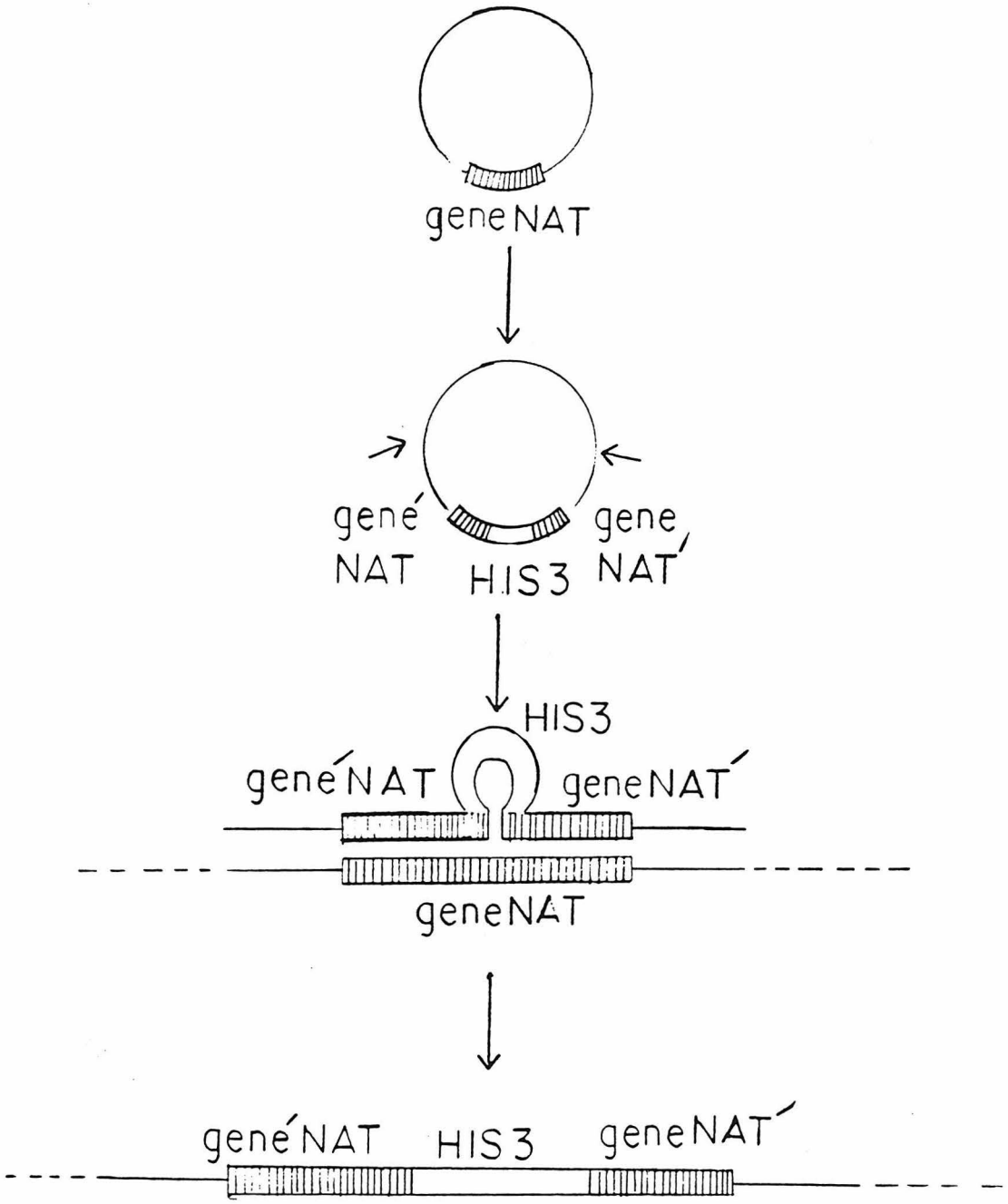
FIGURE I

THE SCHEME FOR ONE-STEP GENE DISRUPTION

The cloned fragment containing GENE NAT is digested with a restriction enzyme that cleaves within the GENE NAT sequence. A fragment containing a selectable yeast gene, HIS3, is cloned into the site. The fragment containing the disrupted GENE NAT is liberated from plasmid sequences, making certain that homology to the GENE NAT region remains on both sides of the insert. Transformation of yeast cells with the linear fragment results in the substitution of the linear disrupted sequence for the resident chromosomal sequence.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