THE CONSTRUCTION AND KINETIC ANALYSIS OF SPECIFIC MUTANTS OF ß-LACTAMASE

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Abstract

Mutants of RTEM-1 ß-lactamase, an enzyme responsible for bacterial resistance to ß-lactam antibiotics, have been constructed by cassette mutagenesis. ß-Lactamases have been classified into three groups based on catalytic and structural similarities. Class A ß-lactamases, of which the RTEM-1 enzyme is a member, maintain a conserved triad of amino acids about the active site serine, Ser-Thr-X-Lys, where X is not conserved. Endonuclease restriction sites flanking the codons of the active site residues were used to remove the portion of the ß-lactamase gene coding for the wild-type active site sequence. A duplex strand of synthetic oligonucleotides spanning the removed region of the gene and coding for the desired mutations was ligated in place of the wild-type sequence. Subsequent chemical sequencing of the mutated plasmids by the Maxam-Gilbert technique confirmed the presence of the mutations.

A mutant converting Thr71 to cysteine, designated Thr71 -->Cys, was constructed and shown to impart to cells a phenotype resistant to high levels of ampicillin (>350 mg/L). Purification of the mutant to homogeneity was accomplished using plasmid pJN which contains the ß-lactamase gene under control of the tac promoter and the gene for kanamycin resistance. Cells were grown at 37°C and induction was carried out at 30°C to reduce protein losses due to the thermal in-

stability of the mutant. The Thr71-->Cys mutant retains 12% of the efficiency of the wild-type enzyme on benzylpenicillin 3% on cephalothin, and 9% on 6-aminopenicillanic acid. The mutant has an altered pH activity profile, with activity declining more rapidly at alkaline pH than for the wild-type enzyme. The mutant is susceptible to inactivation by the sulfhydryl reagent pCMB under conditions where the activity of the wild-type enzyme is not affected. The mutant is inhibited competitively by borate ion. The Thr71-->Cys mutant is less stable to thermal denaturation than the wild-type enzyme, as are all other mutants at position 71.

The conserved lysine at position 73 of ß-lactamase was replaced with all other amino acids and an amber stop codon by cassette mutagenesis using synthetic oligonucleotides containing a mixture of bases in place of the lysine codon. After ligation the mixtures of plasmids were used to transform E. coli to tetracycline resistance. Plasmids derived from one hundred colonies were sequenced by the Maxam-Gilbert method. Because of an uneven mixture of bases in the mixed codon not all twenty amino acids were located in the first one hundred colonies. Mutants Lys73-->Asp, Glu and Pro were constructed separately using the same three fragment ligation scheme. None of the mutants at position 73 were able to confer to cells a phenotype resistant to even low levels of ampicillin. Western blot analysis of whole cell extracts revealed mutants with all nineteen amino acid replacements

present in amounts not significantly different from the amount of the wild-type enzyme.

Purification of mutants Lys73-->Arg and Cys was accomplished with the use of plasmid pJN and the tac promoter. Kinetic analysis revealed both mutants maintain 0.01% of the activity of the wild-type enzyme on benzylpenicillin. The Michaelis constant, K_M, was not significantly different from the value of the wild-type. The Lys73-->Cys mutant was slightly less stable to thermal denaturation than the wild-type enzyme. The pH-activity profile of the Lys73-->Cys mutant showed a maximum at pH 8.3 while the wild-type activity is maximal at pH 6.5. Treatment of the Lys73-->Cys mutant with ethylenimine, which converts cysteine to aminoethylcysteine, under mildly denaturing conditions resulted in a dramatic increase in specific activity of the mutant. Purification of the derivatized mutant by anion exchange on FPLC and kinetic analysis showed the derivatized mutant regained 62% of the catalytic efficiency of the wild-type enzyme.

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Nomenclature

The numbering system for ß-lactamase is that proposed by Ambler, (1980) Phil. Trans. Roy. Soc. London Ser. B 289, 321-331.

Expressions of the form, Lys73-->Cys, indicate a mutant in which the lysine residue at position 73 has been replaced by cysteine. Expressions of the form, pBR322K71C, indicate a plasmid in which the codon for the amino acid at the 73rd position of the protein, originally the codon for lysine, has been replaced by the codon for cysteine. The one letter notation used is the one 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; Y, tyrosine.

Abbreviations:

IPTG, isopropylthiogalactopuranoside; bp, basepairs; Tet^R, tetracycline resistant; Amp^R, ampicillin resistant; pCMB, parachloromercuribenzoate; DTT, dithiothreitol.

Chapter 1

Introduction

Applications of Oligonucleotide-directed Mutagenesis

The linear sequence of amino acids composing a protein determines the protein's three dimensional structure. The structure of a protein dictates its catalytic function. The contribution of individual amino acids to the overall structure and thus to the function of a protein is a subject of extreme importance and one that is currently under investigation in a wide variety of biochemical systems. Perturbations of the amino acid sequence by single site substitutions is one approach to assessing the importance of individual amino acid residues to the structure and function of an enzyme.

Studies of the relationship between the structure of a protein and its function have been greatly facilitated by the development of oligonucleotide-directed mutagenic techniques. These techniques allow the change of one or more specific amino acids of a protein to any other amino acid, a helpful addition to the classical method for obtaining mutants which consisted of screening for spontaneous variations in phenotype, and also of increasing the rate of variation by random mutagenesis. Differences in the functions of the wild-type and mutated proteins are attributed to the difference in the amino acid being studied. Thus, the role of a particular residue in a protein may be inferred by the study of one or more mutants at that site (for recent reviews see (1) and (2)).

Several general techniques have been developed for the

creation of site-specific mutations. The most widely used method involves a single-strand vector, generally M13 phage (3). Briefly, the part of the gene coding for the protein of interest and containing the site to be mutated is subcloned into one of the M13 cloning vehicles. Single strand phage DNA (ssDNA) is isolated. An oligonucleotide primer, typically 12 to 20 bases, containing one or two mismatches creating the mutation, is annealed to the ssDNA and the large (Klenow) fragment of DNA polymerase I is added and DNA polymerization is carried out. This gives rise to the double stranded, replicative form (RF) of the phage DNA which is used to transfect E. coli. The resulting plaques are screened for the presence of the mutated gene by one of several methods. The most widely used method involves screening by hybridization to the radioactive oligonucleotide primer. The melting temperature of the primer annealed to the mutated sequence will be distinguishably higher than that of primer annealed to the original, mismatched sequence. Putative mutations thus identified may be confirmed by DNA sequencing. Improvements designed to increase the frequency of mutation have been reported (4-7), and summarized (8,9).

Another, similar method requires the gene of interest be located on a double stranded vector (10,11). Generally, double-stranded vectors are the usual expression system and can reduce the frequency of unwanted, secondary mutations observed with single-stranded vectors (12). These methods in-

volve nicking the double-stranded vector with a restriction endonuclease or DNAse I at a site adjacent to the site to be mutated. The nicked circles are subjected to exonuclease digestion to create gapped duplexes. The mutagenic oligonucleotide is annealed and polymerization carried out. Transformation and screening are performed in a manner analogous to that for M13 mutagenesis.

An important improvement on the standard oligonucleotide-directed mutagenic strategies has been termed site-saturation or cassette mutagenesis (13-15). This technique allows the simultaneous generation of at least twenty mutations at a single amino acid residue, thus eliminating the need for predicting which mutations may be of interest. By standard mutagenic techniques restriction endonuclease sites are created that flank the codon to be mutated and that are separated by a short distance (one that can be spanned by synthetic oligonucleotides). The intervening sequence is excised by endonuclease digestion and replaced by ligation with a linear duplex sequence made by annealing complementary synthetic oligonucleotides that contain a mixture of codons in place of the wild-type codon. The mixture of mutated plasmids is used to transform E. coli and the resulting mutations characterized either by a phenotypic screen or by sequencing individual colonies.

A variety of systems have been studied using oligonucleotide-directed mutagenesis, and a wide range of questions

have been addressed. The following discussion attempts to provide examples of some approaches using oligonucleotide-directed mutagenesis to answer questions about the structural requirements for protein function. Specific problems that have been addressed include determination of the roles of specific amino acids in catalysis and in substrate binding, in allosterism, in protein stablility, in protein-protein interactions, and in protein-DNA interactions. In general, target residues for mutagenic experiments are decided on the basis of existing structural or kinetic information. Data from x-ray structures, chemical modification experiments, or from sequence comparisons may indicate sites that are of interest.

The role of an individual amino acid in catalysis has been assessed using oligonucleotide-directed mutagenesis on triosephosphate isomerase (TIM). TIM interconverts dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP). Glu165 was postulated on the basis of chemical modification experiments (16) and the crystal structure (17) to participate in catalysis as a general base, first accepting the pro-R hydrogen of DHAP and then protonating the resulting enediol at C-2 to form DGAP. The mutant Glu165-->Asp was constructed and found to have k_{cat} for the forward reaction reduced by a factor of 240 while K_M for the forward reaction was increased by a factor of 2, confirming that Glu165 is important for catalysis but involved only slightly in the

binding of substrate (18). A more detailed study of the kinetics of catalysis by the Glu165-->Asp mutant has been carried out by measuring the isotope effect on incorporation of solvent protons into the product. An increase relative to the wild-type enzyme of 4 kcal/mole in the energies of the transition states between the enediol intermediate and enzyme bound substrate and product was calculated (19).

The energetics of substrate-active site interactions and how they affect catalytic efficiency have been investigated using a series of mutations of tyrosyl tRNA synthetase (TStyr, reviewed in ref. 22). TStyr from B. stearothermophilus is a dimer of 47.5 kdalton subunits whose crystal structure has been solved to 3.0 Å resolution with (20) and without (21) substrate bound to the active site. The enzyme catalyzes the transfer of tyrosine to tyrosyl tRNA, after forming a tyrosyl-AMP intermediate. Examination of the crystal structure implicated several residues as being involved in hydrogen bonds to the intermediate, tyrosyl adenylate. Thr40 and His45 are involved in hydrogen bonds to the α-phosphate of the tyr-ATP transistion state. The energetics of this interaction were determined by creating mutations which removed the hydrogen bonds causing an increase in the energy of the transition state but no significant change in the binding of substrates. Cys35 was observed to contribute a good hydrogen bond to the C(3) hydroxyl of the ribose ring of ATP. Mutants Cys35-->Ser and Cys35-->Gly were con-

structed and shown to have reduced affinities for substrate ATP because of the complete removal of the hydrogen bond (Cys35-->Gly) or its slight displacement (Cys35-->Ser)(23). The Cys35-->Ser mutant had a lower affinity for ATP than the Cys35-->Gly, a difference that was attributed to the ability of Cys35-->Ser to hydrogen bond to water in the absence of substrate. Energy would be required to break the hydrogen bond to water, decreasing the energy available for binding substrate. A similar effect was reported for mutations at Thr51 which was observed in the crystal structure to be involved in a weak hydrogen bond to the ribose ring oxygen (O-1). The Thr51-->Ala mutant showed a slightly raised affinity for ATP (24). Interestingly, a Thr51-->Pro mutant showed a 25-fold increase for the rate of the first reaction catalyzed (the formation of tyrosyl adenylate) by TStyr due mainly to a lower K_M for ATP (24), and explained by local distortions in the peptide backbone, which influenced the position of His48 so that it had an improved hydrogen bond to O-1 (25).

Oligonucleotide-directed mutagenesis has also been used to study serine proteases. The binding of substrate to trypsin from rat pancreas has been investigated. Trypsin catalyzes the cleavage of peptide bonds with a specificity for peptides containing lysine or arginine. Mutations converting Gly216 and Gly226, both located in the substrate binding site, to alanine, separately and as a double mutation affected the specificity (k_{cat}/K_M) of the enzyme for arginyl

and lysine-containing substrates (26). The wild-type enzyme shows a specificity for arginyl substrates. The Gly216-->Ala mutant shows an enhanced specificity for arginyl substrates, but the Gly226-->Ala mutant has a higher specificity for lysine-containing substrates. The double mutant, Gly216--> Ala/Gly226-->Ala, had a lowered specificity for arginyl substrates.

For subtilisin, the variation in activity with pH was altered by introducing a mutation converting Asp99 to serine. The mutant showed a reduced catalytic efficiency at alkaline pH, despite the location of the mutation 14-15 Å from the active site. The authors concluded that the results were due to long range electrostatic effects (27).

The roles of active site residues in the catalytic mechanism of dihydrofolate reductase (DHFR) have been investigated. DHFR catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate. The carboxyl group of Asp27 is believed to stabilize a protonated transition state. An Asp27-->Asn mutant was constructed and found to have only 0.1% of the activity of wild-type enzyme (28). Structural requirements for catalysis were also studied by removal of a cis peptide bond between Gly195 and Gly196, by creating a Gly195-->Ala mutant, which resulted in the total loss of activity. A Pro39-->Cys mutant was able to form a disulfide bond with Cys85 wich caused a reduction in activity (28). The role of Leu54 was investigated by cre-

ating a mutant converting it to glycine. The mutant had an increased rate of substrate release and a decrease of 10^4 in the rate of hydride transfer indicating a change in the rate-determining step from product release for the wild-type to hydride transfer for the mutant, despite the location of Leu54 10 Å from the active site (29).

One of the most striking examples of the effects of point mutations on biological activity is the activation of proto-oncogenes. The cellular analog of the transforming gene of Harvey murine sarcoma virus (c-Ha-ras) is able to transform cells when mutations in codon 12, normally coding for glycine, are present (30). All 19 mutations at residue 12 were constructed by oligonucleotide-directed mutagenesis and tested for their ability to transform NIH 3T3 cells. All but Gly12 and Gly12-->Pro were able to transform cells. The authors suggested that these two mutants affect an α-helical structure in that region of the protein necessary for the protein's ability to cause transformation (31).

Investigations of the importance of phosphorylation of tyrosines on the transforming properties of oncogene products have utilized oligonucleotide-directed mutagenesis. The middle-T antigen of polyoma virus can transform cultured rat cells (32). Middle-T antigen maintains a tyrosine-kinase activity probably due to its association with $pp60^{c\text{-}src}$, a cellular proto-oncogene product (33). The association causes an increase in the specific activity of the kinase (34).

Middle-T antigen is itself a substrate for the kinase activity, and the effect of phosphorylation of several tyrosines on its transforming ability has been determined by the use of site-directed mutagenesis to convert known tyrosine phosphate acceptors to phenylalanine. Tyrosines 315, 250, 372, and 297 are known to be phosphate acceptor sites. Deletion of Tyr322 or Tyr297 had no apparent effect on transforming activity (35). A mutation converting Tyr250 to Phe caused a reduction in transforming ability (36) as judged by a focus formation assay. Mutations converting Tyr315, the major site of phosphorylation, to phe have produced conflicting results, either causing a drastic reduction (36) or little change (35,37) in transforming ability. Differences in the assays and cell lines used probably account for the different results. Similar experiments on the oncogene of Rous sarcoma virus, $pp60^{v\text{-}src}$, have shown that phosphorylation of Tyr416 is not essential for transformation since the mutant Tyr416-> Phe could cause transformation (38). An autophosphorylation site, Tyr1073, of $P130^{gag\text{-}fps}$, the oncogene product of Fujinami sarcoma virus, was mutated to serine and threonine. Neither ser nor thr were autophosphorylated and the mutants had lowered enzymatic and oncogenic activities (39). The ability of the c-ras-encoded protein, p21, the cellular analog of the transforming gene of Harvey murine sarcoma virus, to bind GTP was abolished by conversion of Asn116 to either lysine or tyrosine. The mutants also lost their

ability to autophosphorylate and to transform NIH3T3 cells (40).

Oligonucleotide-directed mutagenesis has proven useful in the study of the roles of individual amino acids in the allosteric regulation of activity. Mutants of aspartate transcarbamoylase (ATCase) have been constructed. ATCase catalyzes the conversion of carbamoyl phosphate and aspartate to carbamoylaspartate and inorganic phosphate, the first committed step in pyrimidine biosynthesis. The enzyme consists of six 34 kdalton catalytic subunits and six 17 kdalton regulatory subunits. Homotropic cooperativity is exhibited by both substrates and feedback inhibition is accomplished by the heterotropic inhibitor, CTP. ATP acts as an heterotropic activator to ensure the equal production of purines and pyrimidines. Biophysical techniques and x-ray structures on the enzyme alone and in the presence and N-phosphonoacetyl aspartate (41)(PALA, a competitive inhibitor) indicate a substantial restructuring of the holoenzyme on binding of the allosteric effectors. The roles of several individual amino acids in mediating the conformational changes of the enzyme have been investigated. Target residues were identified by examining data from chemical modification studies in addition to the xray data. A mutant converting Gln133, known to reside near the contact region between the catalytic and regulatory subunits, to Ala caused a marked increase in cooperativity without effecting activity

(42). From the x-ray crystal structure, His134 was shown to be in proximity to the phosphate binding site and was suggested as a possible proton donor to the transition state. Mutation of His134 to Ala resulted in a mutant with 5% of the catalytic activity of the wild-type, considerable cooperativity and increased substrate binding constants (42). Mutation of Tyr165, which was thought to be in the substrate binding site from chemical modification studies, caused an increase in binding constants for both aspartate and carbamoyl phosphate, and V_{max} decreased to about 30% of the wild-type value. Cooperativity was also decreased (43). Lys84 was suggested on the basis of inactivation by pyridoxyl phosphate and $NaBH_4$ to be essential for catalysis (44). Substitution of Arg or Gln for Lys 84 gave a mutant possessing only 0.05 or 0.01 % of the activity of the wild-type enzyme, respectively. Chemical modification of Lys83 caused considerable inactivation. However, substitution of Gln for Lys83 caused little change in activity (42). A loop of the protein containing residues 230-245 was shown to undergo a large movement on changing from the T to the R state. Tyr240 was converted to Phe in a mutant that showed altered homotropic interactions and a different reactivity towards p-hydroxymercuribenzoate in the unligated state suggesting an altered conformation (45).

The contribution of disulfide bonds to the thermal stability and conformation of proteins has been studied.

Successful attempts to engineer disulfides into proteins to increase the thermal stability have taken into consideration not only the proximity of the two sites for placing cysteines but also their relative orientations in the three-dimensional structure of the protein since not all dihedral angles are conducive to disulfide formation. An intramolecular disulfide bond was "engineered" into T4 lysozyme by replacing Ile3 with Cys which could then form a bond with Cys97 (46). The disulfide-containing mutant had a substantially increased thermal stability. An intermolecular thiol-disulfide exchange reaction was shown to occur with unpaired Cys54, leading to production of inactive oligomers of the Ile3-->Cys mutant. This reaction was prevented by converting Cys54 to Thr or Val (47). Human interleukin-2 contains a disulfide bridge between Cys58 and Cys105 and a free Cys at position 128. Mutant Cys58-->Ala showed 250 times less activity than wild-type while mutant Cys105-->Ala showed 8-10 times less activity. Cys128-->Ser showed comparable activity to wild-type (48). The lac permease of E. coli catalyzes the H^+/β galactoside symport. A secondary structural model of the single polypeptide chain of the protein based on hydropathic analysis and circular dichroic measurements proposes that 12 α-helical domains span the membrane. A mutation converting Gln60, which resides in the middle of the second proposed α-helix from the N-terminus, to Glu caused a decrease in the thermal stability. The mutant lost activity at 45°C with a

half-life of 20 minutes while the wild-type had a half-life of 50 minutes (49). Removal of the disulfide bond of RTEM-1 ß-lactamase, the enzyme responsible for bacterial resistance to penicillins, by conversion of Cys77 to serine caused a decrease in the thermal stability and stability at alkaline pH of the enzyme though the catalytic parameters at lower temperatures and neutral pH had not measurably changed (50). General methods for increasing the thermal stablility of proteins have been proposed (51). Comparison of the primary sequences of the neutral protease of thermophilic and mesophilic bacteria suggested the construction of a mutation containing the substitution Gly144-->Ala, which is of higher thermal stability than the wild-type enzyme (52). Individual mutants of kanamycin nucleotidyl transferase that enhance the thermostability of the enzyme have been identified (53). The combination of two separate mutations into a double mutant resulted in a cumulative enhancement of stability (54). The stability of subtilisin to oxidative inactivation has been improved by mutagenesis (55). Mutation of Met222, the site of oxidation, to nonoxidizable amino acids, such as Ser, Ala, or Leu, prevented inactivation by peroxide. A mutant of α_1-antitrypsin, a protease inhibitor, which converted Met358 to valine was more resistant to oxidative inactivation by N-chlorosuccimide than the wild-type enzyme (56).

Alterations in the interactions at the interface of protein dimers have been made. Phe164 of tyrosyl tRNA syn-

thetase interacts with its partner Phe164 in the active dimer. A mutant converting Phe164 to Asp showed a shift in the pH dependence of activity. At pH 6, where dimerization is favored, the mutant was fully active. At pH 7.8, dissociation of the dimer was favored and the activity was lowered (57). Mutagenesis of λcI repressor converted Tyr88, known to interact with its partner Tyr88 in the dimer, to Cys created a mutant which could then spontaneously form a disulfide bridge in the dimer without perturbing the structure of the dimer. The mutant was shown to have an increased binding affinity for its operator sequence. In contrast, a mutant converting Tyr85, which was known to be distant from its partner Tyr85 in the dimer, to Cys created a mutant which could not spontaneously form a disulfide bond. Reaction with dithio-2-nitro benzoic acid caused dimer formation and the dimers had a 10-fold reduced affinity for the operator sequence (58).

The mechanisms of recognition and binding interactions between sequence specific DNA-binding proteins and their DNA substrates have been explored using oligonucleotide-directed mutagenesis. A synthetic gene for the λ cro protein was constructed by ligation of a series of oligonucletoides, including conveniently located sites for restriction endonucleases (59). Excision of segments of the wild-type sequence and replacement with a duplex strand of oligonucleotides allowed the rapid production of several single amino acid

replacements. Several mutations in the α-3 helix, one of the helices thought to determine DNA sequence specificity, altered the affinity of cro for its operator sequence. Tyr26 was suggested on the basis of the xray structure to donate a hydrogen bond to O4 of a thymine (60). Conversion of Tyr26 to Asp caused a substantial reduction in affinity for the O_R3 operator sequence, while conversion of Tyr26 to Phe caused an intermediate level of affinity. Interestingly, converting Tyr26 to lysine had little effect on the binding affinity, results explained by an ionic interaction between the lysine and the phosphate backbone substituting for the lost hydrogen bond. Gln27 was thought to be involved in two hydrogen bonds as both a donor and acceptor with an adenine. Substitution of Leu, Cys , or Arg for Gln27 resulted in a large decrease in binding affinity. Serine 28 was thought to be involved in two hydrogen bonds to an adenine. Removal of the hydroxyl of serine by conversion to alanine caused a pronounced decrease in binding affinity (60). The DNA-binding specificity of bacteriophage 434 repressor was changed to the specificity of the repressor from bacteriophage P22 by altering five amino acids on one side of an α helix, the side thought to form specific contacts with the operator sequence (61).

Although the use of oligonucleotide-directed mutagenesis is still in its infancy, it has proven helpful in resolving questions about the relationship between structure and function in proteins. Applications of the method to other sys-

tems and further refinement of the technique should amplify its usefulness.

The work of this thesis involves the use of oligonucleotide-directed mutagenesis to study the roles of highly conserved amino acids in the active site of RTEM-1 ß-lactamase [EC(3.5.2.6)], the enzyme encoded by the bla gene of plasmid pBR322. The conserved Thr at position 71 was replaced with cysteine as described in Chapter 2. The "saturation" of Lys73 with all possible single site mutations is described in Chapter 3, and the kinetic characterization of two mutants at position 73, Lys73-->Arg and Lys73-->Cys is documented in Chapter 4.

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Chapter 2

Construction and Kinetic Characterization of a Thr71-->Cys Mutant of RTEM-1 ß-lactamase

Introduction

ß-lactamases catalyze the hydrolysis of the ß-lactam ring of penam and cephem antibiotics (1). They are principally responsible for resistance to these antibiotics in prokaryotes and therefore have been subjects of interest to biochemists and medical researchers alike. ß-lactamases from different bacterial sources have been classified into three groups, A, B, and C, on the basis of structural and catalytic similarities (2). Class A ß-lactamases have molecular weights of approximately 28,000 daltons, catalyze the hydrolysis of penams at higher efficiency than cephems, and include the S. aureus PC1, B. licheniformis 749/c, B. cereus 569/H1, and the RTEM enzymes. Class B consists of the metallo-enzymes from B. cereus and P. maltophilia (3). Class C enzymes have molecular weights of approximately 38,000 daltons, hydrolyze cephems at higher efficiency than penams and include the enzymes from P. aeruginosa and E. coli K12 (4). Class C enzymes also show the ability to transfer the hydrolyzed ß-lactam antibiotic to an exogenous nucleophile such as methanol and catalyze the hydrolysis of the resultant ester (5).

The ß-lactamase used in this work is the RTEM-1 enzyme encoded by pBR322. The RTEM enzymes are named after the strain of E. coli from which they were first isolated. The RTEM-1 enzyme has an isoelectric point of 5.4 and the RTEM-2 enzyme, encoded by plasmid RP1, has an isoelectric point of

5.6, which reflects a single amino acid difference. The RTEM-1 enzyme has glutamine at position 39, where the RTEM-2 enzyme has a lysine. The RTEM-1 enzyme is synthesized as a preprotein and secreted from the cytosol to the periplasm with the proteolytic loss of a 23 amino acid leader sequence. The mature form of the enzyme has a molecular weight of 28.5 kdaltons.

Class A ß-lactamases maintain a conserved triad of amino acids, Ser-Thr-Xaa-Lys (2). The serine of the conserved triad has been implicated as the active site nucleophile by studies of its interaction with an irreversible inactivator 6-ßbromopenicillinanic acid (6,7,8). The role of the conserved Thr has been investigated by cassette mutagenesis (9) to replace the Thr71 of RTEM-1 ß-lactamase with the other nineteen amino acids. Cells harboring all mutants except Thr71-->Lys, Arg, Tyr, Trp, and Asp showed a resistant phenotype to ß-lactam antibiotics and all were apparently less thermally stable than the wild-type enzyme. The physical and catalytic properties of the mutants Thr71-->Ser and Ile have been studied in some detail (10,11). These results have supported the general conclusion that Thr is conserved at position 71 of RTEM-1 ß-lactamase more for purposes of protein stability and less for binding of substrate or for catalysis.

This chapter describes the construction and kinetic characterization of the mutant Thr71-->Cys of RTEM-1 ß-lac-

tamase. The mutant was constructed by cassette mutagenesis using the three fragment ligation scheme (9) to ligate a duplex strand of synthetic oligonucleotides containing the desired double mutation ACT-->TGT, replacing the Thr71 codon with that of Cys into a modified form of pBR322. Cells harboring the mutant have a resistant phenotype to ß-lactam antibiotics. Purified mutant enzyme is active against benzylpenicillin, cephalothin, and 6-aminopenicillanic acid (6-APA), although at lower efficiency than the wild-type enzyme. The mutant has an altered pH-activity profile, showing increased sensitivity to activity loss at alkaline pH. The mutant is sensitive to inactivation by parachloromercuribenzoate (pCMB) under conditions where activity of the wild-type is not affected. The mutant is less stable to thermal denaturation than the wild-type enzyme as are all other mutants at position 71 (10,11).

Materials and Methods

Enzymes and Chemicals

Restriction enzymes and the large (Klenow) fragment of DNA polymerase I were purchased from Boehringer Mannheim. T4 polynucleotide kinase and T4 DNA ligase were from Bethesda Research Laboratories. Antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.) The [α-^{32}P]dTTP, 3000 μCi/mmol was obtained from Amersham, and the [γ-^{32}P]ATP was from ICN (Irvine, CA). Tryptone and yeast extract were from Difco (Detroit, MI). All other chemicals used were reagent grade.

Bacterial strains

E. Coli strain LS1 (12) was used as host of plasmid pBR322 and the derivatives of pBR322. E. Coli strain D1210 was used as the host strain for plasmid pJN and is a lac I^q derivative of HB101.

DNA

Oligonucleotides were synthesized by using the phosphoramidite chemistry (13) on the Applied Biosystems (Foster City, CA) DNA synthesizer, model 380A and purified by preparative polyacrylamide gel electrophoresis, followed by desalting by G-25 chromatography.

DNA fragments were purified by electrophoresis on 1.2% agarose gels and electroeluted using either DEAE-cellulose paper or an International Biotechnologies, Inc. (New Haven, CT) UEA electroeluter.

Plasmid DNA was prepared by the alkaline lysis method (14). The numbering system for pBR322 is the one commonly used (15).

Reactions and Mutagenesis

The complementary mutagenic oligonucleotides were phosphorylated using standard procedures (15), and were annealed by mixing 0.4 pmole/μL of each strand and heating to 95°C in 10 mM $MgCl_2$, 50 mM Tris-HCL, pH 8, followed by slow cooling to room temperature over a one hour period.

The three fragment ligation scheme was used (9). A sample of plasmid pBR322ss (a derivative of plasmid pBR322 containing an additional AvaI restriction site at 3972 bp and an additional ScaI restriction site at 3937 bp, and missing the ScaI restriction site at 3846 bp) was digested with 7 units each of SalI and ScaI restriction endonuclease and the resulting 3286 bp fragment was isolated from a 1.2% agarose gel as described above. Another sample of pBR322ss was digested with 7 each units of AvaI and SalI restiction endonucleases and the resulting 1042 bp fragment was isolated. The following mutagenic sequence,

5'CC GAG GAA CGT TTT CCA ATG ATG AGC (TGT) TTT AAA GT 3'

C CTT GCA AAA GGT TAC TAC TCG (ACA) AAA TTT CA 5',

was kinased and annealed as described. For the ligation, approximately 0.04 pmole of each restriction fragment and 0.4 pmole of the synthetic fragment were added in 10 mM $MgCl_2$, 50 mM Tris-HCl, pH 8, 0.5 mM ATP, 5 mM dithiothreitol

with 10 units of T4 DNA ligase and incubated at 15°C for approximately 16 hours. The ligation was terminated by heating to 65°C for 10 minutes and a 10 μl aliquot of the ligation mixture was used to transform E. coli LS1 to tetracycline resistance, using standard transformation procedures (16).

Screening

To ensure the presence of the desired mutation, plasmid DNA derived from an individual colony was digested with EcoRI restiction endonuclease in medium buffer (15) and then electrophoresed on a 1.2% agarose gel. A control sample of plasmid pBR322 was also digested with EcoRI and electrophoresed in an adjacent lane. DNA was transferred to nitrocellulose (Schleicher and Schuell, BA 85) by standard techniques (15), and fixed by heating to 80°C in vacuo for 90 minutes. The following mutagenic oligonucleotide was kinased with [γ-^{32}P]ATP and used for screening.

5'TG ATG AGC TGT TTT AAA G 3'

The nitrocellulose was washed with prehybridization buffer (0.2% SDS, 10x Denhardt's solution (3), 0.1 mg/ml sonicated denatured salmon sperm DNA, 1 mM sodium orthophosphate, 1 mM sodium pyrophosphate, 0.5 mM ATP, and 6x SSC buffer (15)) for 30 minutes. The filter was then hybridized at room temperature in a solution containing 0.2% SDS, 10xDenhardt's solution, 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 buffer three times for three minutes each wash, first at room temperature and then at 5 degrees below the estimated melting temperature, Td, as determined by the following equation, Td = 4(# GC bps) + 2(# AT bps). Autoradiograms were taken after each wash.

Sequencing

To confirm the presence of the desired mutation, plasmid DNA was sequenced by the following procedure. Plasmid was digested with AvaI restriction endonuclease and the resulting 2962 bp fragment isolated as described before. The fragment was labeled at nucleotide T-3973 using [α-^{32}P] dTTP and the large (Klenow) fragment of DNA polymerase I, as shown in Figure 2. The labeled fragment was precipitated from the polymerase solution with 2.5 volumes of ethanol and pelleted by centrifugation. The pellet was washed with 70% ethanol and dried in a Speedvac. The labeled fragment was sequenced by the method of Maxam and Gilbert (17). The sequencing gels were 40 cm long and contained 20% polyacrylamide (wt/vol) and 40% urea and were run using standard conditions (15).

Subcloning into pJN

Plasmid pBR322T71C and plasmid pJN were digested with EcoRI and PvuI restriction endonucleases and the resulting 626 bp and 4800 bp fragments isolated by electroelution, respectively. The 626 bp EcoRI-PvuI fragment of plasmid

pBR322T71C was ligated to the 4800 bp PvuI-EcoRI fragment of plasmid pJN using procedures described above. Transformation of E. Coli D1210 to kanamycin resistance was performed as described. To confirm the presence of the mutated gene, plasmid derived from a kanamycin resistant colony was digested with AvaI restriction endonuclease and run on a 1.2% agarose gel. The additional AvaI restriction site from pBR322ss was confirmed.

Purification of Thr71-->Cys

The purification procedure used was a slight variation of the published procedure (10).

A frozen stock of E. coli strain D1210 harboring plasmid pJNT71C was used to inoculate 300 ml of L broth (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter) containing 25 mg/L kanamycin. The culture was grown at 37°C with shaking for 7 to 9 hrs. Fifty mls of the culture was used to inoculate 10 L of FB media (25 g tryptone, 7.5 g yeast extract, 50 ml of 1 M Tris, pH 7.5, 30 g NaCl per L) containing 25 mg/L kanamycin. The culture was grown in a New Brunswick Scientific Co. fermenter at 30°C with vigorous aeration for 12 to 14 hours. The temperature was dropped to 30°C and IPTG was added to 0.1 mM. The induction was allowed to continue for 2 to 3 hours with aeration and then the cells were collected by centrifugation. The osmotic extrusion was performed as described (18). Solid ammonium sulfate was added to the extrudate to reach 20% (w/v). The

solution was stirred at 4°C for several hours. Occasionally a precipitate appeared and was removed by centrifugation at 10 Krpm in a GSA rotor for 30 minutes. Solid ammonium sulfate was added to the supernatant to reach 60% (w/v), and the solution stirred at room temperature for 12-16 hours. The precipitate was collected by centrifugation at 10 Krpm for 45 minutes and dissolved in 200 mls distilled deionized water and dialyzed against water containing 0.02% sodium azide using a Diaflo fitted with a PM-10 membrane. After removal of ammonium sulfate, the solution was changed to 25 mM triethanolamine, pH 7.25 and applied in 30-50 mls to a 2.5 x 25 cm DE-52 column. Protein was eluted using a linear gradient from 25 to 200 mM triethanolamine, pH 7.25. Fractions corresponding to the activity peak were pooled and dialyzed into 20 mM Tris, pH 6.75, and applied to a 1 x 82 cm Ultragel ACA 54 column in several 0.5 ml portions. Yields using this procedure were generally poor, about 1 to 2 mgs of protein probably due to the thermal instability of the mutant.

Assays

Protein concentrations were determined by absorbance at 281 nm using the conversion factor, 29,400 $M^{-1}cm^{-1}$ (19). Activity assays were done at 30°C (unless otherwise indicated) in 0.1 M potassium phosphate, pH 7, using either a Beckman Acta CIII or DU-7 spectrophotometer with 1 cm pathlength cells for assays of activity on benzylpenicillin and 6-amino-

aminopenicillanic acid. For assays of activity on cephalothin an optical quartz block was inserted into the cuvet to reduce the pathlength to 1 mm. Hydrolytic cleavage of cephalothin was followed at 265 nm, and at 240 nm for benzylpenicillin and 6-aminopenicillanic acid (19). The $\Delta\epsilon$ values for cephalothin, benzylpenicillin, and 6-aminopenicillanic acid were 7900, 500, and 500 $M^{-1}cm^{-1}$ respectively (19).

Inactivation by pCMB

To 125 μL mutant and wild-type ß-lactamase in 20 mM Tris, pH 6.75 and 7, respectively was added 2.5 μL of a 5 mM parachloromercuribenzoic acid in 0.01 M sodium carbonate. Aliquots were removed at timed intervals and assayed for residual activity. To determine the effect of substrate on the inactivation a control sample of the mutant ß-lactamase was made 1 mM in benzylpenicillin and treated as described above.

Activity vs pH

The pH of the assay buffer was varied from pH 5 to pH 8.4. Aliquots of mutant and wild-type ß-lactamase were added and assayed immediately.

Thermal stability

Mutant ß-lactamase was incubated at the indicated temperatures in 80 mM sodium phosphate, pH 7. At timed intervals aliquots were removed and assayed for residual activity on benzylpenicillin at 30°C.

Inhibition by borate

Assay buffer was made from 0 to 10 mM borate. Reaction progress curves at low substrate concentrations, those approaching the K_M of benzylpenicillin, were determined using a constant amount of mutant ß-lactamase.

Results

The three fragment ligation scheme used to create the mutant, Thr71-->Cys, is outlined in Figure 1. Plasmid pBR322SS is a derivative of pBR322 containing an additional restriction site for AvaI at 3972 bp from the EcoRI site and an additional site for ScaI at 3936 bp, sites that flank the active site serine-70 codon of the ß-lactamase gene. The ScaI site of pBR322 at 3846 bp was removed (9). Ligation of the annealed, kinased oligonucleotides to the two plasmid fragments followed by transformation by the method of Hanahan (16) gave approximately 200 tetracycline-resistant colonies. Seven of the eight Tet^R colonies that were plated onto L agar plates containing 200 mg/ l ampicillin showed a resistant phenotype. Plasmid prepared from one of the Tet^R, Amp^R colonies was screened for the presence of the desired mutation using a ^{32}P-labeled hybridization probe containing the TGT codon for position 70. Thus, the probe has a double mismatch on binding to the wild-type sequence leading to a large decrease in the melting temperature compared to the probe bound to the mutated sequence, a difference distinguishable even at room temperature as shown in Figure 2. To confirm further the existence of the mutation plasmid pBR322T71C was sequenced by the Maxam-Gilbert technique (17). The sequencing strategy outlined in Figure 3 takes advantage of the assymmetric overhangs left on digestion by AvaI restriction endonuclease. Labeling at the end closest to the

active site is accomplished by the addition of [α-^{32}]dTTP to that end by the Klenow enzyme.

Purification of the mutant protein was accomplished using plasmid pJN, which contains the tac promoter (20, 21) and the gene for kanamycin resistance. The mutant Thr71--> Cys gene was ligated into pJN as an EcoRI-PvuI fragment and its placement was confirmed by the presence of the additional AvaI restiction site at 3969 bp. The host strain for pJN was E. coli strain D1210 which expresses the lac repressor constitutively, shutting off expression of ß-lactamase until the addition of IPTG. Induction was carried out at 30^{o}C to minimize activity loss due to the known thermal instability of the Thr71-->Cys mutant. Despite this precaution yields were generally low, about 1 to 2 mgs for a 10 L growth. This quantity of enzyme is sufficient for kinetic studies. The final buffer pH was kept below 7 due to the discovered instability of the mutant at alkaline pH. The DE-52 column profile is shown in Figure 4. The protein used in the kinetic studies was determined to be homogenous by 12% SDS-PAGE (22).

The Michaelis-Menten parameters were determined for the substrates benzylpenicillin, cephalothin, and 6-aminopenicillanic acid and are shown in Table 1. The values for k_{cat} and K_M were determined from Lineweaver-Burk (23) plots generated by following reaction progress curves and using [S], the average substrate concentration, and v, the average

reaction rate for a region of the reaction progress curve according to published procedures (24). The value of k_{cat}/K_M, a measure of the efficiency of enzymatic catalysis, of the mutant is 12% for benzylpenicillin, 3% for cephalothin and 9% for 6-aminopenicillanic acid of the value of k_{cat}/K_M for the wild-type enzyme.

The activity of the Thr71-->Cys mutant was determined at various pH values ranging from 5.5 to 8.4 and the results are shown in Figure 6. The mutant shows an increased sensitivity to activity loss at alkaline pH compared to the wild-type enzyme. This activity loss is completely reversible on the assay time scale (20 minutes) up to a pH of approximately 8.2. At pH greater than 8.2 the assay was not linear but decreased gradually suggesting the denaturation of the mutant. By contrast the activity of the wild-type enzyme was linear up to the highest pH tested, pH 8.4. The Michaelis-Menten parameters for the Thr71-->Cys mutant were determined at pH 8.1 for PenG and are shown in Table 2.

The Thr71-->Cys mutant was shown to be sensitive to inactivation by pCMB at a concentration (100 μM) that does not affect the activity of the wild-type enzyme, as shown in Figure 7. As a control reaction to show that the inactivation is due to specific reaction at the active site, benzylpenicillin was added to the inactivation reaction. The presence of 1 mM benzylpenicillin in the inactivation reaction decreased the initial rate of activity loss and the inactiv-

ation ceased at about 60% of the original activity. This "protection" against inactivation is most probably due to formation of an adduct between the benzylpenicillin and the pCMB, possibly between the thiazolidine ring sulfur and the mercury of the pCMB. Under the conditions employed for the inactivation the substrate is in a 10:1 excess over the pCMB and would halt inactivation by reacting with all of it. However, the wild-type enzyme serves as a sufficient control demonstrating inactivation occurs by reaction at Cys71 since it differs from the mutant at this position only.

The thermal stability of the Thr71-->Cys mutant is reduced relative to that of the wild-type enzyme as show in Table 3 and in Figure 7. The mutant shows a significant and rapid loss of activity above 40°C while the wild-type enzyme is relatively stable until the temperature reaches 50-55°C.

Borate ion competitively inhibits both the wild-type and Thr71-->Cys mutant. The K_I of borate is 1 mM for the wild-type enzyme (25). The K_I for borate and the Thr71-->Cys was determined to be 2.5 mM by measuring reaction progress curves for the hydrolysis of benzylpenicillin in the presence of from 2 to 10 mM borate ion, using substrate concentrations ranging from 1 to 3 times the K_M value.

Discussion

Cassette mutagenesis provides a rapid and efficient method for the production of structural mutants of a protein, when restriction sites flanking the codon of interest are present. Cassette mutagenesis has distinct advantages over oligonucleotide mutagenesis; more than one mutant may be generated at the same time at nearly equal frequencies, a large number of base changes can be incorporated generating changes in more than one codon simultaneously, and the tedious screening procedures of oligonucleotide-directed mutagenesis are eliminated. Cassette mutagenesis has been used to produce the mutation Thr71-->Cys in RTEM-1 ß-lactamase, placing a cysteine adjacent to the active site serine.

The replacement of Thr71 with cysteine was carried out in order to complete the permutations of Cys, Ser, and Thr at positions 70 and 71 of ß-lactamase. The mutants Cys70--> Thr71, Ser70-->Ser71, Thr70-->Ser71, have already been constructed and their physical and catalytic properties examined (26, 10). Since Thr70-Ser71 shows no catalytic activity, the construction of Thr70-Cys71 was deemed unnecessary. The mutant Cys70-Cys71 was also constructed but showed no phenotypic resistance to ampicillin and was not further characterized (data not shown).

Except for its increased sensitivity to alkaline pH and its thermal instability, the mutant Thr71-->Cys behaved similarly to the wild-type enzyme during its purification, in

that the osmotic extrusion released activity from the cells into the extrudate. This indicates that processing and secretion of the mutant is not severely affected by the presence of the additional cysteine six residues away from Cys77 which is involved in a disulfide bond with Cys123. Assuming K_M reflects the intrinsic binding constant of substrate to the enzyme, binding is not significantly changed relative to the wild-type enzyme, indicating no gross structural changes occurred. The mutant Ser70-->Cys shows similar reductive behavior on treatment with DTT as the wild-type enzyme (27).

The Thr71-->Cys mutant shows appreciable catalytic activity on penams and cephems, as shown in Table 1. The mutant does not hydrolyze 6-aminopenicillanic acid with as high an efficiency as it does benzylpenicillin and its catalytic efficiency on cephalothin is even poorer. Due to the thermal instability of the mutant and the necessity of exposing it to room temperature during the ammonium sulfate precipitation step, the absolute values for k_{cat} may contain a systematic error due to limited thermal denaturation but since the kinetic parameters were all obtained over a two day period the relative rates of catalysis on the different substrates should be accurate. The lower efficiency of catalysis on cephalothin is consistent with that seen with other mutants at position 71 (10,11) and may reflect a more stringent protein structural requirement for turnover of cephems. The K_M for cephalothin for both the mutant and wild-type enzymes is

about ten times that for benzylpenicillin. Thus, the binding orientation of cephems to the class A ß-lactamase is not optimal for catalysis and is even less so for mutants at position 71. The molecular basis for the decreased efficiency of catalysis of 6-APA is not clear but may be due to an interaction between the positive charge of the amino group of the substrate and the sulfhydryl group of the Cys71. The decreased efficiency of the mutant on 6-APA is reflected in its phenotypic behavior. Cells harboring pBR322T71C grow as well as cells containing the wild-type enzyme on all other concentrations of antibiotics but will only grow on up to 100 mg/L 6-APA at 37°C where the wild-type is resistant to concentrations in excess of 250 mg/L (9).

Replacing Thr71 with Cys generates a mutant with several novel properties. As the pH of the assay buffer becomes more basic the mutant shows a more rapid decrease in activity compared to the wild-type which does not begin to show a substantial decrease in activity until the pH is above 8.4. The midpoint of activity loss for the mutant is about pH 8 and since the pKa of cysteine is 8.33 the loss of activity probably reflects the development of anionic character of the sulfhydryl adjacent to the active site serine. The value of K_M increases from 40 to 166 μM from pH 7 to 8.1 for the mutant on benzylpenicllin. The value of K_M for the wild-type also increases over the same pH range from 20 to 67 μM (28). If K_M is assumed to reflect the intrinsic binding constant of

substrate to enzyme the larger increase for the mutant may mean a conformational change associated with the anionic character of the sulfhydryl of Cys71. The decrease in k_{cat} of the mutant over that pH range may result from the conformational change or from the effect of the anion adjacent to the active site serine on the nucleophilicity of the serine hydroxyl. Of interest is the decrease in k_{cat} and increase in K_M of the mutant Ser70-->Cys at basic pH, although the change is less dramatic and occurs at the same pH as for the wild-type enzyme (27). By contrast, thiolate anion is more reactive towards benzylpenicillin at higher pH (29). A change in the surface charge of a mutant of subtilisin (Asp99 -->Ser), a distance of 14-15 Å from the catalytic triad, changed the pH dependence of enzymatic activity, decreasing catalytic efficiency. The authors concluded the change was due to long range electrostatic effects (32).

Phenotypic data on the resistance of mutants Thr71--> Asp and Thr71-->Glu are consistent with the incompatibility of an anion adjacent to the active site serine. Thr71-->Asp shows no phenotypic resistance to any antibiotic tested. However, Thr71-->Glu with the additional methylene group acting as a spacer shows appreciable growth on low levels of benzylpenicillin (9).

Another novel property of the Thr71-->Cys mutant is its sensitivity to inactivation by the organic mercurial reagent, pCMB at concentrations where the activity of wild-type ß-

lactamase is not affected. This result indicates the side chain of the residue adjacent to the active site is available to reaction with small molecules and that covalent incorporation of the bulky pCMB group in the active site causes inactivation, probably by sterically preventing the binding of substrate or by causing a conformational change. The almost complete loss of activity suggests the activity associated with the Cys71 mutant is due to the mutant and not to contamination by the wild-type enzyme.

The thermal stability of the mutant is decreased relative to that of the wild-type. The cysteine side chain substitutes a sulfhydryl group for the hydroxyl of threonine and does not have the corresponding methyl group. The mutant Thr71-->Ser has been shown to be less thermally stable than the wild-type and is also more easily denatured in the presence of urea (10). Therefore the methyl group also contributes to the thermal stability of the enzyme. The overall energy of stabilization of a protein is in the range of 10-40 kcal/mole (30). The loss of 2-5 kcal/mole of stabilization energy from the hydrogen bonding of the hydroxyl and favorable hydrophobic interactions of the methyl group represents a substantial fraction of the overall energy of stabilization and leads to the observed thermal instability of the mutant. In an interesting contrast the Ser70-->Cys mutant shows a 3-fold greater resistance to inactivation by trypsin at 40°C, which infers an 0.6 kcal/mole greater energy of stabilization

compared to wild-type enzyme. The authors concluded the greater stability was due to replacing an hydroxyl group with the sulfhydryl group in the apolar interior of the enzyme (27).

Borate ion competitively inhibits both the wild-type and mutant ß-lactamases with K_I's of 1 and 2.5 mM respectively. Borate also competitively inhibits the Thr71-->Ser mutant with a K_I of 1 mM but only noncompetitively inhibits the Ser70-->Cys mutant at high borate concentrations. Therefore interaction of borate with the active site hydroxyl is required for competitive inhibition but some interaction with the hydroxyl on the adjacent residue may occur lowering the inhibition constant. Alternately, the K_I of borate may be increased in the mutant because of electrostatic interactions between the sulfhydryl of Cys71 and the negatively charged borate ion.

Summary

Replacing the conserved threonine residue adjacent to the active site serine of RTEM-1 ß-lactamase with a cysteine by cassette mutagenesis generates a mutant with several novel properties. The mutant shows phenotypic resistance to ß-lactam antibiotics. The efficiency of catalysis of the mutant is reduced relative to the wild-type to 12% on benzylpenicillin, 3% on cephalothin, and 9% on 6-APA. The mutant is much more sensitive to activity loss at alkaline

pH, losing 50% activity at about pH 8.1, than the wild-type which loses 50% of its activity at about pH 9. The activity loss is probably due to the development of anionic character at the sulfhydryl causing a small conformational change or affecting the ionic character of the active site. The mutant is sensitive to inactivation by pCMB under conditions where the wild-type enzyme is not affected. The mutant is substantially less thermally stable than the wild-type enzyme suggesting the methyl group and the hydroxyl group of the wild-type threonine are important for stability of the enzyme though not required for catalysis. Borate ion inhibits both the wild-type and mutant enzymes although the K_I for the mutant is slightly elevated.

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Figure 1

Three fragment ligation scheme

for the construction of mutant

Thr71-->Cys

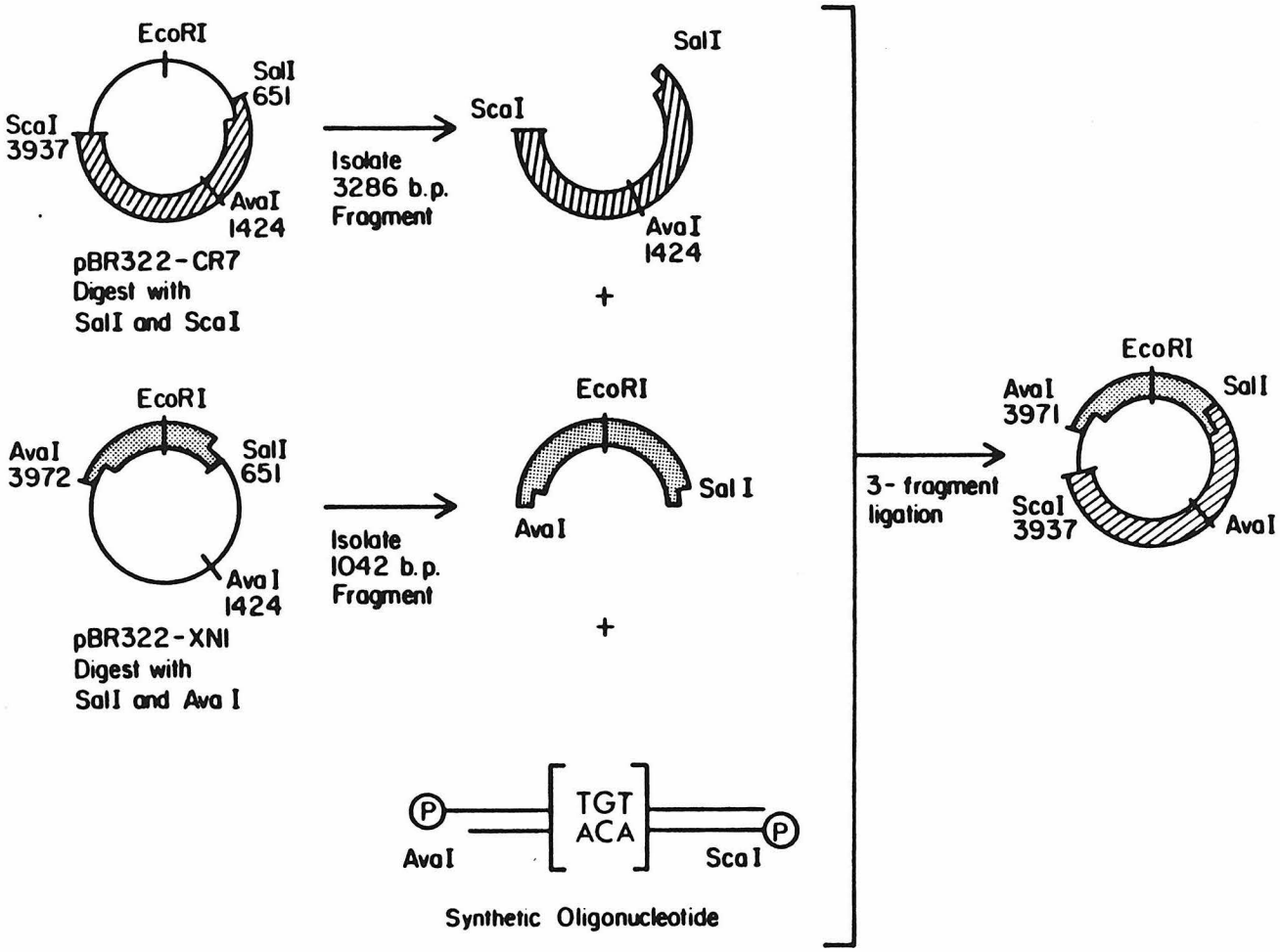

EcoRI
SalI
651
ScaI
3937
AvaI
1424
pBR322-CR7
Digest with
SalI and ScaI
Isolate
3286 b.p.
Fragment
SalI
ScaI
AvaI
1424
+
EcoRI
AvaI
3972
SalI
651
AvaI
1424
pBR322-XNI
Digest with
SalI and Ava I
Isolate
1042 b.p.
Fragment
EcoRI
SalI
AvaI
+
P
TGT
ACA
P
AvaI
ScaI
Synthetic Oligonucleotide
3-fragment
ligation
EcoRI
AvaI
3971
SalI
ScaI
3937
AvaI

Figure 2

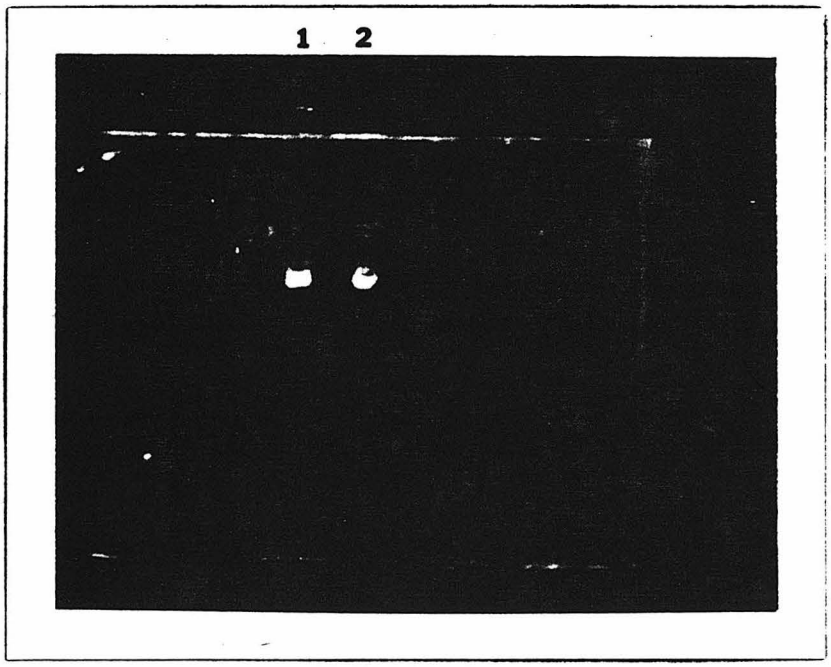
Hybridization screening of plasmid containing the mutation AGC-->TGT in codon 71 of the ß-lactamase gene

A. Agarose gel electrophoresis of EcoRI digests of plasmid pBR322T71C (lane 1) and pBR322ss (lane 2)

B. Autoradiogram of the hybridization of a mutagenic probe to a nitrocellulose replicate of A., room temperature wash

C. as in B., 39°C wash

A)



B)

C)

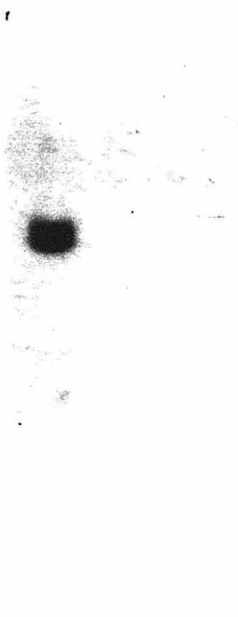
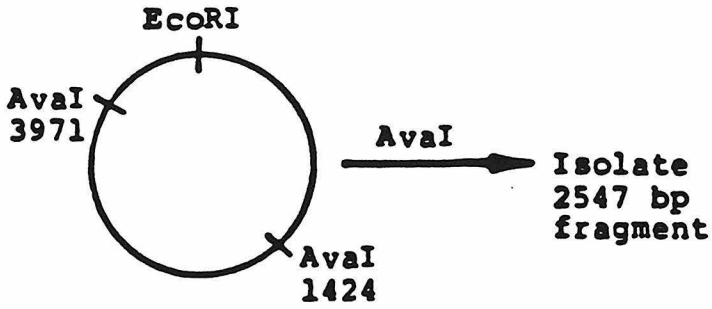
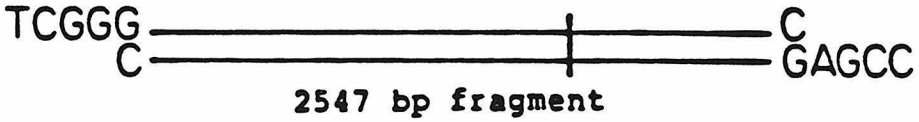


Figure 3

Labeling scheme for Maxam-Gilbert sequencing





2547 bp fragment

$[\alpha\text{-}^{32}P]$TTP
dGTP
Klenow

Maxam-Gilbert
sequencing

thr-71 codon 23, 24, 25 bp in length

Figure 4

DE-52 column profile of the purification of mutant Thr71-->Cys (●-activity, o-A_{281})

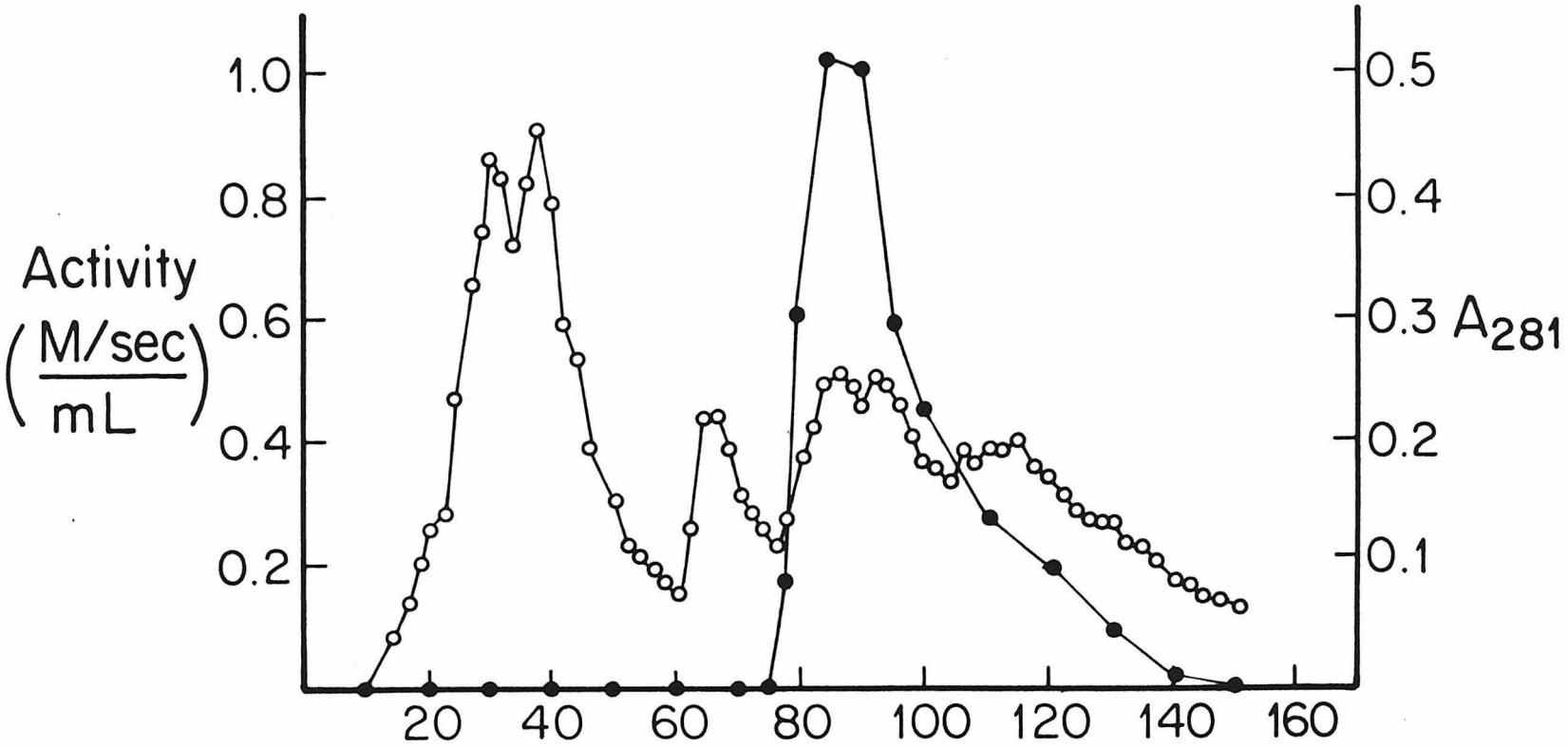

Activity
(M/sec / mL)
1.0
0.8
0.6
0.4
0.2
0.5
0.4
0.3
0.2
0.1
A281
20
40
60
80
100
120
140
160

Figure 5

Activity vs. pH for Thr71-->Cys (o) and for wild-type (●)

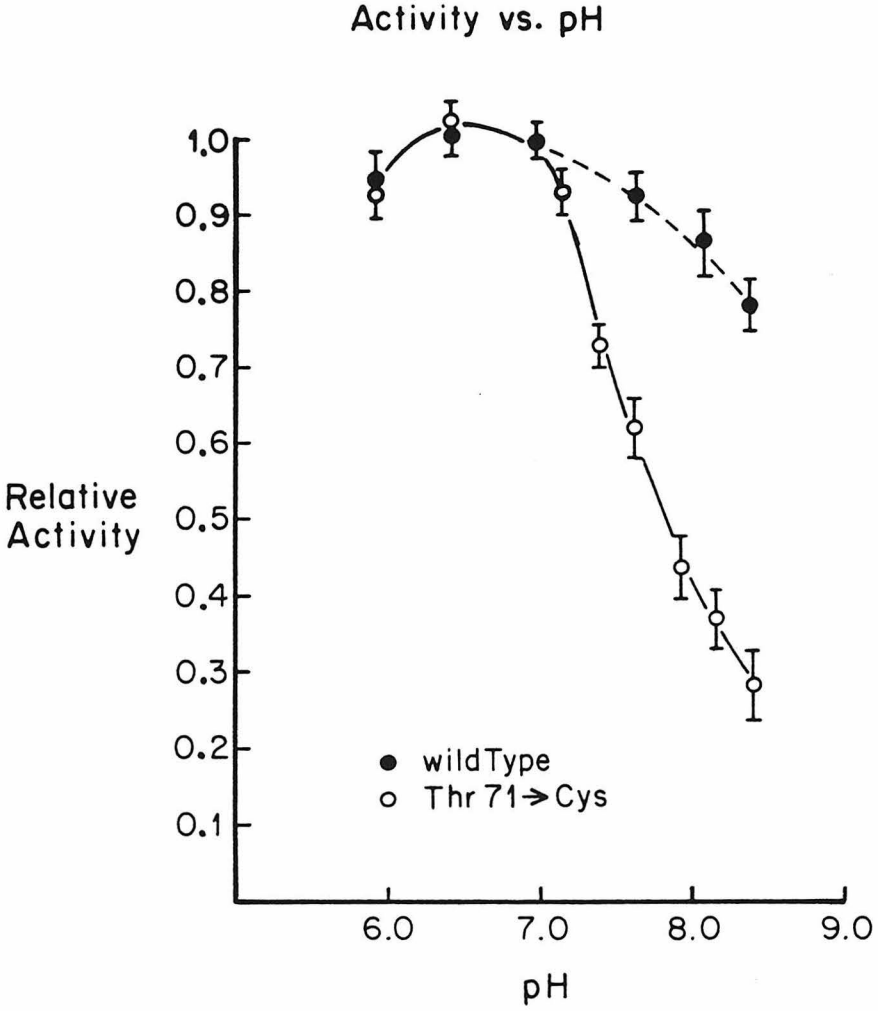

Activity vs. pH
Relative Activity
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
6.0
7.0
8.0
9.0
pH
wild Type
Thr 71→Cys

Figure 6

Inactivation of Thr71-->Cys by pCMB

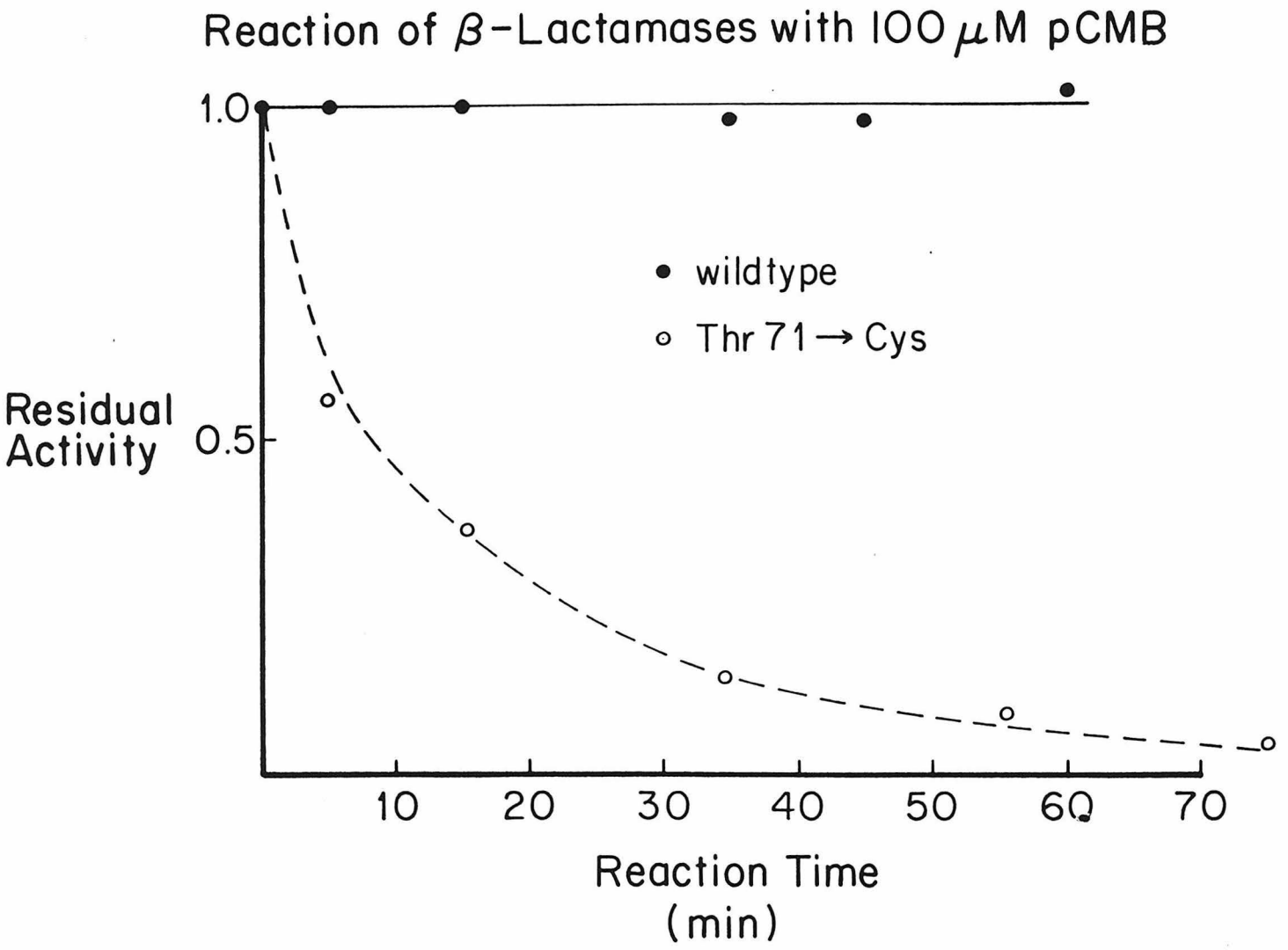

Reaction of β-Lactamases with 100 μM pCMB
1.0
0.5
Residual Activity
wildtype
Thr 71 → Cys
10
20
30
40
50
60
70
Reaction Time (min)

Figure 7

Initial turnover rates at increasing assay temperature

(●-wild-type, o-Thr71-->Cys)

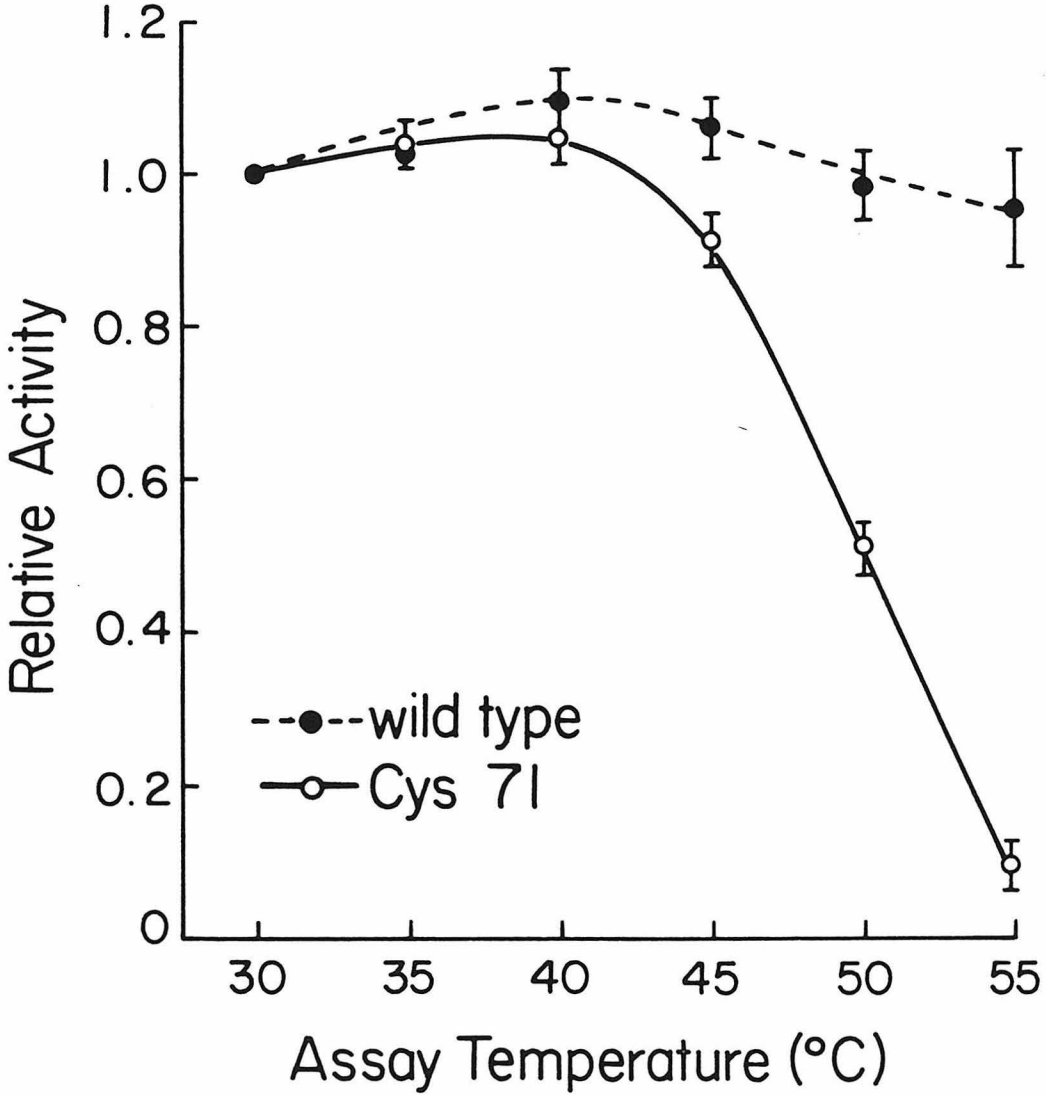

1.2
1.0
0.8
0.6
0.4
0.2
0
Relative Activity
wild type
Cys 71
30
35
40
45
50
55
Assay Temperature (°C)

Table 1. Kinetic Parameters

	Thr71-->Cys (wild-type)		
	k_{cat} (sec^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1}sec^{-1}$)
Penicillin G	470±45 (2000)	40±10 (20)	$1.2x10^7$ (10^8)
Cephalothin	13±1 (120)	690±60 (190)	$1.9x10^4$ ($6x10^5$)
6-aminopen-cillanic acid	166±14 (1500)	240±20 (195)	$6.9x10^5$ ($7.6x10^6$)

	k_{cat}/K_M Thr71-->Cys/wild-type
Penicillin G	.12
Cephalothin	.03
6-APA	.09

	$[(k_{cat}/K_M)$ Cephalothin$]/[(k_{cat}/K_M)$ Pen G$]$
Thr71-->Cys	0.2 %
Wild-type	0.6 %

	$[(k_{cat}/K_M)$ 6-APA$]/[(k_{cat}/K_M)$ Pen G$]$
Thr71-->Cys	5.8 %
Wild-type	7.6 %

Table 2

Thermal stability of wild-type and Thr71-->Cys

T (^{o}C)	$t_{1/2}$ (min) wild-type	Thr71-->Cys
40	-	15
45	-	7
50	>60	3
55	15	
60	2	

Table 3

Kinetic parameters at pH 8.1

	k_{cat} (sec^{-1})	K_M (μM)	k_{cat}/K_M
Wild-type	1800	67	2.7×10^7
Thr71-->Cys	260	166	1.6×10^6

	$[k_{cat}/K_M]$pH 8.1/$[k_{cat}/K_M]$pH 7
Wild-type	.27
Thr71-->Cys	.13

Chapter 3

Site Saturation of Lys73 of RTEM-1 ß-lactamase

Introduction

Studies of the relationship between the linear sequence of amino acids of a protein, its three-dimensional structure and its function have been greatly facilitated by the advent of site-directed mutagenesis (1,2,3). This technique allows the introduction of a specific change in the linear sequence of the protein. Differences in the structure or function of the mutagenized protein can then be ascribed to the single mutation.

Oligonucleotide-directed mutagenesis has been used to study the roles of individual residues in catalysis by several enzymes. The contributions of the side chains of active site residues to the binding energy of substrate and the effect of changes in binding energy on catalysis have been investigated in a series of mutations of tyrosyl tRNA synthetase (4-7). Amino acid residues of cytochrome c previously thought to be necessary for electron transfer were found to be nonessential (8). The active site serine of alkaline phosphatase was changed to cysteine and the resulting mutant retained 2-10% of the wild-type activity, depending on the substrate (9). Oligonucleotide-directed mutagenesis has also been used to study lac carrier protein (10), aspartate transcarbamoylase (11), triose phosphate isomerase (12), and ß-lactamase (13).

Mixtures of oligonucleotides have been used to generate more than one mutation at a time (14,15,16). However, a more

efficient method for simultaneously generating more than one mutant either at the same site or at different positions of the same protein is cassette mutagenesis (17,18). With this technique a cassette of duplex oligonucleotides that may contain a mixture of codons at one or more positions is ligated between two restriction sites flanking the region of interest. Cassette mutagenesis has been used to generate all nineteen mutations at a single site in ß-lactamase (17) and in subtilisin (18). The specificity of DNA binding of the 434 repressor was converted to that of the P22 repressor by substituting five amino acids of the P22 sequence for the corresponding amino acids of the 434 repressor along a 39 bp duplex sequence by cassette mutagenesis (19). Cassette mutagenesis was used to investigate the role of the 5' ends of mRNA in the CYC1 gene of Saccharomyces cerevisiae (20).

Cassette mutagenesis becomes even more rapid and powerful when applied to a system with a convenient phenotypic screen. Large numbers of mutants may be generated simultaneously and then screened for a particular function. Mutants of interest can be selected on the basis of their phenotypic behavior and investigated further. The existence of a phenotypic screen for enzymatic activity of ß-lactamase makes it a convenient system for such studies using cassette mutagenesis.

Class A ß-lactamases, of which the RTEM-1 enzyme is a member, maintain a conserved triad of amino acids, Ser-Thr-

Xaa-Lys, where the serine is the active site nucleophile (21-24). The role of the conserved Thr has been investigated using oligonucleotide-directed mutagenesis (25,26), and cassette mutagenesis (17). Thr71 of RTEM-1 ß-lactamase appears to be conserved more for reasons of protein stability and less importantly for catalysis or substrate binding. A Lys residue three amino acids to the carboxy side of the active site Ser is conserved not only in Class A ß-lactamases but also in Class C ß-lactamases, the related D,D-carboxypeptidases-transpeptidases, and in all higher molecular weight penicillin-binding proteins sequenced to date (21,27,28). This fact alone suggests a critical role for this conserved lysine. Data from chemical modification experiments using methyl acetimidate indicate the existence of an essential amino group that is protected from reaction by the presence of substrate (29). Inactivation by phenylpropynal (30) and phenylglyoxal (31) is consistent with an essential lys or arg. Studies of the inactivation of ß-lactamase by the mechanism-based inactivators penicillanic acid sulfone (32), clavulanic acid (33) and 6-acetylmethylene penicillanic acid (34) indicate the involvement of a nucleophile other than the serine close to the active site. The conserved lysine is a logical possibility for such a nucleophile.

This chapter describes the use of cassette mutagenesis to replace the conserved lysine of RTEM-1 ß-lactamase with the other nineteen amino acids. A duplex synthetic oligo-

nucleotide containing a mixture of codons in place of the lysine codon was ligated to restriction fragments of pBR322. The ligation mixture was used to transform E. coli to tetracycline resistance. Plasmids derived from one hundred Tet^R colonies were sequenced. Due to an uneven mixture of bases in the mixed codon the mutants Lys73-->Asp, Glu and Pro were not located and were constructed separately. None of the nineteen mutants conferred resistance to cells to low levels of ampicillin. Western blot analysis of whole cell extracts showed each of the mutants was present in the cells in amounts comparable to the wild-type enzyme.

Materials and Methods

Enzymes and Chemicals

Restriction enzymes and the large (Klenow) fragment of DNA ploymerase I were purchased from Boehringer Mannheim. T4 polynucleotide kinase and T4 DNA ligase were from Bethesda Research Laboratories. Antibiotics were obtained from Sigma Chemical Co. The [α-^{32}P]dTTP, 3000 μCi/mmol was obtained from Amersham, and the [γ-^{32}P]ATP was from ICN. All other chemicals used were reagent grade.

DNA

Oligonucleotides were synthesized by using the phosphoramidite chemistry (35) on the Applied Biosystems (Foster City, CA) DNA synthesizer, model 380A and purified by preparative polyacrylamide gel electrophoresis, followed by desalting by G-25 chromatography. The mixed codon was synthesized by using an equimolar mixture of the four nuleotide phosphoramidites or an equimolar mixture of G and C phosphoramidite in a normal coupling reaction.

DNA fragments were purified from 1.2% agarose gels using either DEAE-cellulose paper or an International Biotechnologies, Inc. (New Haven, CT) UEA electroeluter.

Plasmid DNA was prepared by the alkaline lysis method (36). The numbering system for pBR322 is the one commonly used (37). *E. coli* strain LS1 (38) was used as host for pBR322 and its derivatives.

Reactions and Mutagenesis

The complementary mutagenic oligonucleotides were phosphorylated using standard procedures (37), and were annealed by mixing 0.4 pmole/μL of each strand and heating to 95°C in 10 mM $MgCl_2$, 50 mM Tris-HCl, pH 8, followed by slow cooling over a one hour period to room temperature.

The three fragment ligation scheme was used to generate the mutants (17). The mutagenic oligonucleotides shown in Figure 2 were kinased and annealed as described. For the ligation approximately 0.04 pmole of each restriction fragment and 0.4 pmole of the synthetic fragments were mixed in 10 mM $MgCl_2$, 50 mM Tric-HCl, pH 8, 0.5 mM ATP, 5 mM dithiothreitol and 10 units of T4 ligase and incubated at 15°C for approximately 16 hours. The ligation was terminated by heating to 60°C for 10 minutes and a 10 μL aliquot was used to transform E. coli LS1 to tetracycline resistance, using standard transformation procedures (39).

Growth on antibiotics

Transformation resulted in approximately 450 Tet^R colonies. One hundred of these were picked with sterile toothpicks onto defined positions on L agar plates containing either 15 μg/mL tetracycline or 25 μg/mL ampicillin and incubated at 37°C for 12 to 16 hours. Thirteen of the one hundred colonies grew on ampicillin. Plasmid was purified from each of the one hundred colonies as described and used for sequencing.

Sequencing

Plasmid was digested with AvaI restiction endonuclease and the resulting 2962 bp fragment isolated as described before. The fragment was labeled at nucleotide T-3973 using [α-^{32}P]dTTP and the large (Klenow) fragment of DNA polymerase I, as shown in Figure 2. The labeled fragment was precipitated from the polymerase solution with 2.5 volumes of ethanol and pelleted by centrifugation. The pellet was washed with 70% ethanol and dried in a Speedvac. The labeled fragment was sequenced by the method of Maxam and Gilbert (40). The sequencing gels were 40 cm long and contained 20% polyacrylamide (w/v) and 40% urea (w/v) and were run using standard conditions (37).

Western blotting

Cells containing plasmids for each of the nineteen mutations were grown to late log phase in LB broth with 15 μg/mL tetracycline. One ml of cells was pelleted by centrifugation and resuspended in 50 μL of SDS-PAGE sample buffer (10% (v/v) glycerol, 3% (w/v) sodium dodecyl sulfate, 62.5 mM Tris-HCl, pH 6.8, 1 mM EDTA, 0.1 % bromphenol blue) and lysed by incubation in boiling water for 10 minutes. Aliquots of 20 μL were loaded onto a 15 cm 12% polyacrylamide gel with a 2 cm 3.5% stacking gel and electrophoresed for 12 to 16 hours at 5 mA until the dye had run off the gel.

The protein was transferred from the gel to nitrocellulose (BA85, Schleicher and Schuell) using a Biorad Transblot cell. The gel was soaked in transblot running buffer (25 mM

glycine, 193 mM Tris, 20% methanol, pH 8.3) for 20 minutes. The gel was then sandwiched between the nitrocellulose and Whatmann No. 1 filter paper and electrophoresed for 5 to 7 hours at 40 to 60 volts.

ß-lactamase was visualized using a staining system based on anti-ß-lactamase antibody. After electrophoresis the nitrocellulose was washed in a solution of TPBS (10 mM sodium phosphate, 0.9% NaCl, pH 7.5, 0.05% Tween 20), 1% normal goat serum, 1% BSA for 12 hours at room temperature. The nitrocellulose was rinsed with TPBS and then transferred to a solution containing TPBS and a 1:1000 dilution of primary anti-ß-lactamase antisera. The antisera was the generous gift of J. Neitzel, and was raised in rabbits by injection of complete Freund's adjuvant containing denatured ß-lactamase that had been purified from a SDS-PAGE gel(25). Subsequent booster shots were made with ß-lactamase purified as described (25). The nitrocellulose was then stained using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA), according to the manufacturer's instructions (41).

Results

The three fragment ligation scheme used to generate mutants of ß-lactamase at position 73 is illustrated in Figure 1 of Chapter 2. One sample of plasmid pBR322ss was digested with AvaI and SalI restriction endonucleases and the 1042 bp fragment was isolated. Another sample of plasmid was digested with SalI and ScaI restriction endonuclease and the resulting 2692 bp fragment was isolated. The purified fragments were mixed with the annealed, kinased oligonucleotides shown in Figure 1 and ligated by the addition of T4 DNA ligase. The resulting ligation mixture was used to transform E. coli LS1 to tetracycline resistance, generating approximately 450 transformants. Of these 100 were picked with sterile toothpicks onto defined positions of L agar plates, containing either 15 mg/L tetracycline or 25 mg/L of ampicillin. Thirteen of the one hundred colonies grew on ampicillin.

Figure 3 in Chapter 2 describes the labeling scheme used for DNA sequencing. Plasmid derived from each of the 100 colonies was digested with AvaI restriction endonuclease and the 2962 bp fragment was isolated. Since AvaI restriction endonuclease leaves assymmetric overhangs, it was possible to label the fragment assymmetrically by adding radioactive T to one end with the Klenow enzyme. To suppress the 3'-5' endonuclease activity of the Klenow enzyme from digesting the opposite end of the fragment and exposing an A which could

lead to labeling of that end and confusion of the sequencing, a small amount of dGTP was added to the labeling reaction.

The sequences of the mixed codon from the 100 colonies are shown in Table 1. All thirteen of the colonies resistant to ampicillin contained lysine at position 73. Five of those contained the wild-type codon for Lys, TTT, and the remainder contained the codon TTC resulting from the incorporation of the synthetic oligonucleotides. All colonies containing the codon for lysine at position 73 were resistant to ampicillin. None of the colonies containing codons for other amino acids at position 73 were resistant.

The quality of the oligonucleotide synthesis is evidenced by the low frequency of point mutations in the synthetic sequence. Two transition errors, one insertion error and one deletion error were found. Seven of the one hundred colonies contained deletions at the initial base of the ScaI restriction fragment, probably the result of incorrect digestion by ScaI restriction endonuclease, and incorporation of the blunt end.

The mixed codon of the synthetic oligonucleotides did not contain an even mixture of the four bases. In the first two positions, A and T were present twice as often as G and C. Zon, et al (42), have shown that the G phosphoramidite decomposes more rapidly than the other three base phosphoramidites. Since the synthesis is done by adding equal amounts of all four phosphoramidites, a lower amount of G in

the synthesis will result in fewer G-containing oligonucleotides. When the complementary strands are annealed for ligation into the vector, the number of oligonucleotides containing G in the mixed codon will limit the complementary C-containing oligonucleotides resulting in a comparably lower percentage of C in the mixed codon as is observed in Table 2. Because of the uneven percentages of the bases in the mixed codon, not all twenty amino acids were found in the first one hundred colonies. Lys73-->Asp, Glu and Pro were not found and were constructed separately using the oligonucleotides shown in Figure 1, and the same three fragment ligation scheme.

To determine if cells harboring mutated plasmids produce ß-lactamase protein, western blots were run. The results are shown in Figures 2, 3, and 4. All nineteen single amino acid replacements had little apparent effect on the intensity of the staining of the ß-lactamase band of these blots, demonstrating the mutants were produced in amounts comparable to the wild-type enzyme and were of comparable stability. LS1 cells without plasmid showed no band corresponding to ß-lactamase. The presence of unidentified proteins from E. coli that cross-react with the anti-ß-lactamase antibody serves as a useful internal control demonstrating that comparable amounts of the extracts were loaded onto each lane of the gel.

Discussion

Cassette mutagenesis is an enormous improvement in mutagenic techniques because it provides a rapid, efficient method for the production of one or more mutants at one or more positions across a segment of a gene. The size of the segment is determined by the placement of convenient unique or nearly unique restriction sites and the limit of efficient synthesis of oligonucleotides, typically one hundred bases or less. In this work, cassette mutagenesis has been used to generate nineteen amino acid replacements and one amber mutation at the 73rd amino acid of ß-lactamase.

The quality of the oligonucleotide synthesis is of paramount importance when using cassette mutagenesis to produce more than one mutation simultaneously. Of the one hundred plasmids sequenced only four contained errors in the synthetic oligonucleotides: one insertion, one deletion and two transversions. By contrast, in the production of twenty mutants of subtilisin approximately 30% of the plasmids sequenced contained errors (18). Seven deletion errors at the initial base of the ScaI-SalI restriction fragment were found, probably the result of incorrect restriction by ScaI endonuclease or incorrect ligation. Similar deletions were observed in the ligation of synthetic DNA into the PstI site of the subtilisin gene (18).

Close to equimolar ratios of the four bases at the

position of the mixed codon are required for easy, rapid location of the twenty mutants if a site is to be saturated. This requirement is even more stringent if saturation is to be extended to two or more sites simultaneously to ensure production of all permutations of amino acids at the two sites. Unfortunately the mixed codon of the oligonucleotides used to saturate Lys73 did not contain an even mixture of all four bases. The ratio of A and T to G and C was about 2:1 in the first two positions of the mixed codon. The reason for the uneven mixture probably is the reported greater instability of the G phosphoramidite (39). Since C will be opposite G in the complementary strand the lower amount of G will be the limiting factor reducing the number of C-containing sequences. Because of the uneven mixture of bases in the mixed codon only 25 of the 32 codons were found (Table 4) and not all twenty amino acids were found in the first one hundred colonies; accordingly the mutations Lys--> Asp, Glu and Pro were made separately. The third position of the mixed codon contained only G and C since this cuts in half the number of codons produced without eliminating any amino acid and also provides a means of distinguishing between the Lys codon of the wild-type sequence (AAA) and that of the synthetic oligonucleotides (AAG).

Several factors can effect the level of phenotypic resistance to antibiotics including plasmid copy number, transcription, mRNA stability, secretion and processing of

pre-ß-lactamase, thermal stability, stability to native proteases, and enzymatic activity. Plasmid copy number is controlled by a region of the plasmid far removed from the site of mutation. Additionally no significant change in the yield during plasmid purification for sequencing was observed. Codon usage can effect the level of translation (43). However all of the codons found at the site of mutation except that for Phe are used elsewhere in the ß-lactamase gene (44). Western blotting shows that all nineteen mutants were present in cell extracts at levels comparable to the level of wild-type enzyme indicating that the stability of the mutants to thermal denaturation and proteolysis is not significantly affected. Western blotting also shows, at least in most lanes, a faint band 2-3 kdaltons above the ß-lactamase band that is absent in the LS1 extract. This band probably corresponds to pre-ß-lactamase and, since it stains only faintly compared to the ß-lactamase band, it infers that most of the preprotein is processed correctly. Mutations may effect the secretion of the protein, as is the case with maltose binding protein (43). Mutants Thr71-->Ser and Ser70--> Thr are processed and secreted normally in S. typhimurium (25). The mutant Cys77-->Ser is also secreted normally (46).

The inherent enzymatic activity of the mutants is therefore the most likely reason for the absence of phenotypic resistance to ß-lactam antibiotics. The Ser70-->Cys mutant maintains 1-2% of the wild-type activity on ampicillin and

shows phenotypic resistance to the antibiotic at a concentration of 10 mg/L which is approaching the selection limit. A mutant showing approximately 0.1% of the wild-type activity on ampicillin is not phenotypically active (26). Thus, if one assumes the stability and production of the mutant and wild-type proteins are equivalent, the activity cutoff for phenotypic selection on ampicillin falls somewhere between 0.1 and 1% of the activity of the wild-type enzyme. Presumably, the activity all of the mutants at Lys73 is below this cutoff value.

Such a drastic reduction in catalytic efficiency is not without precedent in mutagenesis. The mutant Thr71-->Ile of the ß-lactamase from S. aureus PC1 is inactive (47). Structural studies of the mutant indicate that it is improperly folded. A mutant of tyrosyl tRNA synthetase with efficiencies less than $1/10^5$ that of the wild type enzyme has been constructed (48). A mutant of dihydrofolate reductase, Leu54-->Gly, shows a 10^4-fold reduction in the rate of hydride transfer (50).

A reduction in catalytic efficiency of 10^3 or greater compared to the wild-type enzyme may still mean the mutants are true enzymes. Catalysis is defined by an enhancement of the rate over the background rate and the turnover of more than one molecule of substrate per enzyme molecule. The background hydrolysis rate for benzylpenicillin at pH 7 is 0.2 M^{-1} sec^{-1} (49). The wild-type catalyzes the hydrolysis

of benzylpenicillin with an efficiency of 10^8 $M^{-1}sec^{-1}$. Therefore a reduction of three orders of magnitude would still mean a rate enhancement of 10^6 over the background rate.

Summary

The lysine residue at position 73 of RTEM-1 ß-lactamase has been replaced by the other nineteen amino acids and an amber stop codon by cassette mutagenesis. Due to an uneven mixture of bases at the mixed codon not all nineteen mutants were located in the first one hundred plasmids that were sequenced. Therefore the mutants Lys73-->Asp, Glu and Pro were constructed separately by cassette mutagenesis. None of the mutants confer to cells resistance to low levels of ampicillin indicating a reduction in catalytic efficiency to at most 1% that of the wild-type enzyme. Western blots of whole cell extracts indicate that all nineteen mutants are produced in the cell in amounts comparable to the wild-type. Therefore the lack of phenotypic resistance to ß-lactam antibiotics is not due to problems of transcription or translation.

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Figure 1

Oligonucleotides used for mutagenesis

1. Oligonucleotides used for the attempted saturation
2. Oligonucleotides used to create the mutant Lys73-->Pro
3. Oligonucleotides used to create the mutants Lys73-->Asp, Glu

1.

```
                                                    [AA ]
5' CC GAG GAA CGT TTT CCA ATG ATG AGC ACT TTT       [CCC] GT
      C CTT GCA AAA GGT TAC TAC TCG TGA AAA         [GGG] CA 5'
                                                    [TT ]
```

2.

```
5' CC GAG GAA CGT TTT CCA ATG ATG AGC ACT TTT CCG GT 3'
      C CTT GCA AAA GGT TAC TAC TCG TGA AAA GGC CA 5'
```

3.

```
                                                    [ G]
5' CC GAG GAA CGT TTT CCA ATG ATG AGC ACT TTT       [GAC] GT 3'
      C CTT GCA AAA GGT TAC TAC TCG TGA AAA         [CTG] CA 5'
                                                    [ C]
```

Table 1. Sequences of the mixed codon

Sequence Number	Sequence	Amino Acid	Active	Comment
1.	CAC	His		
2.	TTC	Phe		
3.	AGC	Ser		
4.	ACC	Thr		
5.	AAA	Lys	X	
6.	TTC	Phe		
7.	GGG	Gly		
8.	ATG	Met		a
9.	CAG	Gln		b
10.	AAG	Lys	X	
11.	ATG	Met		
12.	GTC	Val		
13.	GGG	Gly		
14.	TGC	Cys		
15.	TCG	Ser		c
16.	TGC	Cys		c
17.	GTC	Val		
18.	TCG	Ser		
19.	TGG	Trp		
20.	AAA	Lys	X	
21.	AAG	Lys	X	
22.	AAG	Lys	X	
23.	CGC	Arg		
24.	ATG	Met		
25.	AAA	Lys	X	
26.	AAA	Lys	X	
27.	AAG	Lys	X	
28.	AAG	Lys	X	
29.	AAG	Lys	X	
30.	AAG	Lys	X	
31.	AAA	Lys	X	
32.	TTC	Phe		
33.	CTC	Leu		
34.	GTC	Val		
35.	AAG	Lys	X	
36.	GTC	Val		
37.	GCC	Ala		
38.	TAC	Tyr		d
39.	AGC	Ser		
40.	TGC	Cys		

Table 1, continued.

Sequence Number	Sequence	Amino Acid	Active	Comment
41.	ACC	Thr		
42.	ATC	Ile		b
43.	CTC	Leu		
44.	CTC	Leu		
45.	TAG	Stop		c
46.	GGG	Gly		c
47.	TAC	Tyr		
48.	TAC	Tyr		
49.	GTC	Val		
50.	TCG	Ser		c
51.	TAC	Tyr		
52.	?			
53.	?			
54.	CAG	Gln		
55.	A?C			
56.	TTC	Phe		
57.	GGG	Gly		
58.	AAC	Asn		
59.	AAC	Asn		
60.	AAC	Asn		
61.	TCG	Ser		
62.	TTC	Phe		
63.	ATG	Met		
64.	TCG	Ser		
65.	CAG	Gln		
66.	TTG	Leu		
67.	?			
68.	CTC	Leu		
69.	TCG	Ser		
70.	ATC	Ile		
71.	GAG	Glu		c
72.	?			
73.	GCC	Ala		
74.	CGC	Arg		
75.	AGG	Arg		c
76.	GTC	Val		
77.	GCC	Ala		
78.	TAC	Tyr		
79.	AAC	Asn		
80.	TAC	Tyr		

Table 1, continued.

Sequence Number	Sequence	Amino Acid	Active	Comment
81.	TTG	Leu		
82.	CAC	His		
83.	TAG	Stop		
84.	?			
85.	TAC	Tyr		
86.	C?G			
87.	ATC	Ile		
88.	CTG	Leu		
89.	AAC	Asn		
90.	TAC	Tyr		
91.	?			
92.	?			
93.	TAG	Stop		
94.	TAC	Tyr		
95.	TCG	Ser		
96.	TCG	Ser		
97.	AAC	Asn		
98.	CCG	Pro		
99.	GAC	Asp		
100.	GAG	Glu		

comments-
a- transversion error
b- deletion error
c- deletion at ScaI site
d- insertion error

Table 2. Percentages of bases in mixed codon.

	Position		
	First	Second	Third
A	34	44	
T	36	26	
G	17	15	43
C	13	15	56

Table 3. Mutants chosen for Western Blotting

Amino Acid	Codon	Growth on 25 mg/L Ampicillin
Lys	AAA(wildtype)	+++
Lys	AAG	+++
Arg	CGC	0
His	CAC	0
Trp	TGG	0
Tyr	TAC	0
Phe	TTC	0
Ser	TCG	0
Thr	ACC	0
Cys	TGC	0
Met	ATG	0
Asp	GAC	0
Glu	GAG	0
Asn	AAC	0
Gln	CAG	0
Ala	GCC	0
Gly	GGG	0
Ile	ATC	0
Leu	CTC	0
Val	GTC	0
Pro	CCG	0

Table 4. Codons found

Codon	Number found
CAC	2
TTC	5
AGC	2
ACC	2
AAA	5
GGG	4
ATG	4
CAG	3
AAG	8
TGC	3
TCG	8
TGG	1
CGC	2
CTC	4
GTC	6
GCC	3
TAC	9
ATC	3
TAG	3
AAC	6
TTG	2
GAG	2
AGG	1
CTG	1
GAC	1
CCG	1

Codons not found-
ACG GTG GGC GCG CGG CCC TCC

Figure 2

Western Blots of whole cell extracts containing the indicated mutants of ß-lactamase at position 73. The blots were stained according to the procedures described in the Materials and Methods section.

β-Lac
Lys
LSA
Gly
Ala
Val
Leu
Ile
Asp
Glu

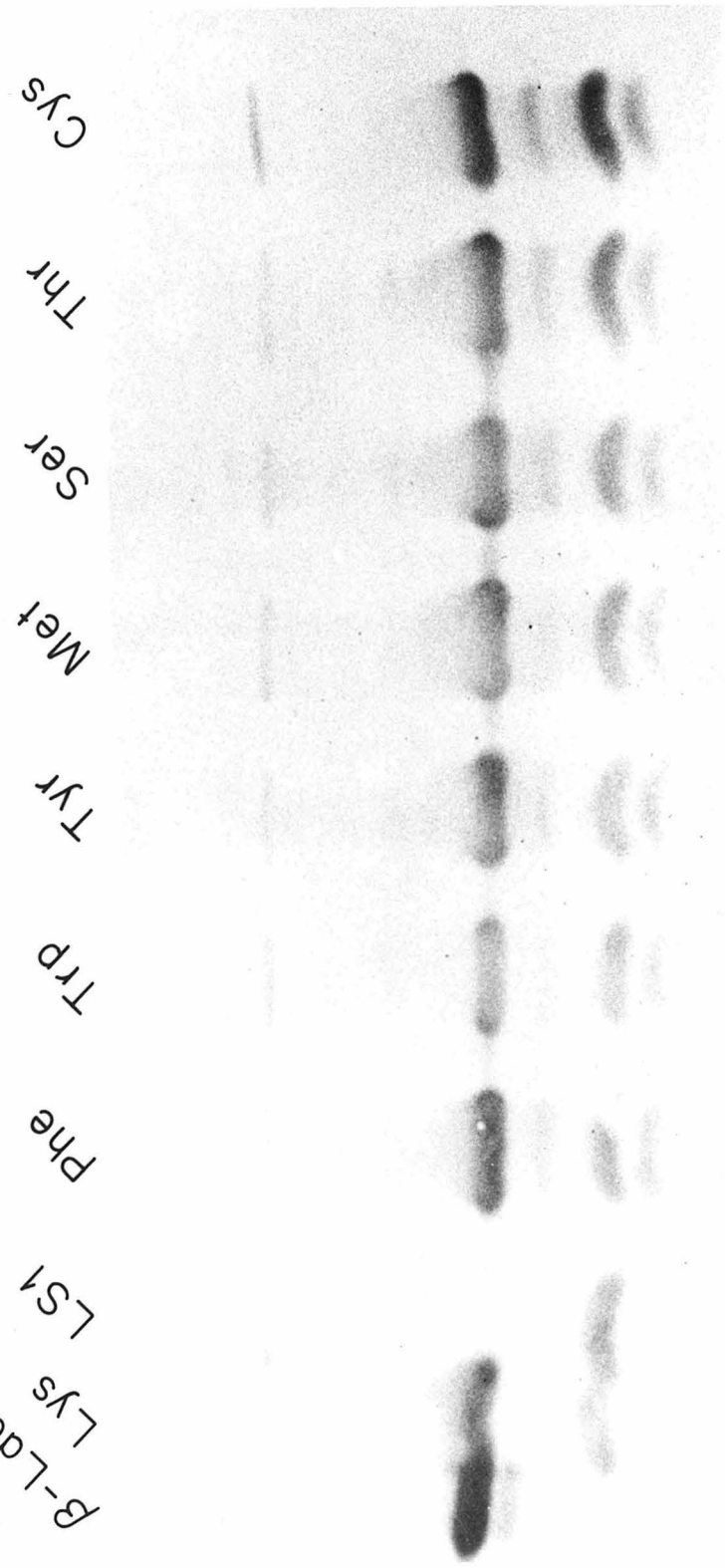
Figure 3

Western Blots of whole cell extracts containing the indicated mutants of ß-lactamase at position 73. The blots were stained according to the procedures described in the Materials and Methods section.

β-LAC
Lys
LSA
Asn
Gln
Arg
His
Pro

Figure 4

Western Blots of whole cell extracts containing the indicated mutants of ß-lactamase at position 73. The blots were stained according to the procedures described in the Materials and Methods section.


Cys
Thr
Ser
Met
Tyr
Trp
Phe
LS1
Lys
β-Lac

Chapter 4

Purification and Characterization of Two Mutants of RTEM-1 ß-lactamase at Lysine 73

Introduction

ß-lactamases catalyze the hydrolysis of the amide bond of the ß-lactam ring of penam and cephem antibiotics (1). Two general mechanisms have been proposed for the catalytic mechanism of ß-lactamases. The enzyme may act either through general acid-base catalysis directing the nucleophilic attack of water on the ß-lactam ring or through attack by a nucleophile of the enzyme itself on the ß-lactam ring. The preponderance of evidence with Class A ß-lactamases suggests the existence of a covalent acyl-enzyme intermediate, consistent with direct nucleophilic catalysis by the enzyme. The first evidence for the acyl-enzyme intermediate came from studies of the hydrolysis of the poor substrate cefoxitin (k_{cat}/K_M = 6 $M^{-1}sec^{-1}$) (2). The slow turnover of cefoxitin by the RTEM-2 enzyme allowed the observation of the buildup and breakdown of a putative acyl-enzyme intermediate. Denaturation of the intermediate followed by gel filtration and Fourier transform IR spectroscopy led to the identification of the intermediate as containing an ester linkage. The existence of an acyl-enzyme intermediate during the catalysis of the good substrates dansyl-penicillin and dansyl-cephalosporin was proven by low temperature and pH studies (3,4). The active site serine nucleophile was identified by reaction with a mechanism-based inactivator, 6-ßbromopenicillanic acid (5,6,7).

Evidence for the involvement of other amino acids in catalysis by ß-lactamases is sparse. Chemical modification

experiments have implicated most amino acid side chains as important in catalysis (9). Oligonucleotide-directed mutagenesis experiments have concentrated on the conserved amino acids about the active site serine. Saturation mutagenesis of Thr71 of RTEM ß-lactamase has shown it to be conserved more for protein stability than for catalysis (10,11). As discussed in Chapter 3 of this work, no other amino acid coded for by DNA can substitute for Lys73 of RTEM ß-lactamase and give rise to an enzyme that can impart to cells a phenotype resistant to low levels of ampicillin.

Evidence that the conserved lysine is in the vicinity of the substrate binding site comes from x-ray crystallographic studies of a related carboxypeptidase. One of the proteins involved in bacterial cell wall biosynthesis is D-ala,D-ala carboxypeptidase, the enzyme responsible for cleaving the terminal D-ala from the crosslinking peptide and regulating the amount of crosslinking in the cell wall. D-ala,D-ala carboxypeptidase binds penicillin but deacylates only very slowly. A relatively detailed crystal structure of the carboxypeptidase from Streptomyces R61 has been solved (12). The significant similarities between the secondary structural characteristics of the carboxypeptidase and the best crystal structure of a ß-lactamase, that of the enzyme from B. licheniformis 749/c as shown in Figure 1A, support the theory that the two enzymes are evolutionarily related (13,14). The similarities between the two structures and the

significant sequence homology also allow the use of the active site structure of the carboxypeptidase as a working model for that of the ß-lactamase. D-ala,D-ala carboxypeptidases maintain a conserved triad, Ser-Xaa-Thr-Lys, about the active site serine (15). As shown in Figure 1B the conserved lysine is in the vicinity of the substrate binding site and is apparently located on the opposite side of the bound substrate molecule from the serine. Thus the position of the conserved lysine may allow the interaction of its side chain with the bound substrate molecule.

This chapter describes the purification and kinetic characterization of two of the mutants at position 73 of the RTEM-1 ß-lactamase, Lys73-->Arg and Lys73-->Cys. These two mutants were chosen because arginine is the most conservative amino acid change from lysine and because cysteine is chemically versatile. The EcoRI-PvuI fragments of the two mutated genes were transferred to plasmid pJN for expression. Induction by the addition of IPTG allowed the easy identification by SDS-PAGE gel or western blotting of the position of mutant ß-lactamases during the purification procedure. The Michealis-Menten parameters for benzylpenicillin were obtained and showed the mutations caused a 10^4 reduction in k_{cat} for both mutants while K_M was unchanged from the wild-type value. Lys73-->Arg was active on cephalothin but no activity was detected for Lys73-->Cys on cephalothin indicating that the positive charge of the side chain may help position the

bulkier cephems for acylation. Reaction of the Lys73-->Cys mutant with ethylenimine under mildly denaturing conditions caused the reactivation of the mutant after renaturation. The Lys73-->aminoethylCys derivative regained 62% of the efficiency of the wild-type enzyme on benzylpenicillin and 9% of the efficiency of the wild-type enzyme on cephalothin. These results prove that the amino group of the side chain of Lys73 of RTEM-1 ß-lactamase is essential for efficient hydrolysis of penams and cephems but is not essential for binding of substrate.

Materials and Methods

Enzymes and Chemicals

Restriction enzymes were purchased from Boehringer Mannheim. T4 DNA ligase was obtained from Bethesda Research Laboratories. Antibiotics were from Sigma Chemical Co. Tryptone and yeast extract were from Difco (Detroit, MI). Ethylenimine was synthesized from 2-aminoethyl hydrogen sulfate (Aldrich), according to published procedures (16). The fraction boiling at 95-100°C was collected and used immediately for the derivatization. Remaining stock ethylenimine was immediately destroyed by addition of ß-mercaptoethanol.

Bacterial strains

E. coli LS1 (17) was used as host of plasmid pBR322 and the derivatives of pBR322. *E. coli* D1210 was used as the host strain for plasmid pJN and is a lac I^q derivative of HB101.

DNA

DNA fragments were purified from 1.2% agarose gels using an International Biotechnologies, Inc. (New Haven, CT) UEA electroeluter.

Plasmid DNA was prepared by the alkaline lysis method (18). The numbering system for pBR322 is the one commonly used (19).

Subcloning into pJN

Plasmids pBR322K73C and pBR322K73R and pJN were digested

with EcoRI and PvuI restriction endonucleases and the resulting 626, 626, and 4800 bp fragments isolated by electroelution, respectively. The 626 bp fragments of the two mutants were ligated separately to the 4800 bp fragment of pJN using procedures described above. Transformation of E. coli D1210 to kanamycin resistance was performed as described. To confirm the presence of the mutated gene, plasmid derived from a kanamycin resistant colony was digested with AvaI restriction endonuclease and run on a 1.2% agarose gel. The additional AvaI restriction site from plasmid pBR322ss was confirmed.

Purification of Lys73-->Cys and Lys73-->Arg

The purification scheme used was basically that described in Chapter 2 but with a few minor modifications.

Single colonies of E. coli D1210 harboring plasmids pBR322K73C or pBR322K73R were used to inoculate 2 ml cultures of L broth containing 25 mg/L kanamycin which were grown to late log phase. One mL was used to inoculate a 250 mL culture of L broth containing 25 mg/L kanamycin which was incubated at 37°C with shaking for 8-10 hours. Fifty to 100 mLs of the culture was used to inoculate 10 L of FB or XB media containing 25 mg/L kanamycin in a New Brunswick Magnaferm fermenter. The culture was grown at 37°C with vigorous aeration for 12-14 hours. IPTG was added to 0.1 mM and induction continued at 37°C for from 20 to 75 minutes. Cells were harvested in a Beckman J-6B centrifuge at 2900 rpm

and were washed with 50 mM Tris, pH 7. The osmotic extrusion was performed as described (2). The ammonium sulfate precipitation was carried out as described in Chapter 2. The 20-60% AS fraction was dialyzed against deionized, distilled water and concentrated to 30-50 mls in 25 mM triethanolamine, pH 7.25 (TEAC). The samples were then applied to a 2.5 by 30 cm DE-52 column and eluted by using a linear gradient of from 25 to 200 mM TEAC, pH 7.25. Mutant protein was located in the column fractions by either migration on SDS-PAGE gel, western blotting, or activity. Peak fractions were pooled and concentrated to 1-2 mls in either 20 mM Tris, pH 7, or in 25 mM TEAC, pH 7.25. The samples in Tris were applied to a 1 by 82 cm Ultragel ACA 54 column and eluted with the same buffer. The samples in TEAC were purified by FPLC using a Pharmacia Mono Q 5/5 column and the following discontinuous gradient. Buffer A is 25 mM TEAC, pH 7.25. Buffer B is 200 mM TEAC, pH 7.25. At time 0, 100% A; time 15 minutes, 85% A; time 40 minutes, 70% A. Flow rates were 0.8 ml/minute. Overall yields were approximately 5 mgs of mutant protein.

Assays

Protein concentrations were determined by absorbance at 281 nm using the conversion factor, 29,400 $M^{-1}cm^{-1}$ (2). Activity assays were done at 30°C in 0.1 M potassium phosphate, pH 7, using a Beckman DU-7 spectrophotometer with 1 cm path length cells for assays of activity on benzylpenicillin or ampicillin. For assays of activity on cephalothin an optical

quartz block was inserted into the cuvet to reduce the path length to 1 mm. Hydrolytic cleavage of cephalothin was followed at 265 nm, at 240 nm for benzylpenicillin and at 235 nm for ampicillin. The $\Delta\epsilon$ values for cephalothin, benzylpenicillin and ampicillin were 7900, 500, and 825 $M^{-1}cm^{-1}$, respectively (20).

Western blotting

Aliquots of 20 μL of selected fractions from the DE-52 column were electrophoresed on a 15 cm 12% SDS-polyacrylamide gel with a 2 cm 3.5% stacking gel. Protein was transferred to nitrocellulose (Schleicher and Schuell, BA 85) using a Biorad Transblot cell with a 40 to 60 volt field for 5 to 7 hours. ß-lactamase was visualized using a staining system based on anti-ß-lactamase antibody and a Vectastain staining kit (Vector Laboratories, Burlingame, CA) as described in Chapter 2.

Reaction of Lys73-->Cys with ethylenimine

Mutant protein (2.0 mL, 0.36 mg/mL, in 300 mM Tris, pH 8.4, purified by FPLC) was made 4.5 M urea by addition of solid urea. An aliquot of 200 μL of freshly synthesized ethylenimine was added and the solution incubated at RT for 35 minutes. Another 200 μL aliquot of ethylenimine and 0.06 g urea were added and the solution incubated for 35 minutes. A 100 μL aliquot of 3 M Tris pH 7 was added and the solution dialyzed against 3 L of 25 mM Tris, pH 7. The solution was then dialyzed against 25 mM TEAC, pH 7.25, before FPLC

purification using the conditions described in the purification section.

Ethylenimine reaction conditions

Mutant Lys73-->Cys (0.17 mg/ml, in 0.5 M sodium phosphate, pH 8.4) was divided into six 400 μL aliquots. The conditions listed in Table 1 were used for the derivatization reaction. Three 40 μL additions of ethylenimine were made separated by 40 minute reaction times. After the reaction the samples were dialyzed against 25 mM Tris, pH 7, using Spectrapor 10K MW cutoff dialysis tubing. Samples were assayed for protein concentration and ß-lactamase activity.

Attempt to observe catalytic burst

The mutant Lys73-->Arg at protein concentrations of 0.6, 0.9 and 1.8 mg/ml in 25 mM Tris, pH 7 was incubated in a cuvet at 30°C containing an optical quartz block for 10 minutes. The solution was made 2 mM in cephalothin and the change in absorbance followed at 260 nm. After 40 to 60 minutes of reaction a 30 μL aliquot was removed and assayed for activity on benzylpenicillin using standard assay conditions.

Search for active revertants

E. coli LS1 harboring plasmids pBR322K73R and pBR322K73C were grown to saturation at 37°C in L broth containing 15 mg/L tetracycline. An aliquot (100 μL) was plated onto an L agar plate containing 15 mg/L ampicillin and incubated at 37°C. Serial dilutions of cells from the tetracycline

culture were made and plated onto L agar plates containing 15 mg/L tetracycline to quantitate the number of cells plated.

Results

The purification of presumably inactive mutants of ß-lactamase is greatly simplified by the use of a strong inducible promoter such as the tac promoter. The tac promoter is a fusion of the lac and trp promoters and, when used in a strain that constitutively expresses the lac repressor, effectively shuts off expression of ß-lactamase until the addition of IPTG (21). Therefore, the tac promoter may be useful for the expression of lethal proteins. The induction was carried out at 37°C for from 20 to 75 minutes since the thermal and proteolytic stability of mutants of ß-lactamase at Lys73 in vivo as determined by western blotting was shown to be comparable to the stability of the wild-type enzyme. The overproduction of ß-lactamase following the addition of IPTG allows the easy identification of its position in column fractions during the purification procedure. The osmotic extrusion and ammonium sulfate precipitation were carried out without the benefit of an assay, on the assumption that the mutants would behave similarly to the wild-type enzyme. After chromatography on DE-52 cellulose the position of the mutant ß-lactamase was determined by either western blotting, by mobility on SDS-PAGE gels, or, after discovery of low levels of activity on benzylpenicillin, by activity assay (Figure 3). Identification of ß-lactamase bands on SDS-PAGE gels is facilitated by the unusual orange-brown color of the ß-lactamase band on staining by silver (22), when most pro-

teins stain gray to black. Subsequent gel filtration chromatography gave apparently homogeneous protein.

Purification of ß-lactamase by FPLC represents a major simplification and improvement over the use of the gel filtration column. The presence of a small amount of an unidentified contaminating protein that runs on SDS-PAGE gels at about the same molecular weight as ß-lactamase was removed by FPLC purification. The use of the DE-52 column prior to FPLC chromatography will ensure removal of contaminating proteins that might bind irreversibly to the FPLC column. Representative FPLC chromatograms are shown in Figure 9 for the wild-type and Lys73-->Cys mutant using the conditions described in the Materials and Methods section.

Purified mutants Lys73-->Cys and Lys73-->Arg maintain low levels of activity on benzylpenicillin. The values for k_{cat} and K_M shown in Table 1 were obtained from Lineweaver-Burk plots generated from single reaction progress curves using [S], the average substrate concentration, and v, the average reaction velocity, over a region of the curve, according to published procedures (23). The value of k_{cat}/K_M on benzylpenicillin for Lys73-->Cys was .012% of the value for wild-type enzyme and the value of k_{cat}/K_M for the Lys73 -->Arg mutant was 0.015%. The value of k_{cat}/K_M for the Lys73-->Arg mutant on cephalothin is .007 % that of the wild-type enzyme. The upper limit of activity for the Lys73-->Cys mutant on cephalothin is approximately 0.001 sec^{-1}.

The values for $\Delta\Delta G_T^{\ddagger}$, the difference between the binding energies of the transition states of the mutant and wild-type enzymes can be calculated from the following equation,

$$\Delta\Delta G_T^{\ddagger} = -RT\ln[(k_{cat}/K_M)_A/(k_{cat}/K)_B].$$

The increase in the binding energy of the transition state of the mutants is 5.4 kcal/mole compared to the value of the wild-type enzyme on benzylpenicillin, and is 5.5 kcal/mole for the Lys73-->Arg mutant on cephalothin. The activation energy, $\Delta G_T^{\ddagger}$, is the sum of two components, $\Delta G^{\ddagger}$, the activation energy of bond making and bond breaking, and ΔG_S, the substrate binding energy. The Michaelis constant, K_M, is a function of ΔG_S. Since the values of K_M of the mutants on benzylpenicillin are comparable to the value for the wild-type enzyme, ΔG_S has not changed. Therefore the increase in $\Delta\Delta G_T^{\ddagger}$ is due to an increase in $\Delta G^{\ddagger}$, the activation energy involved in carrying out the chemical conversion of bound substrate to the transition state.

The activity associated with the mutants is due to the mutants and not to a small amount of contaminating enzyme of much higher specific activity. The activity cannot be due to a reversion to the wild-type codon at Lys73 since that would require a double mutation, which occurs less than 1 in 10^{12} loci. No phenotypically active revertants were found on plating 10^8 cells of E. coli harboring either plasmid pBR322K73C or pBR322K73R. The specific activity of four different preparations of the Lys73-->Cys mutant were within 20%

of each other, making an active reversion unlikely. Activity coelutes with the Lys73-->Cys mutant on the FPLC under conditions where proteins differing by only a single charge can be separated.

The variation of the rate of catalysis of the Lys73--> Cys mutant with increasing pH and with increasing temperature were determined and the results are shown in Figures 5 and 6, respectively. In an interesting contrast to the behavior of the wild-type enzyme which shows maximal activity at pH 6.5 and a decrease in activity at higher pH, the Lys73-->Cys mutant is most active at pH 8.3 and loses activity sharply below pH 8. Both the wild-type enzyme and the Lys73-->Cys mutant show an increase in activity with increasing temperature. However, the increase for the mutant is much greater than the increase for the wild-type enzyme. The pH of the assay buffer increased by .05 pH units on heating from 30 to 45°C, so the values for enzymatic activity of the mutant have been corrected for the increase that would result from increasing the pH.

The specific activity of the Lys73-->Cys mutant is greatly increased on treatment with ethylenimine. Initial efforts at converting Cys73 to aminoethylCys73 resulted in a mixture of partially derivatized protein with specific activities of about 20 sec^{-1}, compared to 0.25 sec^{-1} for the unreacted mutant. Isoelectric focussing gels of the reaction mixture showed most of the protein remained underivatized.

Since ethylenimine is known to be a highly reactive reagent (24,25) the most likely explanation of low yields of conversion was thought to be steric hindrance of the cysteine side chain at position 73. To increase the percentage of derivatization the reaction conditions were varied to unfold the native protein structure and expose the sulfhydryl to reaction, as described in Table 2. The highest specific activity was obtained with 4 M urea in the reaction mixture suggesting the Cys73 side chain is buried in the native protein and exposed in the partially denatured protein at pH 8.4. While the wild-type enzyme regains activity after exposure to 4 M urea at pH 7.0 when diluted into the assay buffer (10), under the conditions employed in this work at pH 8.4, a 50% irreversible activity loss was observed with the wild-type control reaction which may be due to the combination of the effects of high pH and urea. This activity loss of the wild-type enzyme was not dependent on the presence of ethylenimine. Higher protein concentrations, such as 0.6 mg/ml, than those described in Table 2 were found to increase the specific activity of the reaction mixture. However protein precipitated from the reaction solution during the dialysis. These observations are probably due to a cooperative effect of protein concentration on unfolding, because of favorable intermolecular associations when hydrophobic regions become exposed. The precipitated protein is probably in the form of aggregates of ß-lactamase. Therefore, an intermediate pro-

tein concentration of 0.3 mg/ml was chosen for the reaction. An FPLC chromatogram of the reaction mixture is shown in Figure 8. Though the elution times of the native and derivatized mutant vary slightly from native Cys73 and wild-type, respectively, the difference in elution times agree well (6.6 vs 6.7 minutes). The kinetic parameters of the chemical revertant were determined for benzylpenicillin and cephalothin and are shown in Table 1. The value for k_{cat}/K_M for the chemical revertant is 62% of that for the wild-type enzyme on benzylpenicillin, a 5000-fold increase over the value of the Cys73 mutant. Chromatography of urea-treated wild-type enenzyme that had been inactivated to 54% of its original specific activity on the FPLC caused a gain in specific activity to 69% of the original value. Therefore, chromatography on the FPLC probably removes some but not all of the denatured protein, and the value of the catalytic efficiency of the chemical revertant reflects a minimum value.

To determine at what point in the catalytic mechanism the mutants are defective, the ability of the Lys73-->Arg mutant to give burst kinetics on cephalothin was observed. A small burst of less than 0.003 OD was observed independent of protein concentration. Bursts of this magnitude were observed in the absence of mutant enzyme. Incubation of Lys73 -->Arg with cephalothin followed by 50-fold dilution into assay buffer with benzylpenicillin showed linear kinetics with no evidence of a lag in the assay. If the acyl-enzyme

intermediate is thermodynamically stable relative to the enzyme-substrate complex and if deacylation is the rate-determining step, then burst kinetics with cephalothin and a lag on dilution into a benzylpenicillin solution should have been observed. The acyl-enzyme intermediate is stable relative to the ES complex since the rate of ß-lactam ring closure has been measured to be one third the rate of ring opening (30). Therefore, the absence of a burst with cephalothin or a lag on dilution of cephalothin-treated enzyme into a solution of benzylpenicillin indicates that acylation is probably still the rate-determining step and therefore is the step affected by the mutation.

Discussion

None of the nineteen mutants resulting from amino acid substitutions at Lys73 of RTEM-1 ß-lactamase is able to confer a resistant phenotype to E. coli LS1 cells to low levels of ampicillin. As discussed in Chapter 3, the lack of phenotypic resistance could be the result of one or more of several factors, including problems with transcription, translation, translocation, stability to thermal denaturation or to proteolysis, or intrinsic enzymatic activity. The results of the western blots of Chapter 3, which showed that all nineteen mutants were present in amounts in vivo comparable to the amount of the wild-type enzyme rule out all possibilities except the intrinsic enzymatic activity. The mutants Lys73-->Cys and Lys73-->Arg were purified to prove that the intrinsic enzymatic activity of the mutants is lowered relative to that of the wild-type enzyme and to attempt to define the molecular basis for the defect in activity. Lys73-->Arg was chosen because it is the most conservative change of amino acid from lysine and Lys73-->Cys was chosen because of the chemical versatility of the sulfhydryl.

The purification of presumably inactive mutants of ß-lactamase is made possible by the use of the tac promoter. A fusion of the lac and trp operons, the tac promoter, when induced, can convert as much as 30% of the total cellular protein to the induced protein, allowing the easy identification of the protein by mobility on SDS-PAGE gels of total

cellular extracts (21). In the case of ß-lactamase, the location of the protein in the periplasmic space makes for easy fractionation of cellular proteins by osmotic extrusion which both eliminates cytosolic proteins and, since it releases mutant ß-lactamase into the extrudate, proves the processing and secretion of the mutants are not significantly affected. The identification of the location of the mutant ß-lactamases among the fractions off the column was confirmed by western blots and by low levels of ß-lactamase activity.

Despite the lack of a resistant phenotype to ampicillin in cells containing mutants, the purified mutants maintain a low level of activity on benzylpenicillin. The ratio of k_{cat}/K_M for both mutants to k_{cat}/K_M for the wild-type is $1x10^{-4}$ inferring an increase of 5.4 kcal/mole of activation energy. The Lys73-->Arg mutant maintains k_{cat} on cephalothin at 3% of its activity on benzylpenicillin, and the ratio of k_{cat} on cephalothin to that on benzylpenicillin for the wild-type enzyme is 6%. The upper limit for activity of the Lys73-->Cys mutant is estimated as 0.5% of its activity on benzylpenicillin. Since the wild-type enzyme and Lys73-->Arg mutant have appreciable activity on cephalothin and the Lys73 -->Cys mutant does not, these results indicate the positive charge of the side chains of Lys and Arg may assist in the catalysis of cephems. They also point out the more stringent requirements for hydrolysis of cephems, as evidenced by the finding that mutations at Thr71 also have a greater reduction

of catalytic efficiency on cephems compared to that on penams (10). The decrease of four orders of magnitude in catalytic efficiency for the mutants relative to the wild-type enzyme on benzylpenicillin does not mean the mutants are not enzymes. The background rate for the hydrolysis of penams and cephems has been measured as 0.2 $M^{-1}sec^{-1}$ so the mutants cause a $5x10^4$ increase in reaction rate (26). The value of K_M for both mutants on benzylpenicillin is not significantly different from the value for the wild-type enzyme. If one assumes that K_M reflects the intrinsic binding constant for substrate and enzyme, the binding is not significantly affected in the mutants. Therefore the main effect of the mutations is on k_{cat}, the catalytic rate.

The reactivation or chemical reversion of the Lys73--> Cys mutant was accomplished by reaction with ethylenimine. The addition of the ϵ-amino group to the amino acid at position 73 restores 62% of the catalytic efficiency on benzylpenicillin of the wild-type enzyme. This demonstrates both that the mutants are capable of efficient catalysis and that the only functionality they are missing is the amino group at position 73. The efficient conversion of the Cys73 mutant to aminoethylCys73 could only be accomplished under conditions where the protein was at least partially unfolded, indicating in the native state the side chain of Cys73 is buried in the protein or is sterically or electrostatically hindered to reaction with small molecules. By contrast, the side chain

of Lys73 is probably exposed to solvent in the native state since the wild-type enzyme can be separated from Cys73 by FPLC and the reaction of a Class A ß-lactamase from S. aureus PC1 with methyl acetimidate under nondenaturing conditions leads to inactivation presumably by reaction with an essential amino group (31). The reaction with ethylenimine was carried out at pH 8.4 since the sulfhydryl of Cys73 is negatively charged at that pH which will tend to increase both its exposure and nucleophilicity. Appreciable activity on cephalothin is gained by reaction of Cys73 with ethylenimine. The value of k_{cat}/K_M for the aminoethylCys73 on cephalothin is 9% that of the wild-type enzyme.

The molecular basis for the drastic reduction in the catalytic efficiency of the mutants relative to that of the wild-type enzyme could be the result of several factors. The amino group of the lysine side chain may participate in a hydrogen bond with the active site serine hydroxyl to increase the nucleophilicity of the hydroxyl. However, at pH 7 the ϵ-amino group of the lysine is most likely positively charged, making this possibility unlikely. Also, by the usual criterion of reaction with diisopropyl fluorophosphate the serine hydroxyl is not activated (27). Evidence exists that a conformational change occurs in the RTEM enzymes on binding substrate (28). The lysine side chain may be involved in mediating the conformational change which could be important in catalysis. The amino group may be important in

maintaining the correct tertiary conformation of the enzyme for efficient catalysis in a way that is not reflected by its in vivo stability to proteases. However, the value of K_M is not significantly changed from that of the wild-type enzyme, as might be expected if a conformational change had occurred. The amino group of the Lys73 side chain may interact directly with the bound substrate molecule, by an ion pair interaction with the carboxylate group of the thiazine or thiazolidine ring of cephems or penams, respectively. This interaction may serve to orient the substrate for efficient acylation. The interaction between the amino group and the carboxylate could serve to produce a torque on the ß-lactam ring, increasing the rate of hydrolysis. The ϵ-amino group of the lysine may act to polarize the lactam carbonyl making acylation more rapid. The rate of deacylation for D-alanine carboxypeptidase IA of E. coli was decreased on reaction of the enzyme with sulfhydryl reagents while the rate of acylation was not affected (29). The finding that the Lys73-->Cys mutant has its maximal turnover rate at pH 8.3, the pKa of the sulfhydryl of cysteine, indicates a nucleophilic anion at position 73 increases the activity of the mutant and may be involved in deacylation in a similar fashion. The ϵ-amino group may donate a proton either to the amide nitrogen after the lactam bond is broken or to the active site serine after deacylation.

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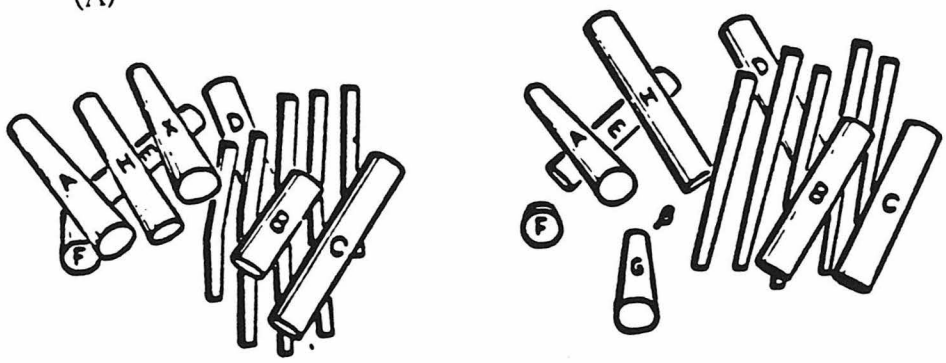
Figure 1

A) Secondary structural elements of ß-lactamase from B. licheniformis 749/c (left) and D,D-carboxypeptidase from Streptomyces R61 (right). Taken from reference 13.

B) The alpha carbon atoms 25-42 of the active site polypeptide with a bound cephalosporin of the D,D-carboxypeptidase from Streptomyces R61. Taken from reference 12. Ser37 of the D,D carboxypeptidase corresponds to Ser70 of ClassA ß-lactamases.

(A)



(B)

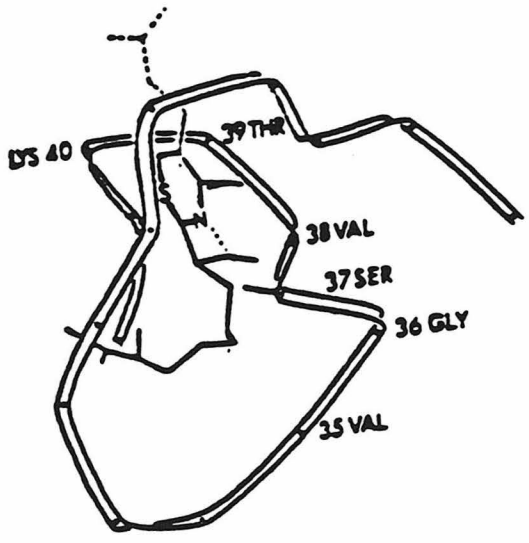
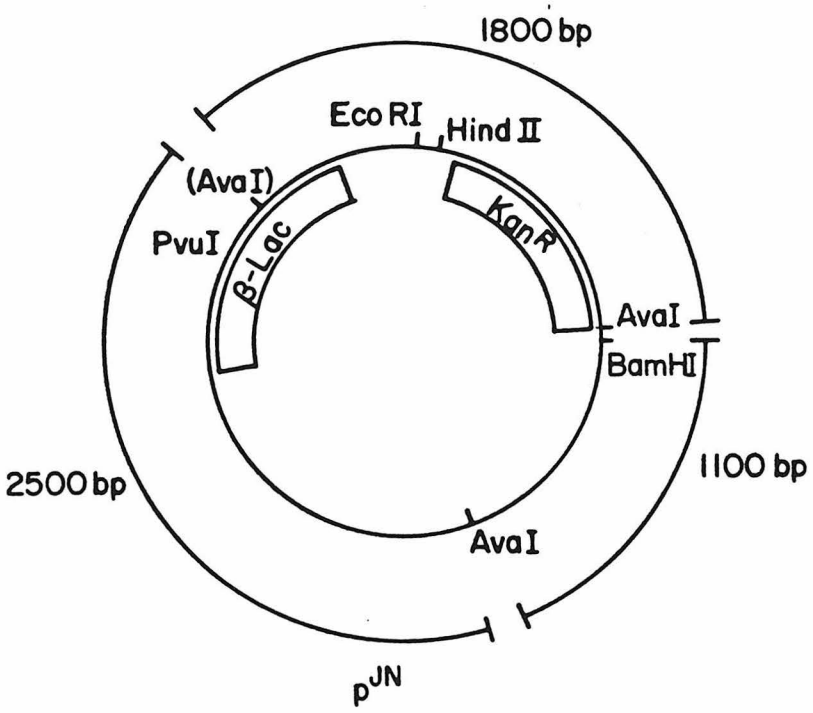


Figure 2

A) Partial restriction map of plasmid pJN

B) Agarose gel electrophoresis of restriction fragments generated by digestion by AvaI restriction endonuclease of plasmid pJN containing no mutation (lane 1), the Lys73 -->Arg mutation (lanes 2 and 3), the Lys73-->Cys mutation (lanes 4 and 5), the Lys73-->His mutation (lanes 6 and 7), the Lys73-->Ala mutation (lane 8).

A)


1800 bp
Eco RI
Hind II
(AvaI)
PvuI
β-Lac
KanR
AvaI
BamHI
1100 bp
2500 bp
AvaI
pJN

B)

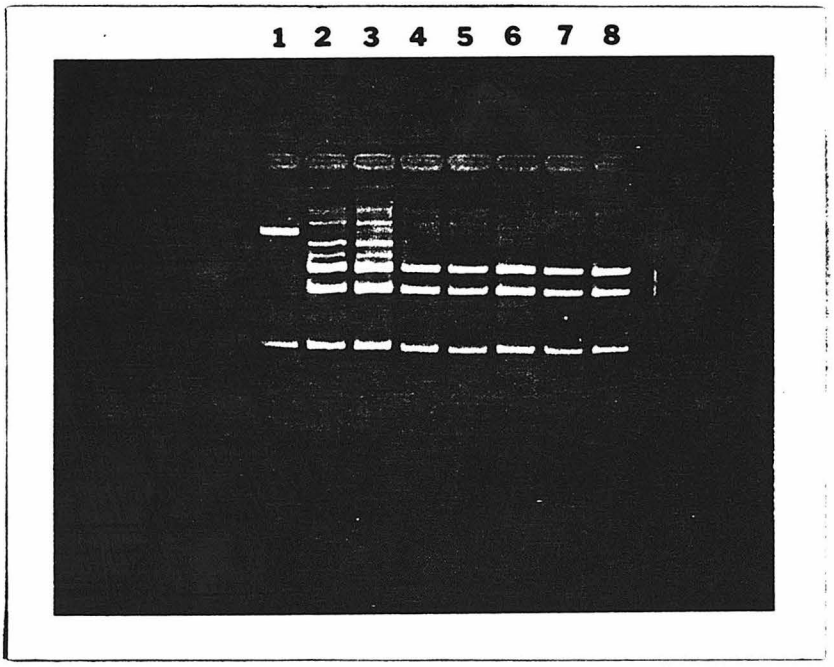

1 2 3 4 5 6 7 8

Figure 3

DE-52 column profile of purification of Lys73-->Cys

(●- A_{280}, o- activity)

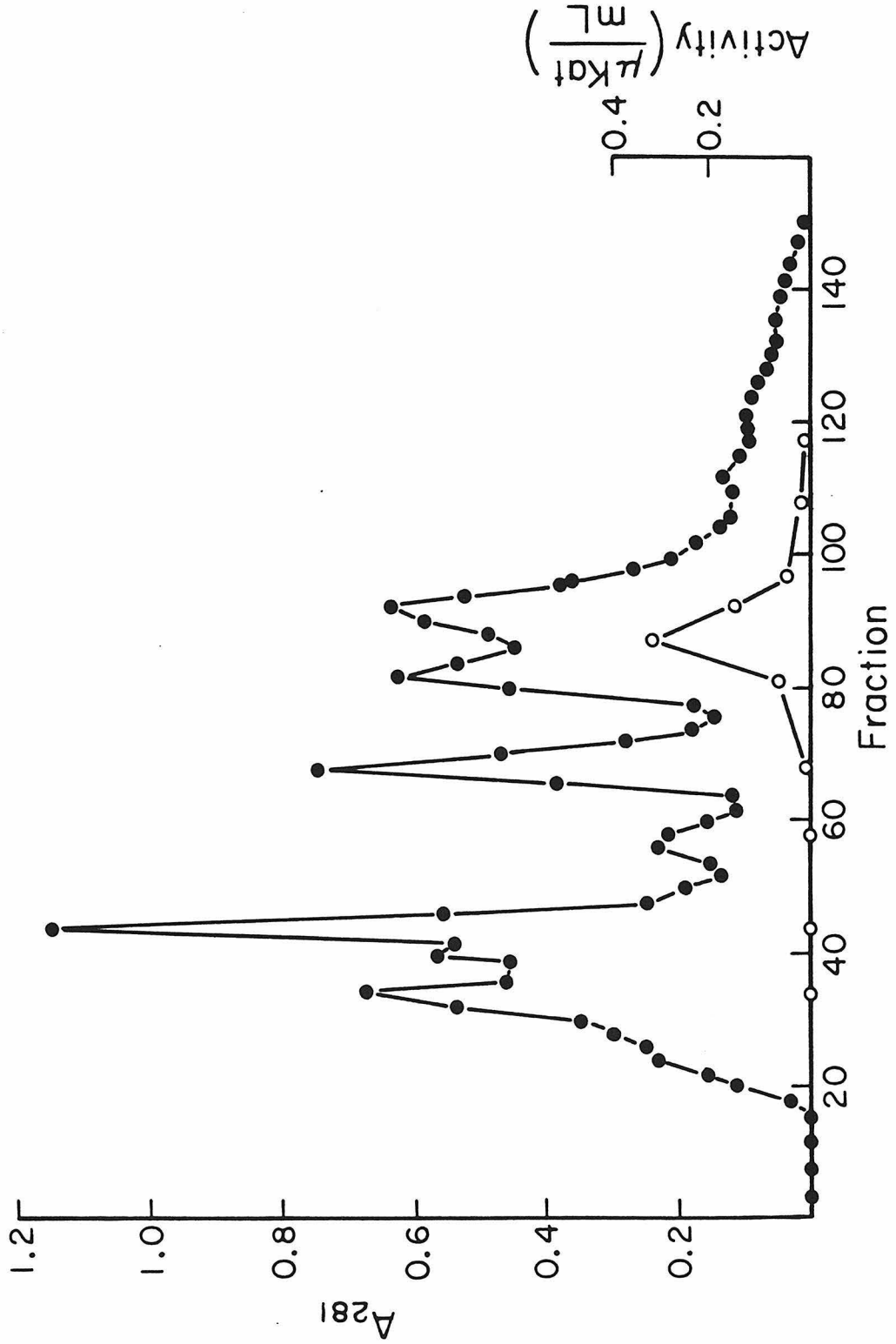

A281
1.2
1.0
0.8
0.6
0.4
0.2
20
40
60
80
100
120
140
Fraction
Activity (μKat/mL)
0.4
0.2

Figure 4

Ultragel ACA 54 column profile of the purification of Lys73-->Cys

(●- A_{280}, o- activity)

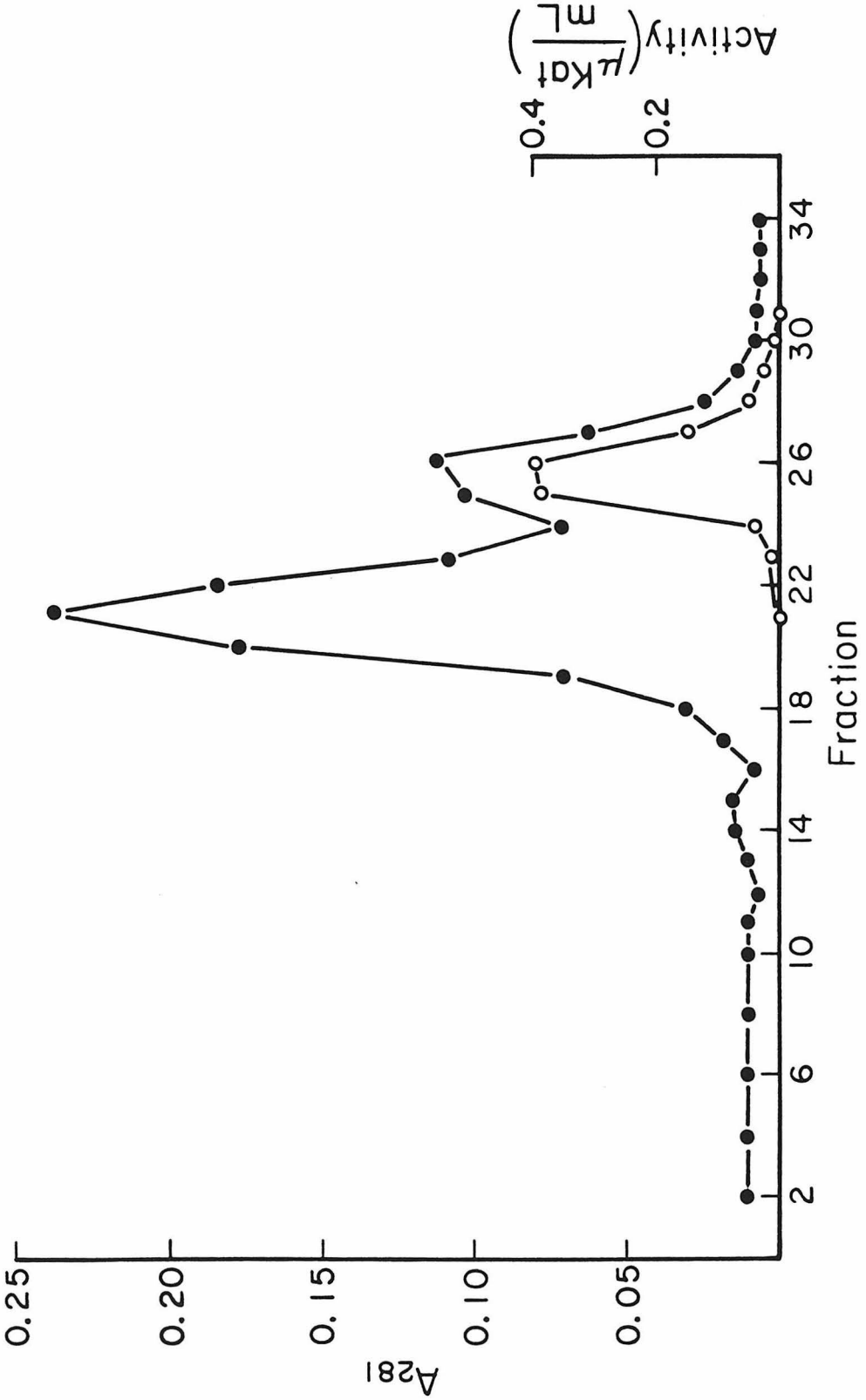

A281
0.25
0.20
0.15
0.10
0.05
2
6
10
14
18
22
26
30
34
Fraction
Activity (μKat/mL)
0.4
0.2

Figure 5

The variation of turnover rate of Lys73-->Cys and wild-type enzyme with pH

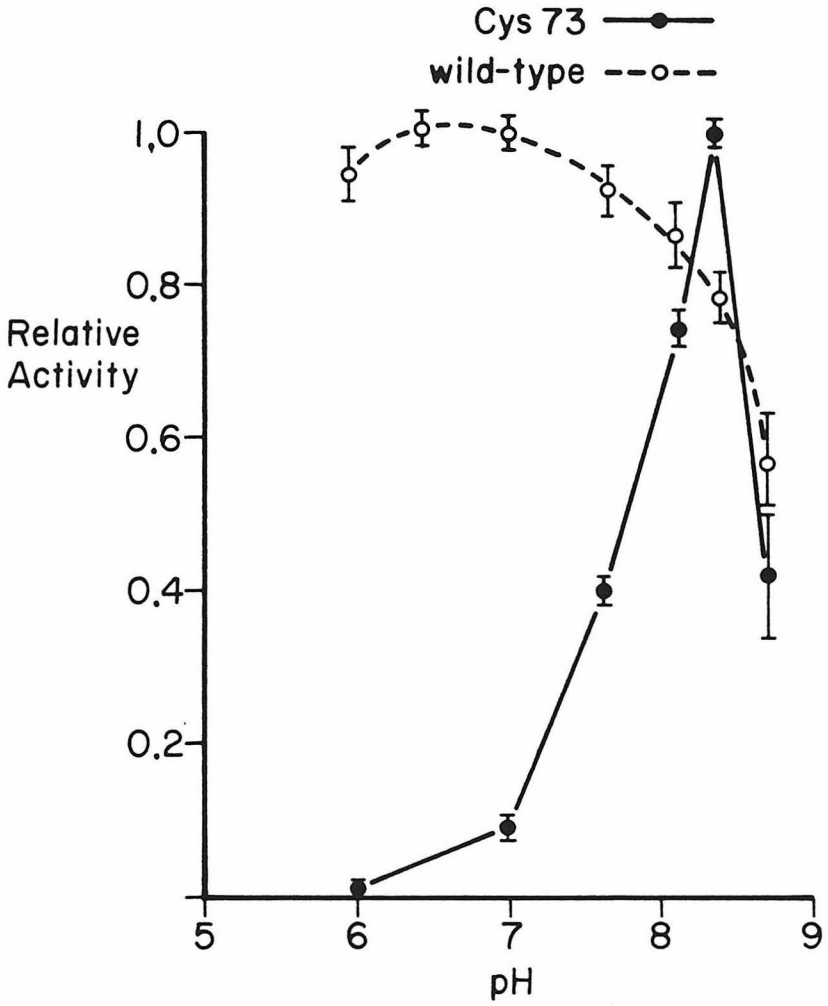

Cys 73
wild-type
1.0
0.8
0.6
0.4
0.2
Relative Activity
5
6
7
8
9
pH

Figure 6

The variation in turnover rate with temperature

for Lys73-->Cys and wild-type enzyme

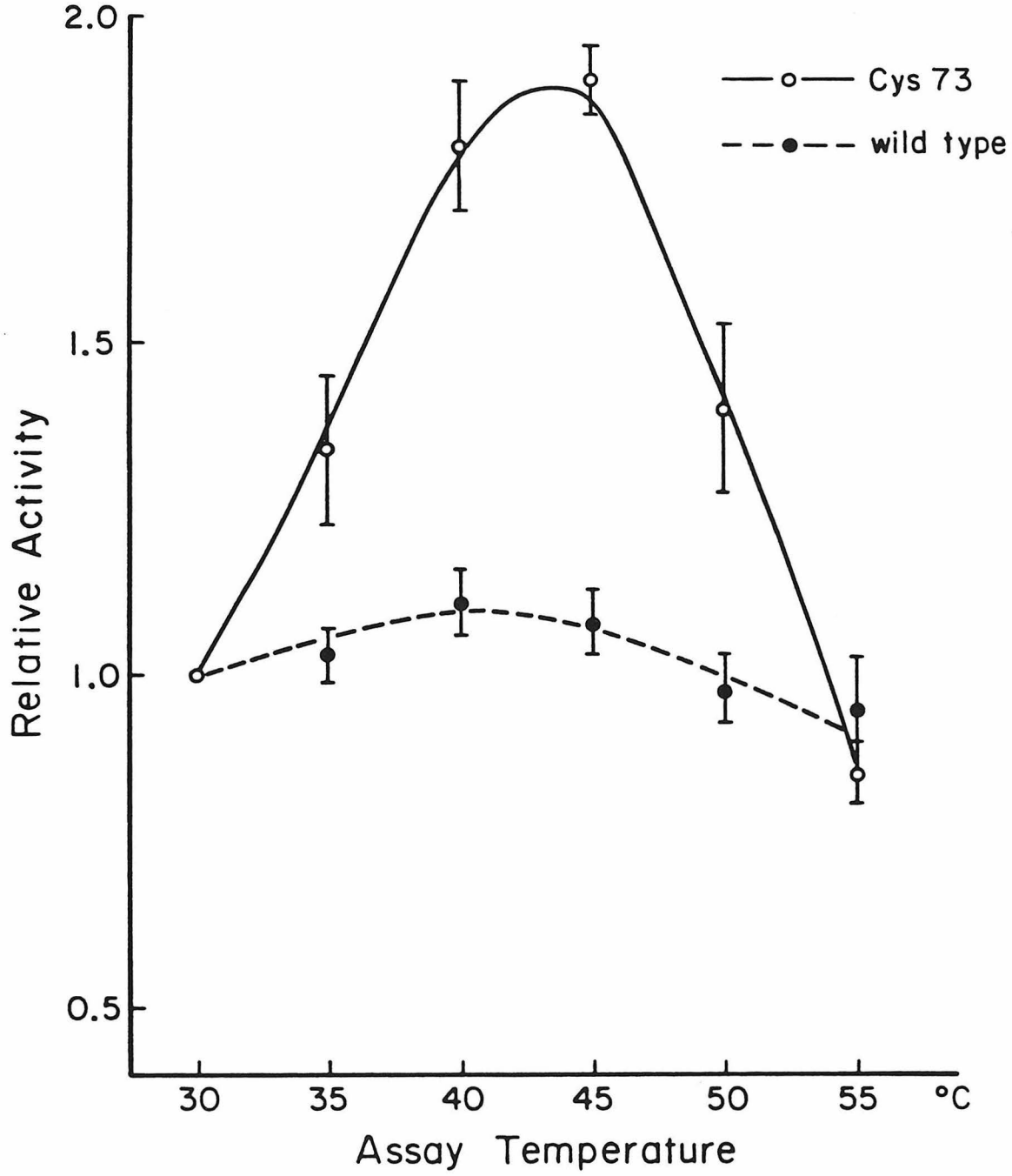

2.0
1.5
1.0
0.5
Relative Activity
Cys 73
wild type
30
35
40
45
50
55
°C
Assay Temperature

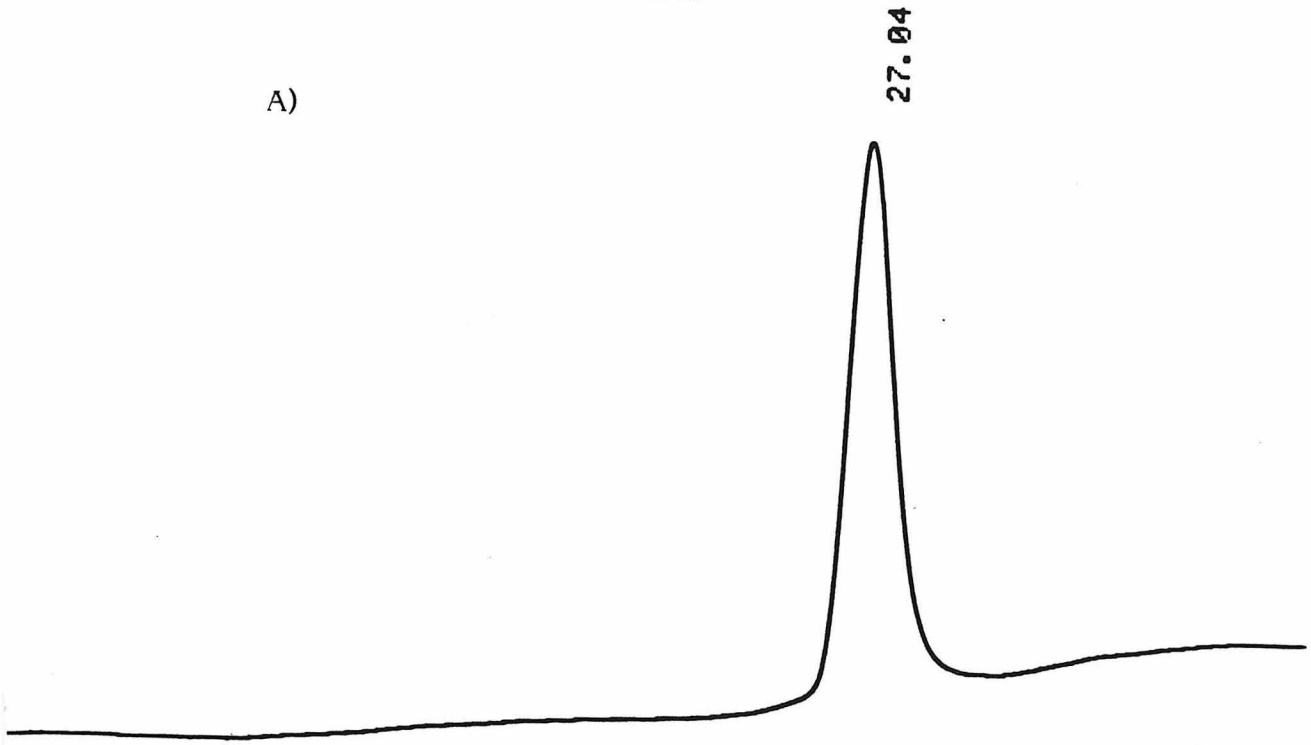
Figure 7

A) FPLC elution trace of wild-type ß-lactamase

B) FPLC elution trace of Lys73-->Cys

(Elution times are given in minutes.)

A)



B)

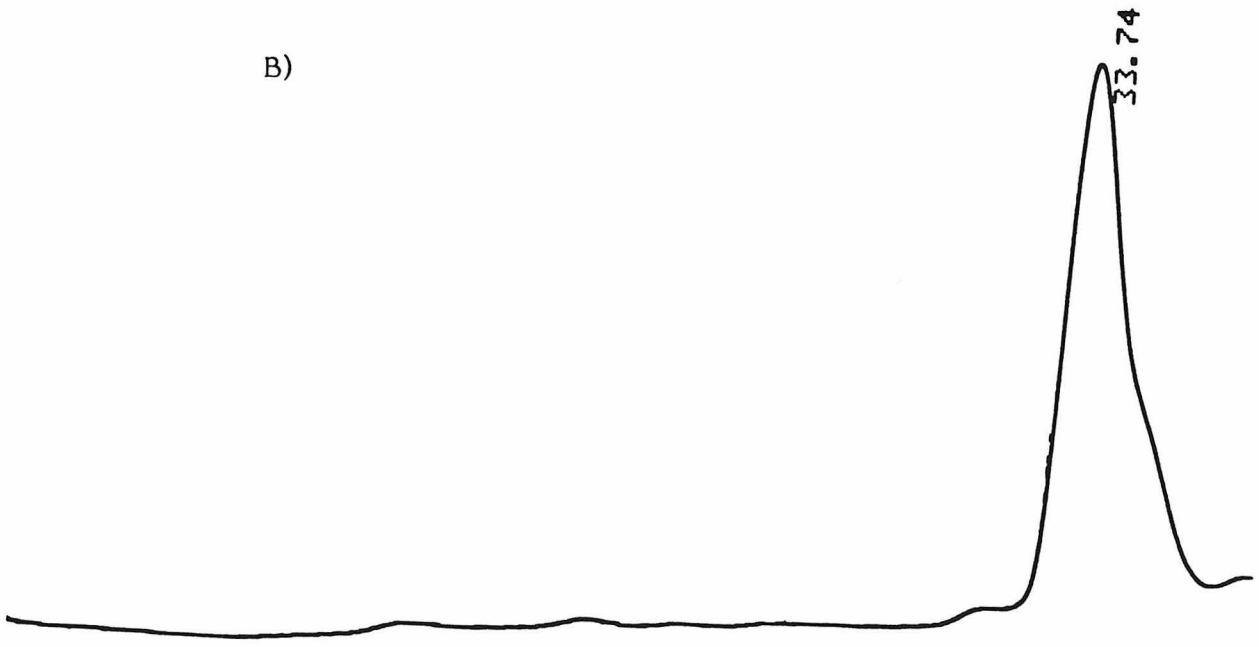


Figure 8

FPLC separation of the ethylenimine and Lys73-->Cys reaction mixture. Upper trace- activity of collected fractions(1 min. per fraction). Lower trace- A_{281} elution profile (0.5 cm per min.).

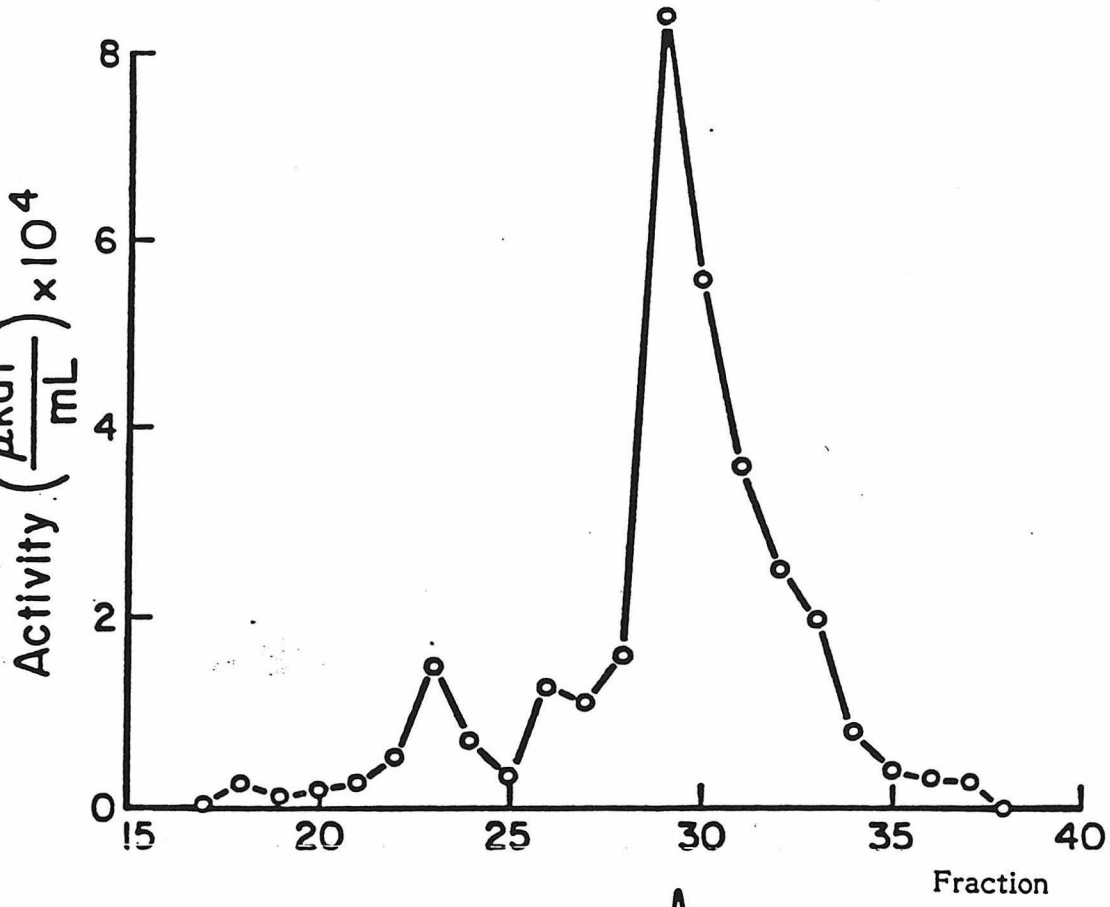

Activity $\left(\frac{\mu kat}{mL}\right) \times 10^4$
0
2
4
6
8
15
20
25
30
35
40
Fraction

Table 1

Michaelis-Menten parameters

Benzylpenicillin

	$k_{cat}(sec^{-1})$	$K_M(\mu M)$	k_{cat}/K_M	Rel.
Lys73-->Arg	0.3±.03	43±11	$6.9x10^3$	$6.9x10^{-5}$
Lys73-->Cys	0.25±.03	21±4	$1.2x10^4$	$1.2x10^{-4}$
wild-type	2000	20	10^8	1
aminoethylCys73	1055±150	17±5	$6.2x10^7$	.62

Cephalothin

	$k_{cat}(sec^{-1})$	$K_M(\mu M)$	k_{cat}/K_M	Rel.
Lys73-->Arg	$7\pm.06x10^{-3}$	136±20	51	$8.6x10^{-5}$
wild-type	120	190	$6x10^5$	1
aminoethylcys73	38±2	660±60	$5x10^4$	.09

Table 2

Conditions of reaction with ethylenimine

	Specific Activity(sec^{-1})	Relative Increase
1. before reaction	0.25	1
2. 0.5 M sodium phosphate, pH 8.4	8.8	35
3. 0.5 M sodium phosphate, pH 8.4	8.8	35
4. condition 2 + 1 mM PenG	6.2	24
5. condition 2, 37°C	31	122
6. condition 2, 4 M urea	44	175
7. condition 2, 37°C, 4 M urea, 1 mM PenG	31	122
8. wild-type, 4 M urea, pH 8.4	1100	(-0.5)

Table 3

Half-lives of Activity for Lys73-->Cys and wild-type as a Function of pH

	$t_{1/2}$ (min)	
T(°C)	wild-type	Lys73-->Cys
45	-	>60*
50	>60*	20
55	15	4
60	2	<1

*Indicates greater than 90% of the original activity was retained during this time period.

Appendix

The Reaction of Sulfhydryl Reagents with Diol Dehydratase, a Coenzyme B_{12}-Dependent Enzyme

Abstract. The reaction of diol dehydratase with several sulfhydryl reagents has been investigated. Apoenzyme was inactivated by phenylmercuric, methylmercuric, and mercuric chloride and iodoacetate. Holoenzyme was protected against inactivation by phenylmercuric ion and iodoacetate but not against methylmercuric or mercuric ion. Radioactive methylmercuric ion labeled only the 60 kdalton subunit, 1 equivalent per subunit. The presence of cyanocobalamin reduced the labeling of the 60 kdalton subunit by iodoacetate from 2 equivalent per enzyme molecule to 1 equivalent per enzyme molecule. The binding of coenzyme was prevented by reaction of apoenzyme with phenylmercuric ion. Coenzyme was released from holoenzyme by reaction with methlymercuric ion. These results are consistent with the hypothesis (Kuno, S., Toraya, T., and Fukui, S. (1981) Arch. Biochem. Biophys. 210, 474-480) that diol dehydratase contains two classes of free sulfhydryls: one essential for enzymatic activity and associated with coenzyme binding and another nonessential for activity and not protected by coenzyme analogs from reaction sulfhydryl reagents. These two free sulfhydryls reside on the 60 kdalton subunit.

Introduction

The structure of vitamin B_{12}, the substance responsible for the abatement of pernicious anemia, was solved in 1955 by x-ray crystallography by Hodgkin and coworkers (1). Their solution capped a monumental effort begun by Minot and Murphy in 1926 directed towards discovering the cause of this fatal disease (2). The vitamin has at its center a cobalt(III) atom ligated to the four central nitrogens of a corrin ring. The D ring of the corrin has as part of one of its side-chains a dimethylbenzimidazole which, in turn, ligates to the cobalt in the α position. A cyanide molecule occupies the remaining coordination site, the ß position. A biologically active form of the vitamin is adenosylcobalamin, where the cyano ligand, an artifact of the purification, is replaced by 5'-deoxyadenosine, one of the few naturally occurring biological compounds with a carbon-cobalt bond. (Figure 1)

Cobalamin is synthesized exclusively by microorganisms. These bacteria contain adenosylcobalamin-dependent enzymes analogous to the mammalian enzymes which participate in the metabolism of fatty acids (3,4) and directly or indirectly in the biosynthesis of deoxyribonucleotides and some amino acids (5). Since bacterial enzymes are generally easier to obtain than mammalian, the bacterial adenosylcobalamin-dependent enzymes have been the more thoroughly studied. There are ten different adenosylcobalamin-dependent enzymes which catalyze the exchange of a hydrogen atom for an adjacent group "X",

where "X" may be alkyl, acyl, OH, or NH_2, reactions that have essentially no counterpart in organometallic chemistry. Propanediol dehydratase [(RS)-1,2-propanediol hydrolyase, EC 4.2.1.28], one of the ten enzymes, catalyzes the rearrangement of 1,2-propanediol to 1,1-propanediol followed by stereoselective dehydration to propionaldehyde (6). An adenosylcobalamin-dependent enzyme, not fitting the above reaction scheme, is ribonucleotide reductase, which catalyzes a reduction (7,8).

The currently accepted mechanism for hydrogen transfer is outlined in Figure 2. Substrate binding is thought to cause a homolytic cleavage of the 5'-carbon-cobalt bond, giving a 5'-adenosyl radical and a Co(II) species. The organic radical abstracts the pro-R hydrogen from C-1 of (R)-1,2 propanediol or the pro-S hydrogen from (S)-1,2 propanediol (9) giving a substrate radical 5'-deoxyadenosine, and Co(II). The hydroxyl of C-2 is transferred to C-1 in an unknown fashion, giving a "product" radical. This radical abstracts a hydrogen from the 5' position of the deoxyadenosine, completing hydrogen transfer.

The role of the protein in the conversion of 1,2-propanediol to propionaldehyde is much less understood. Improvements in the purification procedure of diol dehydratase have recently been reported (10). The previous method (11) gave an enzyme of molecular weight 230 kdaltons, which could be dissociated in the absence of substrate into two

components, F and S, with molecular weights of 26 and 200 kdaltons, respectively. Component F was composed of a single subunit while component S dissociated into four types of subunit with molecular weights 60, 23, 15.5 and 14 kdaltons. Both components were necessary for full activity. The improved method recognizes that diol dehydratase is associated with the bacterial membrane and is susceptible to proteolysis by endogenous proteases. The improved isolation procedure yields an enzyme of higher specific activity in greater quantity than the previous method. The diol dehydratase thus isolated has a molecular weight of 260 kdaltons and is composed of seven subunits; two 60, one 51, two 29, and two 15 kdalton subunits (12).

Sulfhydryl reagents inactivate diol dehydratase. A highly specific reagent for sulfhydryls, p-chloromercuribenzoate (pCMB), inhibits enzymatic activity if added to apoenzyme but has little effect on the holoenzyme, or to enzyme which has bound methylcobalamin or cyanocobalamin (13); however hydroxycobalamin does not protect the enzyme from inactivation by organic mercurials. Iodoacetate and N-ethylmaleimide also inactivate proteolyzed apoenzyme obtained by the method of Poznanskaja et, al. (14). After reaction with iodoacetate, component S could not reform active enzyme with component F (15). Further, the proteolyzed form of diol dehydratase was shown to contain two reactive sulfhydryl groups per enzyme molecule by reaction with pCMB (16). One

of the sulfhydryls was essential for activity and was protected from reaction by the presence of cyanocobalamin, a competitive inhibitor of coenzyme binding. The other sulfhydryl was nonessential and was not protected by the bound vitamin. Conversely, when apoenzyme was reacted with either pCMB or dithiobis-2-nitrobenzoic acid (DTNB) it completely lost the ability to bind cyanocobalamin, but not when it was reacted with N-ethylmaleimide. The pCMB-treated enzyme did not dissociate into subunits. The authors concluded the essential sulfhydryl was located at or near the coenzyme binding site of diol dehydratase.

To help resolve the differences between diol dehydratase isolated by the method of Poznanskaja, et al. (11), and diol dehydratase isolated by the method of McGee and Richards (10), and to gain information about the coenzyme-binding site on the enzyme, we undertook studies on the reactivity of diol dehydratase, purified according to McGee and Richards (10), towards several sulfhydryl reagents. The change in activity of apo- and holoenzyme in the presence of different organic mercurial reagents was observed. Radioactive methylmercuric chloride was used to show that there are two reactive sulfhydryls per enzyme molecule, both associated with the 60Kdalton subunits. On binding cyanocobalamin the number of sulfhydryls available to react with iodoacetate is decreased from two to one. Apoenzyme, after reaction with methylmercuric chloride is unable to bind coenzyme. Coenzyme is

released from holoenzyme in the presence of methylmercuric chloride. These results indicate that at least one of the two free sulfhydryls of the two 60Kdalton subunits is important, either directly or indirectly for coenzyme binding.

Materials and Methods

$^{203}HgCH_2Cl$ was purchased from New England Nuclear, Boston, Massachusetts. The specific activity was originally 0.8 mCi/mg. Calculations of subsequent experiments assumed a half-life of 46.6 days.

1-[^{14}C]-ICH_2COOH (11.1 mCi/mmole) and 2-[^{3}H]-ICH_2COOH (193 mCi/mmole) were from New England Nuclear.

[^{3}H]-Coenzyme B_{12}- was synthesized according to McGee (1982).

Diol dehydratase was isolated by the method of McGee and Richards (1981).

Inactivation of diol dehydratase by organic mercurials- Diol dehydratase (7 units, 16 units/mL) was incubated with either 5 L of a 1 mg/mL solution of coenzyme or with 5 μL of of .01 M K_2HPO_4, pH 8, at 37°C. Ten μL of a 7.8 x 10^{-3} M solution of either CH_3HgCl, phenylmercuric chloride, or mercuric chloride were then added and 5 μL aliquots were removed over a period of hours and assayed.

Reactivation of diol dehydratase inactivated by organic mercurials- Diol dehydratase (3 units, 10units/mL) was inactivated as before. Reaction was allowed to proceed for from 4 minutes to three hours after which 10 μL ß-mercapto ethanol was added to quench the inactivation and effect reactivation. Aliquots of 5 μL were removed and assayed both before and after addition of the ß-mercaptoethanol. The experiments were repeated in the presence of 20 μmoles of

coenzyme for methylmercuric chloride and mercuric chloride.

Activity assay- The method used was that of Bachovchin, et al. (18), employing yeast alcohol dehydrogenase (Sigma) and NADH (Sigma) to reduce product propionaldehyde to 1-propanol. The production of propionaldehyde was measured by following the decrease in absorbance of the solution at 340 nm, due to oxidation of NADH to NAD^+ in an excess of alcohol dehydrogenase activity. Sample volumes were 2.0 mL and reaction was initiated by the addition of either coenzyme or holoenzyme in the dark at 37°C. One unit of activity is defined as that amount of enzyme activity that will catalyze the conversion of one micromole of substrate to product per minute at saturating substrate concentrations at 37°C.

Coenzyme binding experiments- Diol dehydratase (100 units) was dialyzed against 2 mM dl-butanediol, .01 M KH_2PO_4, pH 8. One mL (10 units) was incubated with 100 μL 2 mM dl-butanediol, .01 M KH_2PO_4, pH 8 or with 100 μL 1.73×10^{-3} M phenylmercuric chloride, .01 M KH_2PO_4, pH 8, for 20 minutes. [^{3}H]-coenzyme B_{12} (50 μL of a 1.94×10^{-5} M solution, 5.9 dpm/ mole) was added in the dark and allowed to bind 20 minutes. The solution was applied to a 2.5 x 13 cm Biogel P-4 column and eluted with 2 mM dl-butanediol, .01 M KH_2PO_4, pH 8, in the dark. Fractions (2.1 mL) were collected and 1 mL was counted with 10 mL Aquasol II.

Diol dehydratase (1 mL, 10 units) in 2 mM dl-butanediol .01 M KH_2PO_4, pH 8, was incubated with 50 μL of [^{3}H]-coen-

zyme (1.94×10^{-5} M) for 20 minutes. Then, 100 μL of a 1.7×10^{-3} M methylmercuric chloride, .01 M KH_2PO_4, pH 8, solution was added. The solution was incubated for 20 minutes and then applied to the Biogel P-4 column. Fractions were collected and counted as before.

Labeling of the free sulfhydryls of diol dehydratase by $^{203}HgCl$- Diol dehydratase (10 units, 22 units/mL) was incubated at room temperature with stirring for ten minutes with or without coenzyme. Methylmercuric choride (.14 μmole, 10 μCi) was added and the mixture stirred for from 30 minutes to three hours. The solution was then dialyzed against two changes of three L each .01 M K_2HPO_4, pH 8, for several hours. The protein sample was precipitated by the addition of three volumes of spectrograde acetone (Baker Analytical) and was centrifuged in a Sorvall GLC-2 centrifuge at 3000 rpm. The protein was denatured by heating in 1% SDS and the subunits separated by SDS-PAGE (10% acrylamide). The gel was stained with Coomassie Brilliant Blue, destained and the protein bands were sliced out with a razor blade. The slices were counted for gamma radiation in a Beckmann Gamma 4000 counter, on the ^{51}Cr channel. Counting efficiency was determined to be 65% by counting a sample of stock methylmercuric chloride. The effect of the presence of coenzyme on the labeling was investigated by carrying out the same procedure as before except that prior to the addition of the $HgCH_3Cl$, 5 μL of a 1 mg/mL solution of coenzyme were

added to the enzyme and the solution stirred for 20 minutes at room temperature in the dark. Propanediol (1 M) was present in all cases.

Differential labeling experiments-Diol dehydratase (0.5 mL, 25 units/mL) was incubated with or without cyanocobalamin (1 mg/mL) for 10 minutes at room temperature. [^{14}C]-Iodoacetate (10 to 50 μL of a solution made 1 M by the addition of an appropriate amount of cold iodoacetic acid) was added and the solution incubated in the dark for 20 hours. The mixture was diluted to 10 mL in Buffer G in an Amicon Diaflo apparatus fitted with a PM-30 membrane and an aliquot of [^{3}H]-iodoacetate-labeled diol dehydratase added as a tracer. The solution was dialyzed by continuous flow against 80 mL of Buffer G (10% glycerol, 5% propanediol, .01 M sodium phosphate, pH 8) and then concentrated to 1 mL. Three volumes of acetone were added and the protein precipitated by centrifugation at 7000 rpm for 15 minutes. SDS-PAGE sample buffer (50 μL) was added and the sample was run on a 10% acrylamide gel. The gel was stained with Coomassie Brilliant Blue, destained and sliced into 2 mm slices with a razor blade, noting which slices contained the 60 Kdalton subunit. The gel slices were placed in glass scintillation vials to which 1 mL of 30% H_2O_2 was added. The capped vials were incubated at 60°C overnight. Aquasol (10 mL) was added and the samples were counted for radioactivity in a Beckmann LS 9000 scintillation counter.

Results

The results of the inactivation experiments are shown in Figure 3 for reaction with methylmercuric and phenylmercuric ion, and in Figure 4 for mercuric ion. It is apparent that phenylmercuric ion inactivates apoenzyme, but in the presence of coenzyme the inactivation is substantially reduced. However, the presence of coenzyme has no discernable effect on inactivation by either mercuric ion or methylmercuric ion. The rate of inactivation by mercuric ion was much slower than that of methyl- or phenylmercuric ion, an effect that may be explained by the tighter binding of hydroxyl ion to mercuric ion than to methylmercuric ion. The pK of dissociation of $Hg(OH)_2$ is 11.3, while that of $CH_3Hg(OH)$ is 9.5 (19). A difference in pK of 1.8 means at an initially equivalent concentration of $HgCl_2$ and CH_3HgCl there will be approximately 75 times as much free methylmeruric ion as hydroxymercuric ion. Since the rate of inactivation in this case is proportional the the amount of free label, the rate of inactivation by methylmercuric ion should be 75 times that of mercuric ion, as is observed.

The reactivation of diol dehydratase previously inactivated by organic mercurials could be accomplished in some cases, as is shown in Figure 5. The inactivation caused by phenylmercuric ion was almost fully reversible for periods of inactivation of at least one hour. Reaction of apoenzyme with methylmercuric ion caused an inactivation that was

irreversible after 15 minutes of inactivation, and only partially reversible at 5 minutes of inactivation. The holoenzyme inactivated by methylmercuric ion could be 88% reactivated after five minutes of inactivation but could not be reactivated after one hour of reaction with methylmercuric ion. Mercuric ion caused a slower inactivation than the organic mercurials and this inactivation could not be reversed after three hours of inactivation.

Figure 6 shows the results of experiments investigating the binding of coenzyme to enzyme inactivated by phenylmercuric chloride. Measurements of absorbance at 280 nm indicate that holoenzyme and apoenzyme elute in the void volume and are completely separated from unbound coenzyme. No significant difference in the quantity of protein eluted from the column between runs was observed (data not shown). Under conditions when the enzyme was completely inactivated by phenylmercuric chloride, less than .1% of the radiolabeled coenzyme associated with the control (active) chromatogram eluted in the void volume, indicating the inactivated protein does not bind coenzyme. Holoenzyme inactivated by methylmercuric ion retains 11% of the radiolabeled coenzyme associated with the control run, indicating the coenzyme is largely released from the holoenzyme. The radioactivity eluting in the void volume of the control reaction corresponds to .25 equivalent.

The results of a typical labeling experiment of SDS-

denatured diol dehydratase by [^{14}C]-iodoacetic acid are shown in Figure 6. Both the 60 and 51 kd subunits contain substantial amounts of label and are well separated by the SDS-PAGE gel system. The small amount of label associated with the 29 and 15 kd subunits probably corresponds to labeling of histidines and methionines (24).

The results of the radioactive labeling of the enzyme by methylmercuric chloride are shown in Table 1, both with and without coenzyme. The 60 kd subunit was the only subunit containing more than one equivalent of label per enzyme molecule. None of the other subunits contained counts corresponding to more than 0.4 equivalents of label per subunit under the reaction conditions employed. Therefore, it was concluded that neither the 51, 29 or 15 kd subunit contained free sulfhydryl groups susceptible to labeling by methylmercuric ion, under the reaction conditions employed. It is evident that the number of counts associated with the 15 kd subunit is, in general, half that associated with the 29 kd subunit, suggesting nonspecific labeling. To correct for this nonspecific, molecular weight dependent labeling in the other subunits a correction factor of counts per kd of the 29 kd subunit was applied to the 51 and 60 subunits, as is shown in Table 1. The labeling of the 51 kd subunit was never more than .1 equivalent per subunit greater than this background. The results suggest one sulfhydryl per 60 kd subunit is free to react with methylmercuric ion. It is evident that the

presence of coenzyme does not significantly alter the amount of labeling of any of the subunits.

The effects of the presence of cyanocobalamin on the reactivity of the sulfhydryls of the 60 kd subunit towards iodoacetate were investigated. Increasing the concentration of label beyond 30 μL added (60 mM) did not substantially increase the extent of labeling indicating the enzyme is approaching quantitative labeling under these conditions. Under saturating concentrations of cyanocobalamin the 60 kd subunit contains approximately half the amount of label present in the absence of cyanocobalamin when the enzyme is approaching quantitative labeling. Maximal labeling in the presence of cyanocobalamin was about one equivalent label per enzyme and in the absence of cyanocobalamin was about two equivalents label per enzyme molecule. The addition of enzyme labeled by reaction with [^{3}H]-iodoacetate as a tracer allows the calculation of quantitative yield of the 60 kd subunit during the dialysis, precipitation and electrophoresis after its labeling.

Discussion

A new improved method for the isolation and purification of diol dehydratase was recently developed (10) which yields more enzyme of higher specific activity than previous methods (3, 11). Previous methods gave yields of approximately 6% and a specific activity of about 60 units/mg, whereas the mew method gives yields of 40-50% and specific activity of 95 units/mg, making structural studies possible. The enzyme is composed of four different kinds of subunits with molecular weights of 60, 51, 29, and 15 kdaltons. They are present in a ratio of 2:1:2:2 (60:51:29:15) for an overall molecular weight of 260,000 daltons (12). Amino acid analyses show there are eight cysteine residues present per enzyme molecule, three in each of the 60 kd subunits and two in the 51 kd subunit, and none in either the 29 or 15 kd subunits. Sequence data for 40-45 of the N-terminal residues of each subunit have been obtained, but no cysteines have been located. No interchain disulfide bonds exist since the addition of DTT does not effect the migration of the subunits on SDS-PAGE (20).

Organic mercurials are generally the reagent of choice for investigations of free sulfhydryl groups on proteins. A monofunctional organic mercurial, such as methylmercuric chloride, for the quantitation of free sulfhydryls prevents possible complications due to formation of intermolecular dimers on reaction of mercuric ion with proteins (21, 22).

Although nonspecific reactions have been reported (23) for organic mercurials, they tend to be much more specific than other sulfhydryl reagents (24).

That each of the 60 kd subunits should contain at least one free sulfhydryl is easily envisioned in view of the amino acid data. Since there are no inter-chain disulfide bonds, the remaining two cysteines may either be joined by a disulfide bond or they may be hindered to reaction either sterically or by the ionic character of the local environment. Examples of both cases have been presented so neither may be ruled out (25, 26). The small amount of labeling of the 51 kd subunit, never more than .1 equivalent over background may be due to slight proteolysis of the 60 kd subunit giving rise to a fragment of approximately 51 kd molecular weight. This would mean the two cysteines of the 51 kd subunit are either joined in an intrachain disulfide or are not exposed to reaction with water-soluble reagents.

Amino acid analyses of the 29 and 15 kd subunits show no cysteines to be present. Radiolabeling data of these two subunits generally show some small amount of incorporation, never corresponding to more than .2 equivalent per subunit. The labeling of the 15 kd subunit was usually approximately half that of the 29 kd subunit. Nonspecific labeling by mercurials has been reported (23), generally occurring when the mercurial was used in excess, as was the case in these experiments. Both amide and carboxylate groups have been

implicated as the recipients of the reagent. Either of these two modes of labeling may be responsible since the number of carboxylate and amide containing side chains in each subunit is roughly proportional to the molecular weight (12). Generally this non-sulfhydryl binding is weak and the dialysis of the experimental procedure probably removes most of the spurious binding. Consequently twice the number of counts associated with the 29 kd subunit was subtracted from the counts of the 60 kd subunit to represent the methylmercuric ion binding to sulfhydryls.

Experiments on the inactivation of diol dehydratase by mercurial reagents are consistent with the hypothesis that one of the two free sulfhydryls of the 60 kd subunit is essential for enzymic activity (16). All four sulfhydryl reagents tested (mercuric, methyl- and phenylmercuric, and iodoacetate) inactivate apoenzyme. Holoenzyme was protected against inactivation by phenylmercuric ion and iodoacetate. Iodoacetate is prevented from reaction with one of the two free sulfhydryls of the enzyme by the presence of cyanocobalamin, an analogue of the coenzyme. Presumably, the protected sulfhydryl is the essential one, although this could not be proven due to the long reaction times required for quantitative labeling and the faster inactivation of holoenzyme by oxygen and light.

Holoenzyme is vulnerable to inactivation by mercuric ion and by methylmercuric ion. Uv-vis spectra of the coenzyme

alone was identical to that of coenzyme in the presence of either methylmercuric or mercuric ion, casting doubt on the possibility that inactivation occurs by reaction of the inactivator with the coenzyme. Inactivation may occur by the inability of the coenzyme to protect the essential sulfhydryl or by reaction with the nonessential, unprotected sulfhydryl which causes a change in conformation that reduces or abolishes activity. The different responses of the enzyme to these reagents compared to that with phenylmercuric ion and iodoacetate may be the result of differences in size, or hydrophobicity of the reagents.

Experiments on the reactivation of diol dehydratase also show different results for these two classes of inactivator. Apoenzyme inactivated by phenylmercuric ion could be reactivated almost quantitatively by ß-mercaptoethanol for periods of inactivation of at least one hour. Apoenzyme could not be reactivated after reaction with methylmercuric or mercuric ion. Holoenzyme could be partially reactivated after five minutes of inactivation by methylmercuric ion but not after one hour of inactivation. Binding of methylmercuric ion to either the unprotected (against phenylmercuric ion) sulfhydryl or to the protected (against phenylmercuric ion) sulfhydryl causes a change in the enzyme, possibly of the conformation, that is irreversible. Reaction of phenylmercuric ion at either sulfhydryl does not produce this change since inactivation of apoenzyme is fully reversible. The presence

of coenzyme may delay this change slightly since holoenzyme could be reactivated partially after five minutes of inactivation by methylmercuric ion.

The loss of activity is accompanied by the loss of the ability of the enzyme to bind coenzyme. Apoenzyme inactivated by phenylmercuric ion did not bind [^{3}H]-coenzyme by a gel filtration assay. Holoenzyme inactivated by methylmercuric ion released the bound [^{3}H]-coenzyme. Free sulfhydryls must be required, either directly or for the correct conformation of the enzyme, for the binding of the coenzyme.

Another explanation may involve the enzyme's requirement for a monovalent cation for activity (13). Mercuric ion has an ionic radius of .66 Å (27), which is too small to serve any role in retaining catalytic capability (28). It is not known whether divalent ions may bind in place of the monovalent ion, but a divalent ion-enzyme complex would be inactive. This might be a possible mode of inactivation for mercuric ion. It is difficult to judge the effect of increasing the radius and changing the geometry of the ion by the introduction of phenyl and methyl groups. Since so little is known of the role and manner of action of the monovalent cation, little can be said of the importance of this possible mechanism of inactivation.

The basis for the difference in reactivity of the two free sulfhydryls of the 60 kd subunits is unclear. The coenzyme itself is not symmetrical so that binding of the

coenzyme may create asymmetry between otherwise equivalent sulfhydryls. The sulfhydryls themselves may not belong to the same cysteines on each 60 kd subunit. It would be necessary to isolate proteolytic fragments of sulfhydryl-labeled 60 kd subunits for sequencing to determine if equivalent cysteines are labeled. There is one 51 kd subunit per enzyme which may introduce asymmetry to the enzyme molecule. These experiments do not rule out the possibilty that the 51 kd subunit is also involved in the binding of the coenzyme. There remains the remote possibility that the 60 kd subunits are not identical for their entire sequences, although no discrepancies were noted from N-terminal sequences of the first 40 residues (12).

Diol dehydratase isolated by previous methods (11) represents a proteolyzed form of the enzyme. The proteolyzed form could be resolved onto two components, F and S. Component S could not reform active enzyme after reaction with iodoacetate, suggesting the essential sulfhydryl resides in Component S (16). Since the 60 kdalton subunit of diol dehydratase isolated by the improved method (10) contains both reactive sulfhydryls, this subunit is contained in Component S of diol dehydratase isolated by the earlier method.

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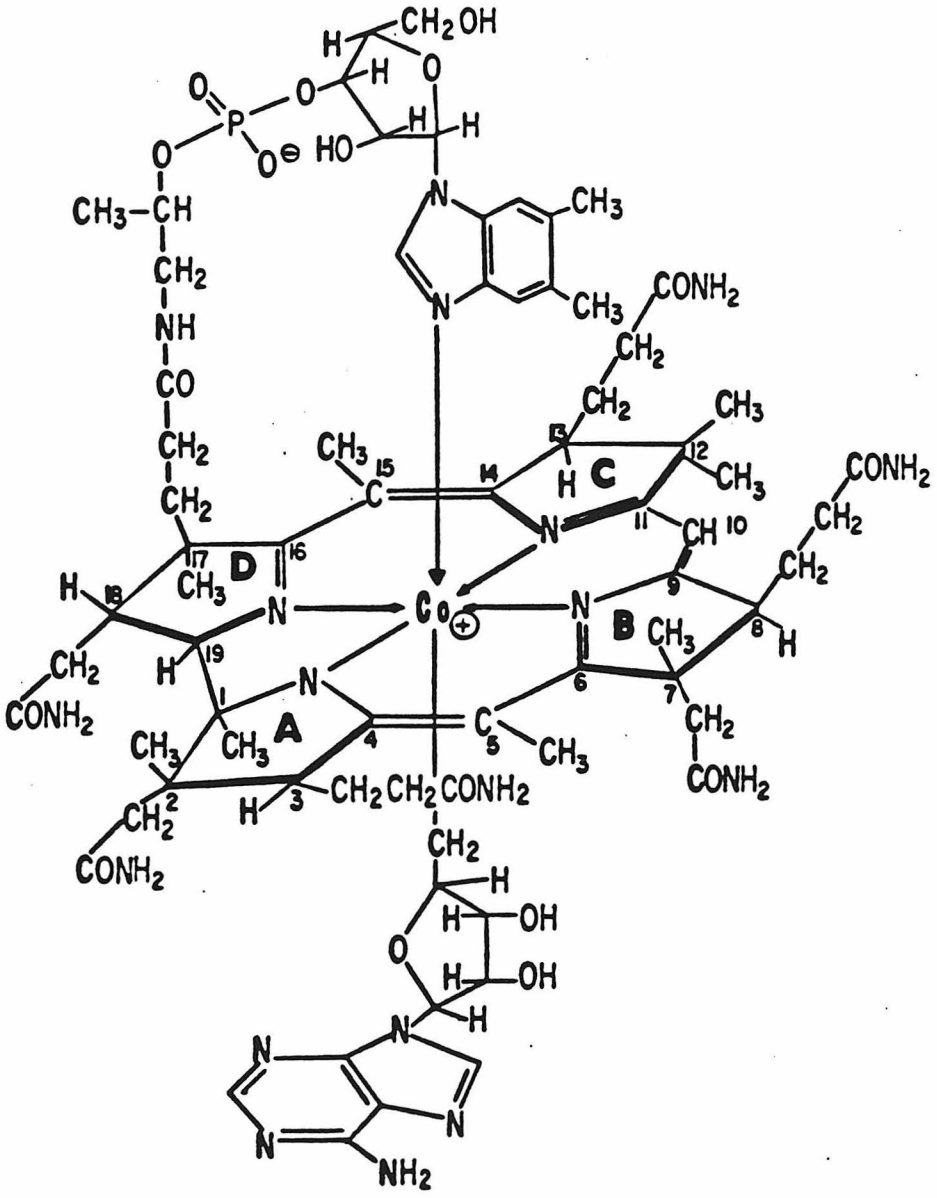
Figure 1

Adenosyl Cobalamin. Dimethyl benzimidazole is the upper (α) ligand, and 5'-deoxyadenosine is the lower (ß) ligand.


CH2OH
H
O
H
H
O
O
P
O
O⊖
HO
H
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O
CH3–CH
CH2
NH
CO
CH2
CH2
N
CH3
N
CH3
CONH2
CH2
CH2
CH3
CH3
15
14
13
12
C
H
CH3
CONH2
CH2
N
11
CH
10
CH2
17
D
16
9
CH3
H
18
N
Co
N
B
CH3
8
H
CH2
19
H
6
7
CONH2
1
N
A
4
C
5
CH3
CH2
CONH2
CH3
CH3
2
3
CH2 CH2 CONH2
H
CH2
CONH2
CH2
H
H
OH
O
H
OH
H
N
N
N
N
NH2

Figure 2

Postulated mechanism for hydrogen transfer. Ad denotes the adenosyl ligand; >Co< and >$\dot{C}$o<, cobalt with and without the bond to carbon, respectively, in the corrin ring; SH and S·, the substrate and substrate radical, respectively; PH and P·, the product and product radical, respectively; ?, the unknown mechanism of hydroxyl migration; and CH_3Ad, the three hydrogen intermediate.

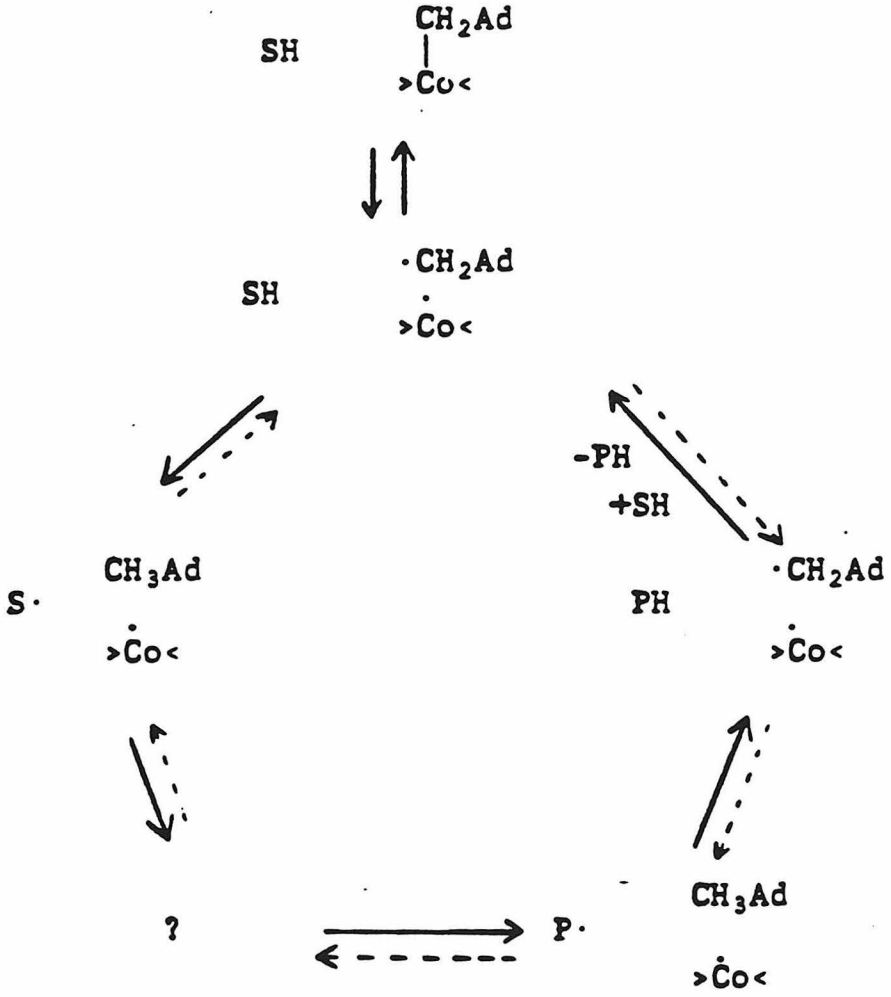
SH
CH2Ad
>Co<
·CH2Ad
SH
>Co<
-PH
+SH
·CH2Ad
PH
>Co<
CH3Ad
S·
>Co<
CH3Ad
>Co<
?
P·

Figure 3

Inactivation of apo- and holoenzyme in the presence of methyl- and phenylmercuric chloride. The percentage of original activity retained after reaction with organic mercurials is plotted for apoenzyme (0.6 μM) with methylmercuric chloride (▲, 0.26 mM) and phenylmercuric chloride (Δ, 0.26 mM) and for holoenzyme (0.6 μM) with methylmercuric chloride (□, 0.26 mM) and phenylmercuric chloride (○, 0.26 mM).

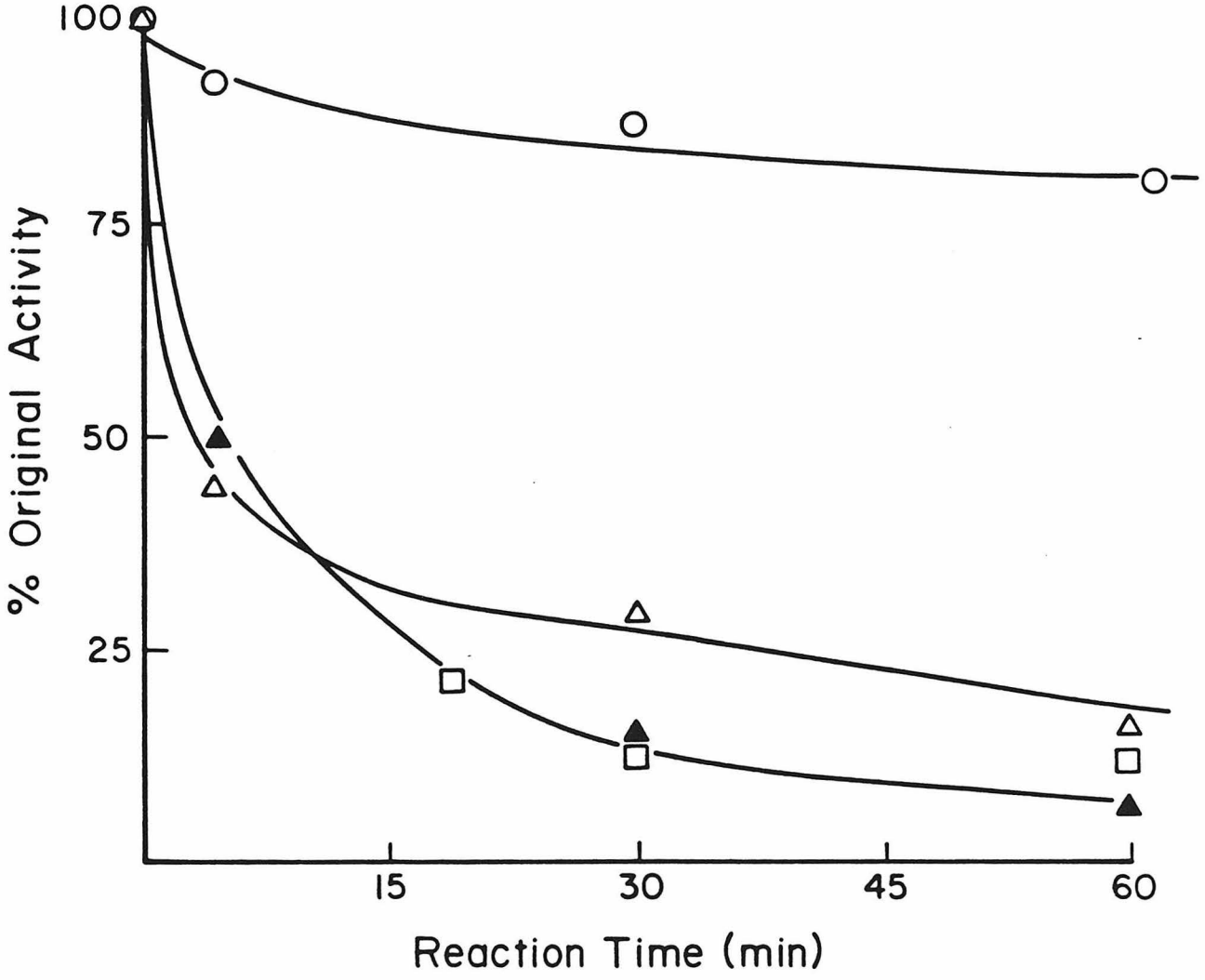
100
75
50
25
% Original Activity
15
30
45
60
Reaction Time (min)

Figure 4

Inactivation of apo- and holoenzyme in the presence of mercuric chloride. The percentage of original activity retained after reaction with mercuric chloride (0.26 mM) for holoenzyme (Δ , 0.6 μM) and for apoenzyme (○ , 0.6 μM) is shown.

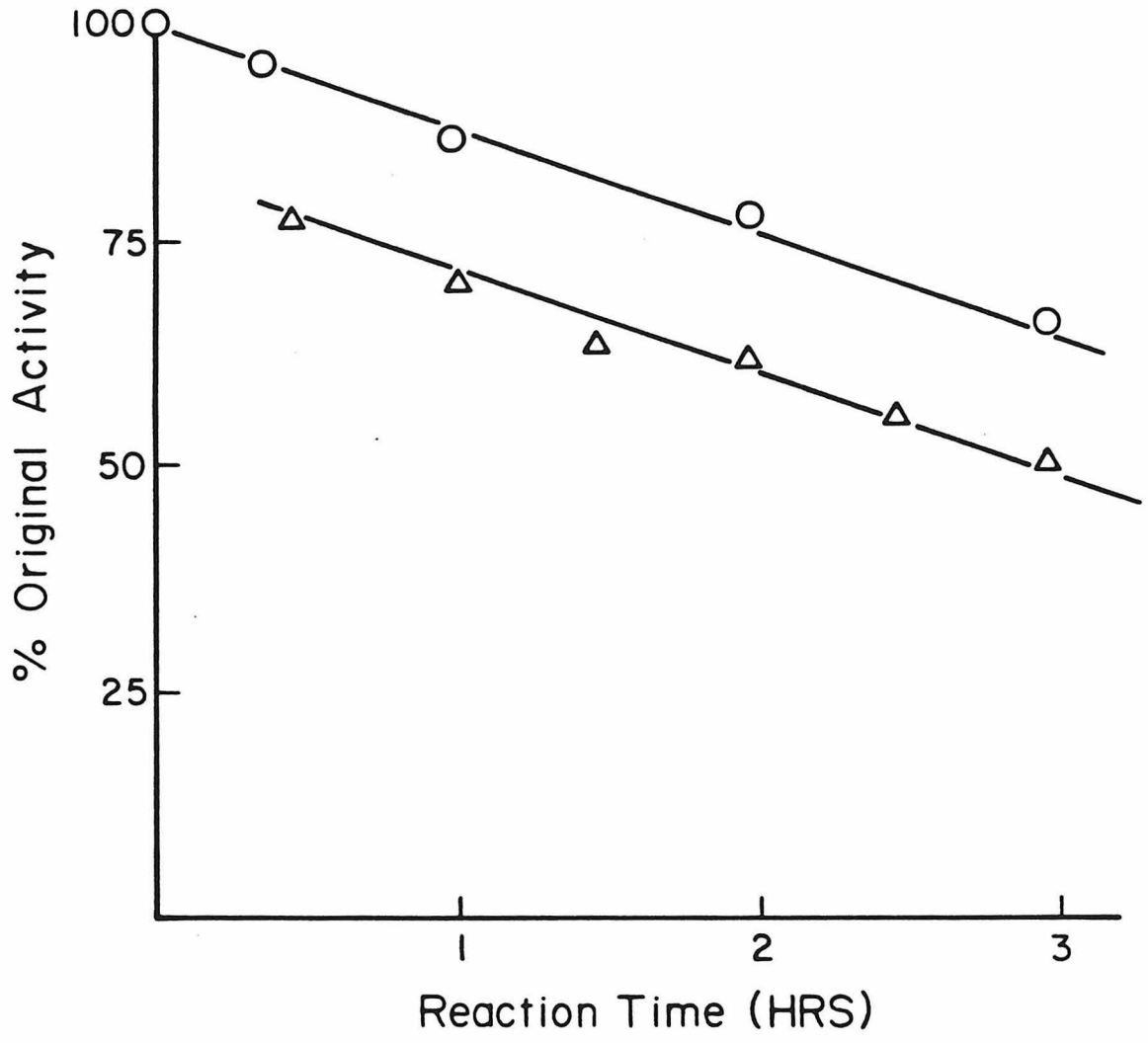
100
75
50
25
% Original Activity
1
2
3
Reaction Time (HRS)

Figure 5

Reactivation of diol dehydratase inactivated by organic mercurials. Apoenzyme was inactivated as before (Figure 3) with phenylmercuric chloride ($_X$), as was holoenzyme with methylmercuric chloride (Δ). After inactivation for from 5 to 60 minutes, ß-mercaptoethanol was added and allowed to react for 20 minutes after which the solution was assayed. The results are plotted for phenyl- (○) and methylmercuric chloride (□).

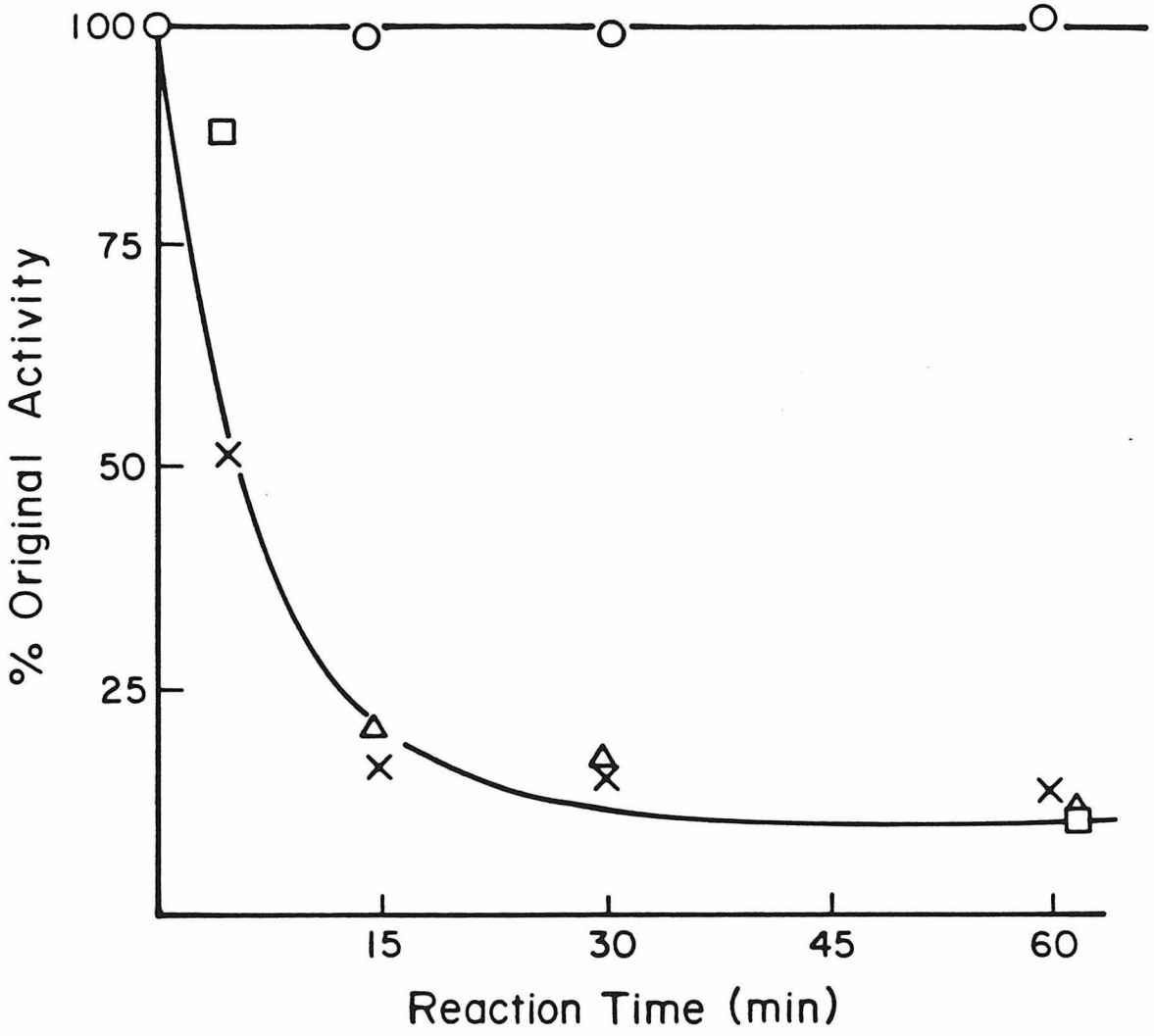
100
75
50
25
% Original Activity
15
30
45
60
Reaction Time (min)

Figure 6

Coenzyme binding by mercurial-inactivated diol dehydratase. Results are shown for unreacted enzyme (o), for apo enzyme inactivated by phenylmercuric chloride (•), and for holoenzyme reacted with methlymercuric chloride (x).

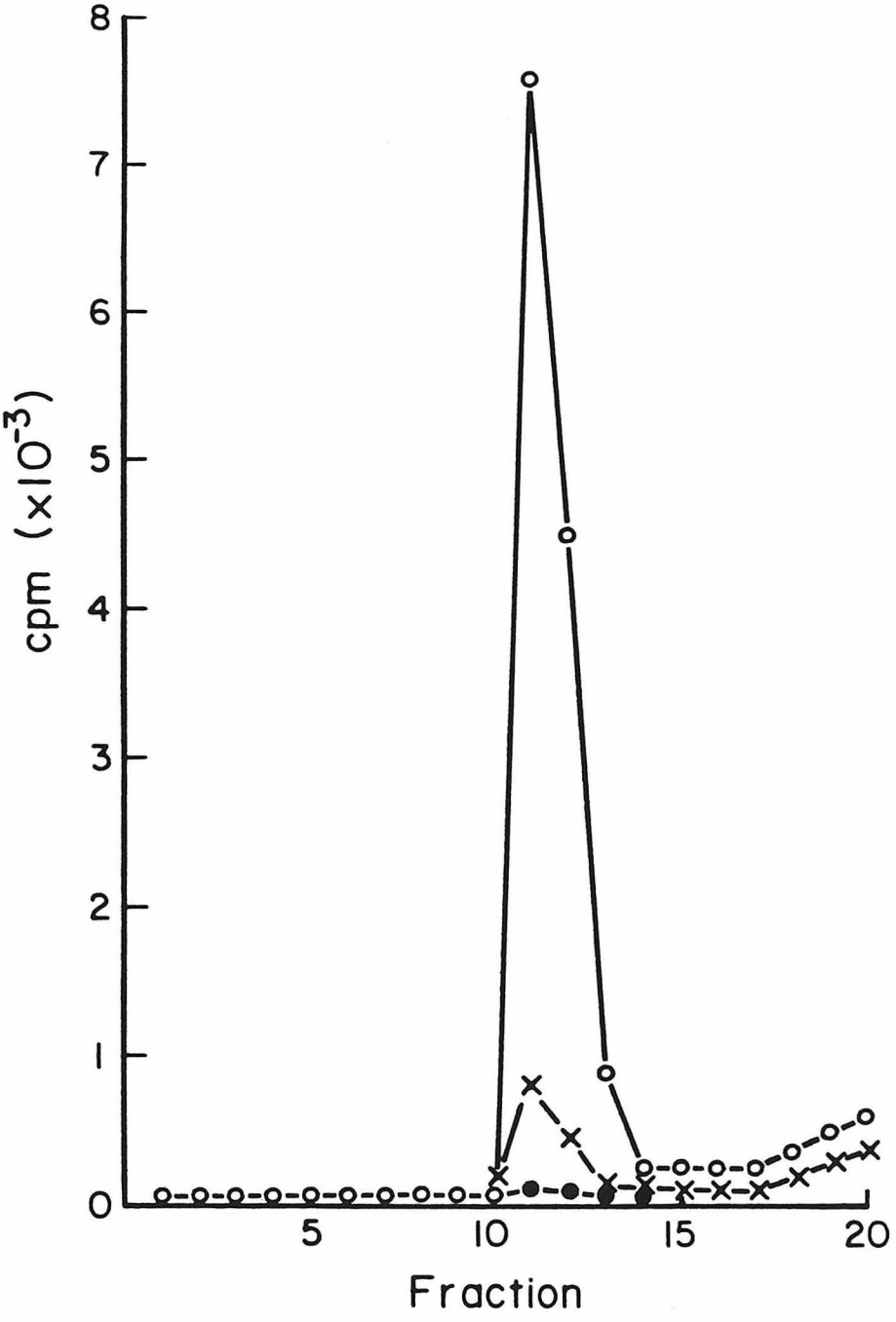
8
7
6
5
4
3
2
1
0
cpm (x10^{-3})
5
10
15
20
Fraction

Figure 7

[^{14}C]-Iodoacetic acid labeling of denatured diol dehydratase. Subunits were separated by electrophoresis on a 10% SDS-PAGE.

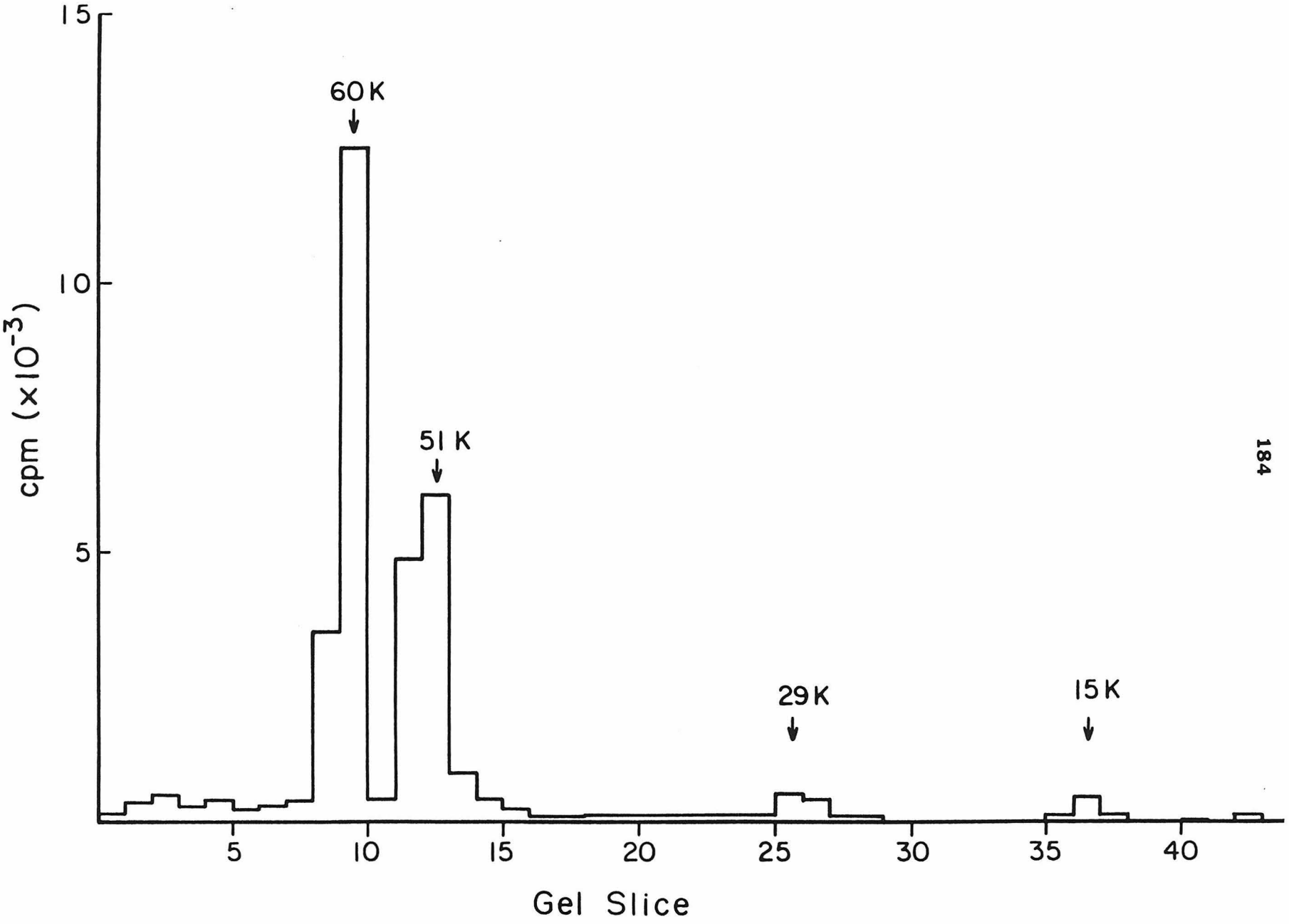
15
10
5
cpm (x10^{-3})
60K
51K
29K
15K
5
10
15
20
25
30
35
40
Gel Slice

Table 1.

Radiolabeling of the subunits of diol dehydratase by radioactive methylmercuric chloride.

Reaction Time		60 kd	51 kd	29 kd	15 kd
30 min.[a]	Total counts	71437	20280	9112	4422
	Corrected[b]	53000	11168	-	-
	Equivalents[c]	.52	.11	-	-
30 min.	Total counts	79768	8447	17060	9237
	Corrected	45708	-	-	-
	Equivalents	.45	-	-	-
30 min.	Total counts	56790	6905	2121	1040
	Corrected	52548	2663	-	-
	Equivalents	.52	.03	-	-
2 hrs.	Total counts	130137	28662	18471	8969
	Corrected	93200	10191	-	-
	Equivalents	.91	.1	-	-
3 hrs.	Total counts	195422	63253	52500	25420
	Corrected	90400	10753	-	-
	Equivalents	.88	.1	-	-

[a] This determination was performed in the presence of coenzyme. The rest were performed in the absence of coenzyme.

[b] The number of counts in the 29 kd subunit were subtracted from those of the 51 kd subunit and twice the number of counts in the 29 kd subunit were subtracted from those of the 60 kd subunit to correct for spurious labeling.

[c] Calculations assume an enzyme specific activity of 95 units/mg, an enzyme molecular weight of 260 kd, and a specific activity of the label of 0.4 Ci/mg.

Proposition 1

Structural and Kinetic Studies of DNA gyrase

Background

DNA gyrase (bacterial topoisomerase II) catalyzes the introduction of negative supercoils into DNA coupled to the hydrolysis of ATP (reviewed in refs. 1-3). Gyrase was discovered as the protein factor necessary for in vitro recombination of relaxed DNA by λ phage int protein on addition of ATP (4). Gyrase catalyzes supercoiling by causing a site-specific double strand break in a duplex DNA substrate, passing another duplex strand through the enzyme-bridged gap in the direction that decreases the linking number, and then resealing the break. The involvement of topoisomerases in several cellular processes has been implicated, including the initiation of replication (5), resolution of pairs of newly replicated DNA (6), transcription (7), and recombination (8).

DNA gyrases from both E. coli and Micrococcus luteus have been purified and the M. luteus enzyme has been shown to have an A_2B_2 subunit structure as determined by chemical crosslinking studies (9). Hybrid proteins formed by mixing subunits from E. coli and M. luteus are fully active, indicating the E. coli enzyme has the same subunit composition (10). The genes for both subunits of the E. coli gyrase have been cloned (11) and the sequence of gyrB has been published (12). The subunits are purified separately and activity is

reconstituted by addition of equimolar amounts of each subunit (11). The A subunit is a homodimer in solution (13) and is the target of the antibiotics oxolinic and nalidixic acid (14) which act to trap an intermediate in which the enzyme is covalently attached to both 5'-ends of the double strand break via a phosphotyrosine bond (15). The B subunit is the target of the antibiotics novobiocin and coumermycin, which interfere with energy transduction by competitively preventing the binding of ATP (16). Therefore, subunit A is involved in strand breakage and subunit B in energy transduction. The molecular weights of the A and B subunits are about 105 and 95 kilodaltons, respectively, giving a mass of 400 kilodaltons for the tetrameric complex (9).

DNase I footprinting studies of the gyrase-DNA complex have shown the enzyme interacts with a region of about 140 basepairs (bp) (18). The central 40 bp are resistant to cleavage by DNAase I centered about the gyrase cleavage site. The sequences flanking the central, protected region show enhanced cleavage by DNAase I every 10 to 11 base pairs with a 2-4 base pair stagger for sites on complementary strands (19-21), suggesting the 50 bp flanking sequences may be wrapped around the protein and exposed to digestion by DNAase I from only one side. Addition of ATP or a nonhydrolyzable ATP analog, ß, ɣ imido-ATP (ADPNP), had no effect on DNAase I cleavage patterns but did change cleavage patterns obtained by digestion with exonuclease III (21). Wrapping of

the DNA about the protein induces the formation of a positive supercoil as demonstrated by the fact that addition of ligase to a complex of gyrase and nicked circular DNA followed by deproteination resulted in an increase in linking number (17). Scanning electron microscopic studies have formed the basis for a model of the gyrase-DNA complex in which subunit A interacts with the cleavage site and the central 40 bp protected region. Surrounding the A subunits, the B subunits interact predominantly with the flanking sequences (13).

The various activities required for this complicated reaction are most easily considered separately.

1. Supercoiling DNA gyrase is responsible for maintaining cellular DNA in a negatively supercoiled state, requiring ATP and Mg^{++} to do so. The reaction is highly processive since after addition of relaxed DNA only supercoiled DNA is observed on agarose gel electrophoresis, no intermediates are seen (16). Each gyrase molecule introduces about 100 supercoils per minute under standard conditions (2). 2'dATP will substitute for ATP but at increased K_M; no other nucleotide triphosphate will stimulate supercoiling. Addition of ADPNP to gyrase and relaxed DNA results in the introduction of 1.4 supercoils per enzyme and then inhibition of further supercoiling, indicating ADPNP causes one round of supercoiling, and implying that hydrolysis of ATP occurs after each round of supercoiling (16).

2. Relaxation In the absence of ATP, gyrase catalyzes the relaxation of negatively supercoiled DNA at a rate approximately 20-40 times slower than the forward reaction. The relaxation is not inhibited by novobiocin or coumermycin but is inhibited by oxolinic acid (23). Topoisomerase II', a form of gyrase containing a fragment of subunit B in place of B is able to catalyze the relaxation of positively supercoiled DNA but is unable to introduce negative supercoils, results explained by the loss of a domain of the B subunit that is responsible for energy transduction (10,22).

3. Double strand breakage Addition of oxolinic or nalidixic acid to gyrase and duplex DNA followed by treatment with SDS results in breakage of the duplex strand and covalent association of gyrase subunit A to the 5' ends of the DNA via a phosphotyrosine bond (15). Gyrase makes a staggered cut leaving four base 5' overhangs. The frequency of cleavage and the pattern of fragments generated is changed by addition of ATP or ADPNP. This change is due to a conformational change of the enzyme on binding of ATP that alters the proportion of enzyme molecules that cleave at their binding position (23). Gyrase, unlike restriction endonucleases, does not cleave at only one defined sequence. Much work has been directed to determining what features of the DNA sequence are recognized by gyrase. Examination of in vivo cleavage sites on addition of oxolinic acid has revealed the

consensus sequence,

5'-RNNNRNRT/GRYCTYNYNYNGNY-3',

where N is any nucleotide, R is a purine and Y is a pyrimidine. Cleavage occurred at the line (24). Mutagenesis of a strong cleavage site on pBR322 has identified some point mutations near the cleavage site that either reduce or abolish cleavage (25).

4. ATPase Gyrase catalyzes the hydrolysis of ATP to ADP and Pi in the presence of DNA with a K_M of about 0.3 mM. The hydrolysis is stimulated much more strongly by duplex than by single strand DNA (3). ATPase activity is inhibited by novobiocin and coumermycin, evidence that the B subunit is responsible for ATPase activity (16). In the presence of urea or on heating the basal ATPase activity of the B subunit is substantially and stably increased, suggesting a conformational change that mimics the effect of binding to DNA. Addition of subunit A to activated subunit B had little effect on ATPase activity (22).

5. Formation and Resolution of catenated and knotted DNA Gyrase catalyzes the formation of catenanes and knotted DNA under conditions of high DNA concentration and low ionic strength which serve to aggregate the DNA, reactions that implicitly require a double strand breaking of closed circular DNA. Gyrase can also relax knots and uncatenate DNA

under normal reaction conditions (3).

The diverse reactions catalyzed by gyrase have been incorporated into several mechanistic models. In one model (27), gyrase binds DNA at specific sites and cleaves both strands. DNA adjacent to the cleavage site wraps around the enzyme in a positive supercoil. ATP binds, causing a conformational change that both separates the strands and drives the wrapped DNA through the opening, decreasing the linking number by two. Hydrolysis of the bound ATP and release of ADP and Pi allow the enzyme to revert to its original conformation. That the hydrolysis follows strand passage is suggested by the experiments with ADPNP which allows one round of supercoiling. This model requires the ends of the DNA not rotate relative to one another. Therefore the two halves of the enzyme-DNA complex must remain in contact while the gap between the ends of the DNA is open. Contact between the B subunits during this phase of the reaction scheme is proposed. Formation of catenanes can be included in the model as transport of a different closed circular DNA molecule. Relaxation of negatively supercoiled DNA in the absence of ATP could occur if DNA binds in the opposite sense and the duplex strand is transported in the opposite direction. A variation of this model requires that the positive supercoiling of the DNA bound to the enzyme not change but that a duplex strand be driven from outside the bound loop to

the inside (13). The sign inversion model proposes that gyrase binds two segments of DNA such that the binding causes the formation of a positively supercoiled node in one part of the ccDNA and a negative node in the remaining part (3). Strand breakage, followed by directional passage of one strand through the break converts the positive node to a negative one.

Experimental Design and Methods

General

E. coli gyrase subunits A and B will be purified separately using the available plasmids pDH24 and pMK461. Plasmid pDH24, which carries the gyrA gene under control of the tac promoter, is maintained in a $lacI^q$ strain, E. coli RB968, and overproduction is initiated by addition of ITPG. Protein from an 11L growth is precipitated with polymin P, resuspended, and precipitated again with ammonium sulfate. Resuspended protein is chromatographed on DEAE-cellulose and then on heparin-Sepharose to yield >95% pure gyrA. Plasmid pMK461 directs the overproduction of gyrB, using a tempera-ture sensitive repressor. Protein from an 11L growth is precipitated with ammonium sulfate followed by chromatography on DEAE-cellulose. Affinity chromatography on novobiocin-Sepharose in which gyrB is eluted with ATP at low ionic strength yields 80% pure gyrB. Further purification by sizing gel chromatography gives essentially pure gyrB. A strong gyrase cleavage site is contained on a 211 bp fragment

of the available plasmid pDH51, which can be excised by digestion with HindIII and purified by agarose gel electrophoresis.

1. Investigation of the requirement for free sulfhydryls

Reaction of gyrase tetramers and DNA with N-ethylmaleimide (NEM) prevented the retention of the complex on a filter binding assay (28). Addition of p-chloromercuriphenylsulfonic acid or NEM to gyrase completely inactivated both the ATPase and supercoiling activities (29). A thorough investigation of the apparent requirement for free sulfhydryls in gyrase may lead to identification of active sites and a better understanding of the mechanism of the enzyme.

The first step will be to determine if one or both of the subunits is susceptible to inactivation by sulfhydryl reagents. Reaction of each subunit separately followed by recombination with unreacted complemetary subunit and assaying will determine if one or both subunits are reactive. Protection against reaction by sulfhydryl reagents using all the various ligands will be attempted, including ATP and its analogs, ADP, P_i, linear DNA of various lengths and the various antibiotics. Protection experiments will be carried out on subunits individually and on the whole tetramer. Inactivation by a number of different sulfhydryl reagents will be carried out since differences in the charge of the reagent and the polarity of the site of reaction can lead to dif-

ferences in the rate of inactivation. The ability of reacted subunits to reform the tetramer can be detected by cross-linking experiments. Quantitation of the number of reactive cysteines will be carried out using either commercially available radioactive sulfhydryl reagents such as iodoacetamide or using the spectrophotometric reagent, DTNB.

There is ample precedent for the involvement of cysteines in the active sites of ATPases. Brain hexokinase (30), Na^+,K^+-activated ATPase (31), phosphofructokinase (32), and aspartokinase L-homoserine dehydrogenase (33) have been shown to contain essential cysteines in the ATPase site. Thus, it is reasonable to suppose that gyrB may contain cysteines in its ATPase site. Affinity labeling of the ATPase sites of gyrB will be attempted using one or more of the available ATP affinity reagents including 8-azidoATP, fluorosulfonyl benzoyl adenosine, and 6-mercapto ribofuranosyl triphosphate (SHTP). SHTP has been shown to inactivate the enyzmes mentioned above by forming a disulfide bond with the cysteines of the ATPase site. The sequence of E. coli DNA gyrase has been determined and shows five cysteines per B subunit (12). Two of the five cysteines are conserved in the gyrB subunit of B. subtilis, making it likely one or both of these cysteines are involved in the ATPase site. Digestion of radiolabeled gyrB subunit by proteases or CNBr will be followed by isolation of labeled fragments by FPLC or HPLC. The labeled fragments will then be sequenced by automated

Edman degradation to localize them in the known gyrB sequence. A complication to be considered may arise if two cysteines are present in the active site. Loss of the covalent label may occur due to displacement of the label from one cysteine by the second cysteine forming a disulfide bond, as has been observed in rabbit muscle pyruvate kinase (34).

2. Investigations of the ATPase activity of gyrase

Analogies have been drawn between the introduction of supercoils by gyrase and catalysis by enzymes involved in muscle contraction, protein synthesis, and oxidative phosphorylation (3). All four systems are proposed to undergo cyclical conformational changes driven by the hydrolysis of ATP. Experiments designed to test the validity of the analogy and to measure rates of individual steps of the proposed mechanism will be carried out.

The reversibility of hydrolysis of ATP has been shown for isolated chloroplast coupling factor 1, demonstrating that proton-motive force is not implicitly required for ATP synthesis (35). This was accomplished using labeled phosphate, $(^{32}P)P_i$, in the medium. $[\alpha\text{-}^{32}P]$ATP was formed and detected by TLC and the ability of hexokinase to utilize the synthesized ATP to phosphorylate glucose. Similar experiments will be carried out with gyrase. If gyrase acts as a conduit between the high energy of ATP and the high energy of supercoiled DNA, incubation of gyrase with ADP, $(^{32}P)P_i$, and

negatively supercoiled DNA should lead to the relaxation of the DNA and the formation of [α-^{32}P]ATP. Control experiments with linear duplex DNA and without DNA will determine if ATP formation is dependent on negative supercoiling. The microscopic rate constants for ATP formation and hydrolysis may be measured by adding $^{32}P_i$ after formation of enzyme bound ATP with cold P_i and measuring the rate of label incorporation. This will also demonstrate enzyme bound ATP isin equilibrium with medium P_i. ATP may be formed on gyrase and then rapidly hydrolyzed, resulting in a futile cycle. This could be detected by carrying out the experiment with ^{18}O-water, as described below.

When the reversibility of ATP hydrolysis has been demonstrated during the relaxation of supercoiled DNA it will be interesting and informative to observe the reversibility of ATP hydrolysis during the forward, supercoiling reaction. A great deal of information regarding steps of the hydrolysis and synthesis of ATP by several ATPases has been gained by analyzing the extent of exchange of oxygen between water and phosphate. Analysis of the amount of ^{18}O incorporated into P_i from ^{18}O-water, as determined by mass spectrometry over a wide range of ATP concentrations during the hydrolysis of ATP by F1-ATPase showed that at low ATP concentrations almost all four oxygens were derived from water indicating nearly infinite reversal of hydrolysis. At high ATP concentrations only one ^{18}O was incorporated in each P_i. These results were in-

terpreted as indicating cooperativity between alternating ATPase sites (36). The release of ADP from one ATPase site required the binding of ATP at another ATPase site. Cooperativity between the two gyrB ATPase sites will be examined using the incorporation of ^{18}O from water into P_i. The extent of ^{18}O incorporation into P_i using various forms of DNA as cofactors, including closed circular DNA, linear DNA of various lengths and no DNA with gyrase tetramers and the B subunit alone over a wide range of ATP concentraitons will be determined. Comparisons of the extent of ^{18}O incorporation and the distribution of ^{18}O-containing P_i species may indicate differences in the rate of release of bound ADP under different conditions. The ratio of gyrase to cofactor DNA will be maintained at a low value to ensure on average one or a few gyrase tetramers per DNA molecule to prevent false cooperative effects due to nearest neighbor interactions on a single DNA molecule (37).

3. Protein-DNA crosslinking

Electron microscopy and patterns from DNase I footprinting studies have provided information on which a model of the gyrase tetramer-DNA complex has been based (13). To test the model and to refine further details of interaction between the enzyme and its DNA substrate, protein-DNA crosslinking studies will be carried out.

Two general approaches will be made. The first involves

the use of the bifunctional protein-DNA crosslinking reagent HSP, which contains a protein-specific moiety, succinate, covalently linked to the photoactivatable DNA-specific group, psoralen (40). The second approach will make use of 5-azido UTP which can be incorporated into the DNA substrate in place of the T of the TG doublet that is the site of cleavage by gyrase (39).

HSP has been used to study the interactions between phage capsid and phage DNA (38). The general method used was addition of HSP to protein in the dark followed by removal of unreacted HSP. The solution is irradiated at 340-380 nm to form psoralen-DNA crosslinks, followed by exhaustive deproteination with proteinase K. The positions of the psoralen crosslinks are determined by denaturing in alkali and rapid separation of single strands from souble strands by agarose gel electrophoresis. The psoralen-crosslinked DNA renatures more rapidly than the denatured single stranded DNA and runs more slowly during electrophoresis. Gyrase subunits will be reacted separately with HSP, synthesized as described (40), and recombined with the unreacted complementary subunit followed by addition of a linear duplex DNA and photolysis. The complex will then be deproteinated by treatment with proteinase K. Psoralen-crosslinked DNA will be separated from noncrosslinked DNA by the alkali-agarose gel system. Restriction endonuclease digestion analysis of the crosslinked DNA and comparison of the double strand fragments

isolated from a second denaturing agarose gel with size standards should allow localization of the positions of the psoralen crosslinks. HSP-treated gyrase B subunit should cause psoralen crosslinks predominately in the flanking regions of the DNA substrate and HSP-treated gyrase A subunit should cause crosslinking in the central, protected region of the DNA substrate. It will be necessary to treat the subunits with HSP under a variety of conditions of differing ratios of reagent to subunit and reaction times to ensure the least amount of perturbation of the structure of the gyrase-DNA complex.

The second approach to protein-DNA crosslinking, which may be less perturbative than the use of HSP, involves the use of 5-azido UTP, a photoreactive analog of UTP, that can be incorporated into linear duplex DNA by DNA polymerase I (39). Radiolabeled 5-azido UTP, synthesized as described, will be added in place of the T of the TG doublet that is the recognition sequence for DNA cleavage. Incorporation of 5-azido UTP in place of T-990 of the major gyrase cleavage site on pBR322 may be accomplished using synthetic oligonucleotides. The 410 bp SphI to NruI restriction fragment from pBR322 can be isolated by agarose gel electrophoresis. The isolated fragment will be ligated to synthetic oligonucleotides encoding a duplex strand with one NruI-compatible end and extending up to T-990 of the TG doublet of the major site of cleavage by gyrase with a 4 base 5' overhang on the

opposite strand. Addition of 5-azido UTP and Pol I will add 5-azido U in place of T-990. Subsequent ligation of a second set of oligonucleotides containing a 3 base 5' overhang compatible with the gyrase cleavage site will generate a linear substrate containing 5-azido U in place of T-990. Since some sequence specificity at this site exists it is reasonable to assume specific protein-DNA contacts are made. Photolysis of the gyrase DNA complex should lead to covalent linkage between subunit A and DNA. Extensive DNase I treatment followed by separation of gyrase subunits by SDS-PAGE and autoradiography should confirm that the A subunit contains the label. Isolation by FPLC or HPLC of labeled protein fragments generated by proteolysis and sequencing of those fragments by automated Edman degradation will identify the residue(s) that contact the critical T. Differences in protein-DNA linkage before and after induction of cleavage by addition of oxolinic acid will be examined.

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Proposition 2

Further Mutagenic Studies of ß-Lactamase

The advent of site-directed mutagenesis has given biochemists a new tool for deciphering the complex relationships between the structure of a protein and its function (1,2). Prior to the development of this technique only fortuitous discovery of random mutants allowed comparisons between the wild-type and mutant to discern the role of the varying amino acid. With site-directed mutagenesis the intelligent design of altered enzymes is possible to provide information on the mechanism of the enzyme, to alter its physical behavior, or to create new activities.

Recent improvements in mutagenic techniques make possible the production of large numbers of structural variants at one or more positions of the protein's primary sequence simultaneously (3,4). Practicality requires a preliminary screen to select those mutants having the desired new activity or altered function from among the large number of variants generated. The existence of a phenotypic screen for the activity of ß-lactamase makes it a good candidate for site-directed mutagenesis.

ß-lactamase catalyzes the hydrolysis of penam and cephem antibiotics (5). These antibiotics act to prevent the biosynthesis of the bacterial cell wall by binding, essentially irreversibly, to the enzymes responsible for the construction of peptidoglycan (6). ß-lactamase confers resistance by hydrolyzing the amide bond of the ß-lactam ring rendering the

antibiotic incapable of acylating its target proteins. Evidence exists that ß-lactamase operates through the nucleophilic attack of an active site serine on the carbonyl of the ß-lactam ring to generate an acyl-enzyme intermediate, which then undergoes hydrolysis. ß-lactamases have been ordered into three general classes based on substrate specificities and physical characteristics. Class A ß-lactamases hydrolyze penams at higher efficiency than cephems, have molecular weights of about 29 kdaltons, and show significant sequence homologies. The Class B enzyme is a zinc-containing lactamase from B. cereus. The Class C enzymes have molecular weights of approximately 39 kdatons and hydrolyze cephems at higher efficiency than penams (7).

Structural information about Class A ß-lactamases exists in the form of primary sequence comparisons (7) as shown below and an x-ray crystal structure of the enzyme from B. licheniformis, resolved to 6 Å (8) (shown in Chapter 4, Figure 1a of this work).

	65					70				
S. aureus PC1	k	R	F	a	y	a	S	T	s	K	a
B. licheniformis 749/c	e	R	F	a	f	a	S	T	i	K	a
B. cereus 569/H	g	R	F	a	f	a	S	T	y	K	a
E. coli RTEM	e	R	F	p	m	m	S	T	f	K	v

Comparisons of the rest of the sequences reveal these four Class A ß-lactamases are strictly homologous in 20% of their

residues but maintain homology by type in a much higher percentage of positions. Mutagenic studies attempted to date have concentrated on the strictly conserved residues about the active site serine (Ser70). The active site serine has been converted to cysteine (9) and threonine (10), the former mutant maintaining about 1% of the activity of the wildtype and the latter being completely inactive. The adjacent threonine (Thr71) has been replaced by the other nineteen amino acids. All mutants except Thr71-->Asp, Tyr, Trp, Lys, or Arg are able to confer resistance to cells to penam antibiotics although all mutants were of reduced thermal stability compared to the wild-type enzyme (3). The saturation of lysine 73 was described in this work. The extension of these saturation mutagenic techniques to more than one residue simultaneously has been proposed (11). Saturating two residues at the same time would require the generation of four hundred different proteins, making location of all four hundred mutants by sequencing individual colonies prohibitively time-consuming. Initial screening for the desired function, resistance to ampicillin, will reduce the number of colonies to be sequenced. If a specific mutant is desired and not found during the sequencing of the active colonies, the mutant may either be located by colony screening using a specifically designed oligonucleotide probe or it may be constructed separately. Since not all mutants will be sequenced it is important to establish that in all probabil-

ity all possible permutations have been screened. Thus, the synthetic oligonucleotides should contain an even mixture of all four bases at the first two positions of the mixed codons and of G and C at the third position to ensure the representation of all 1024 codons generated within a reasonable number of transformants. Zon, et al (12), have shown that even mixtures of oligonucleotides are possible during simple competitive couplings if fresh phosphoramidites are used.

The highly conserved diad, Arg-Phe, at positions 65 and 66 of the RTEM ß-lactamase is a promising choice for two-site saturation mutagenesis. Both amino acids have quite similar substitutes, lysine and tyrosine, respectively. It will be interesting to see if these two amino acids can substitute for the wild-type sequence and if the possible permutations are also phenotypically active. Phe66 is also conserved in Class C ß-lactamases although Arg65 is not. The possibility that Phe66 contributes to the binding energy of the enzyme substrate complex by favorable interactions with the aromatic side chain substituents may be explored by measuring the Michaelis-Menten parameters for substrates with and without aromatic side chains, for instance, benzylpenicillin and 6-amino penicillanic acid, and comparing the values of K_M with those of the wildtype enzyme. The codons for these two residues lie between convenient existing restriction sites allowing easy construction by cassette mutagenesis.

The extension of saturation mutagenesis to a site that

is not strictly conserved but is conserved by type is also proposed. Several candidates close to the active site serine exist such as Phe60. Creation of a unique restriction site 5' to the codon for Phe60 would be required for cassette mutagenesis. It may be possible to acquire information about the reasons behind the greater flexibility of this position. Another position for mutagenesis is Phe72 which lies between the critical active site residues. This site does not appear to be conserved at all. Saturation mutagenesis of this site will determine which amino acids may substitute at position 72 without disturbing the spatial relationship between the essential active site residues, serine and lysine.

Further studies with existing mutants at positions 71 and 73 of ß-lactamase are proposed. The binding of substrate to RTEM-1 ß-lactamase produces a conformational change in the enzyme as judged by differences in the susceptibility to activity loss by proteolysis. Under conditions where in the absence of substrate ß-lactamase was inactivated by proteolysis by Pronase with a half-life of 7 to 9 minutes, addition of either benzylpenicillin or cephalothin increased the half-life to greater than 60 minutes. Addition of oxacillin under the same conditions caused a reduction of the half-life of residual activity to less than one minute (13). The importance of this apparent conformational change to catalysis and the interactions between substrate and residues of the protein that mediate it are not clear. The importance of

Lys73 in the substrate induced conformational change may be determined by examining the proteolytic susceptibility of mutants at position 73, particularly Lys73-->Arg since it is the most conservative change. Any change in the half-life of residual activity or absence of a change in the half-life on addition of substrates would indicate the participation of Lys73 in mediating the apparent conformational change. Since mutants at Lys73 are severely defective in catalysis, if any change in the substrate mediated conformational change is detected, the correlation may indicate the conformational change is important in achieving the maximal turnover rate. If differences are detected it may be interesting to examine the possibility of substrate induced hysteresis, an effect not normally associated with RTEM ß-lactamases but which has been observed with other Class A enzymes.

Adjusting the dependence of enzymic activity on pH is one objective of protein engineering. As discussed in Chapter 2 of this work, substitution of cysteine for Thr71 caused a dramatic decrease in enzyme activity at alkaline pH. The decrease is probably due to the development of anionic character in the vicinity of the active site serine nucleophile. A complementary experiment would be to examine the pH activity profile of the mutant Thr71-->His. The side chain of histidine has a pKa of 6.0. Thus, below pH 6 the side chain of the amino acid adjacent to the active site nucleophile would develop positive charge. The variation in

k_{cat} and K_M at lower pH would give information on the influence of ionic character on the nucleophilicity of the active site serine.

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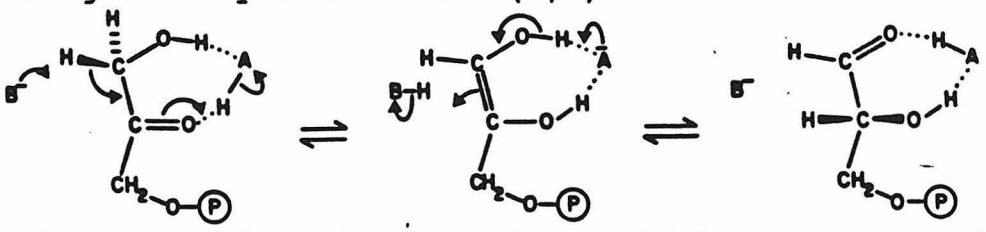
Proposition 3

Mutagenesis of Triosephosphate Isomerase

Triose phosphate isomerase (TIM) catalyzes the interconversion of glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP), which are generated during glycolysis by the splitting of fructose-1,6-diphosphate by aldolase. The interconversion is necessary since the next step in glycolysis converts GAP to 1,3-diphosphoglycerate, so TIM serves to draw DHAP into the useable aldehyde pool. The mechanism of the interconversion by TIM involves the use of general acid-base catalysis as shown below. General base abstraction of the 1-pro-R proton of DHAP by Glu165 of the enzyme from chicken muscle and donation of a proton from an as yet unidentified general acid (either Lys13 or His95 or both) leads to formation of the intermediate enediol. The following step is the donation of a proton to C-2 by the conjugate acid of the general base and abstraction of the hydroxyl proton at C-1 by the conjugate base of the general acid to give enzyme-bound GAP (1,2).

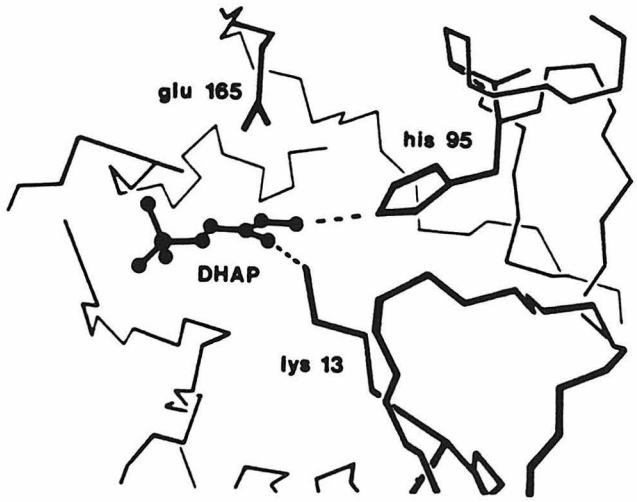


The energetics of catalysis by TIM have been examined in detail, by isotope exchange experiments. The establishment of general base participation was shown by the small but measurable tritium transfer from [1(R)-^{3}H]dihydroxyacetone-3-phosphate to C-2 of product GAP (3). The incorporation of ^{3}H from ^3H_2O into product GAP and into substrate DHAP during the

forward (DHAP-->GAP) reaction was examined, under conditions where released product was trapped by reduction to glycerol phosphate by NADH dehydrogenase (4). The discrimination against tritium incorporation into product as measured by the ratio of the specific activity of ^3H_2O to glycerol phosphate was 1.3, a value too low to represent a primary kinetic isotope effect. Therefore, it was concluded that the intermediate enediol and the enzyme bound product and substrate are in rapid equilibrium and the rate-determining step of the forward reaction is product release. The rate of the increase of the specific activity of the substrate DHAP showed the enediol reverses to enzyme-bound substrate about one third as rapidly as it goes on to product GAP. The reverse reaction (GAP-->DHAP) had a primary kinetic isotope effect of 8 against tritium incorporation into DHAP from ^3H_2O under conditions where the DHAP was trapped by oxidation to 3-phosphoglycerate. Under these conditions release of substrate is not rate-determining and the intermediates were not in rapid equilibrium. Partitioning experiments allowed the determination of the relative rates of the individual reactions. A free energy diagram incorporating the results of the isotope exchange experiments has been developed. The rate determining step of the reverse reaction, the association of GAP and TIM is $3x10^8$ $M^{-1}sec^{-1}$, a value that approaches the diffusion limit. Therefore, TIM is thought to have reached the end of its evolutionary development since increasing the

rate of any of the chemical steps of the catalytic mechanism cannot increase the overall rate of catalysis (5).

A crystal structure of TIM from chicken muscle has been solved to 2.5 Å resolution (6) and the structure of TIM from yeast is known to 1.9 Å without substrate (7) and to 3.5 Å with substrate bound (8). A schematic representation of active site residues is shown below (taken from ref. 10). Because of the detailed existing knowledge of the mechanism and energetics of catalysis by TIM and the high quality of the structural information available, TIM is an excellent system for study by site-directed mutagenesis.



A mutant of TIM, Glu165-->Asp, has been constructed by oligonucleotide-directed mutagenesis. This mutant removes one methylene group from the active site general base and decreases the rate of catalysis 1000-fold (9). A detailed investigation of the changes in the rates of individual steps

in the catalytic mechanism has been carried out. The mutant was first shown to catalyze the interconversion of DHAP and GAP by general acid-base catalysis as does the wildtype enzyme and not by indirect catalysis by a water molecule. The proof consisted of identical stoichiometries of inactivation by the affinity reagent bromohydroxyacetone phosphate and identical solvent D_2O effects. An increase of 4 kcal/mol in the transition state energies of the reactions converting the enediol intermediate to enzyme-bound DHAP and to enzyme-bound GAP was calculated (10).

Catalysis by TIM has an electrophilic general acid component. The role of the general acid is to assist in the polarization of the carbonyl group of DHAP. Proof of the existence of this polarization comes from experiments that show the rate of reduction of the carbonyl of DHAP by borohydride is 8 times faster when the DHAP is bound by the enzyme than when it is free in solution (11), and the fact that FT-IR shows the carbonyl stretching frequency of enzyme-bound DHAP is 19 cm^{-1} less than free DHAP (12). The unambiguous assignment of the electrophilic component of catalysis to a specific amino acid residue is not possible based on existing structural information. However the most likely candidates for the general acid catalyst are the side chains of either Lys13 or His95 or both, as shown in the figure above. Site-specific mutagenesis of these two residues should both determine the relative contributions of these two

residues to catalysis and the importance of the general acid to catalysis by TIM.

Lys13 and His95 will be converted separately and together into alanine(s) which removes the possibility of general acid contributions to catalysis from these residues. Alanine is chosen because it represents the least disruptive mutation possible, substituting an H for the imidazole of the histidine and for $(CH_2)_3NH_3^+$ of the lysine. It may also be interesting to invert Lys13 and His95 to create a double mutant (Lys95,His13). The mutation Lys13-->Arg will also be constructed as it is the most conservative mutation possible. Oligonucleotide-directed mutagenesis will be carried out using the gapped plasmid method on a 1046 bp NcoI fragment encoding the chicken TIM gene (9). The following mutagenic oligonucleotide probes will be synthesized using the phosphoramidite chemistry (13),

```
Lys13-->Ala
           **
5' GGC AAC GCG AAG ATG 3'

His95-->Ala
           **
5' CTG GGC GCC TCA GAG 3'
```

(Asterisks denote the mismatched bases.) The mutagenic probes made radioactive by kinasing using standard procedures (14), and screening for the desired mutations will be carried out as described (15). To create the double mutant, Lys13-->Ala, His95-->Ala, the same mutagenic procedure can be carried out on the single mutant Lys13-->Ala using the His95-->Ala muta-

genic probe. Double mutants will be identified by screening with both probes and by Maxam-Gilbert sequencing. Expression of mutant protein will be carried out in E. coli, using plasmid pX1 (10). Mutant TIM protein will be purified according to published procedures (10). Crystallization of the mutant proteins and comparison of the structures by difference mapping with the known crystal structure of the enzyme from chicken muscle is a relatively rapid procedure and will determine if any gross structural changes accompany the introduction of the mutations.

Measurement of the Michealis-Menten parameters and analysis of the changes in the energetics of the catalytic mechanism will be carried out using the same kinds of isotope partitioning experiments described earlier. An interesting possibility is that both residues His95 and Lys13 contribute to general acid catalysis. The -amino group of the lysine side chain may contribute a proton to the C-2 carbonyl of DHAP while the imidazole ring of His95 accepts the hydroxyl proton of C-1. If this double participation exists then both mutations will show an increased energy of activation for the formation of the enediol. In contrast if only one of the two residues is primarily responsible for the electrophilic component of catalysis then only one of the two mutants will show a substantial decrease in the reaction rate and an increase in the activation energy for enediol formation. If there is no change in the energetics of either singly mutated

enzyme the cause may be the ability of one of the residues to substitute for the other. In this case the double mutation should show a substantial change in the reaction energetics. If there is no electrophilic component of catalysis then none of the mutants should show major changes in the reaction rate or energetics. If perturbations introduced by the Lys13--> Ala mutation leave the mutant completely inactive, it may be interesting to convert Lys13 to cysteine and react the resulting mutant with ethylenimine as described in Chapte 4 of this work. Slight differences in bond distances and angles may be reflected in the reaction energy profile.

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Proposition 4

Kinetic Studies of the T4 Replication System

The rapid, efficient replication of prokaryotic DNA with high fidelity is carried out by multicomponent protein systems which catalyze a complex series of reactions. In general, these reactions include initiation of replication at specific sites on the chromosome by nicking of one strand or by unwinding of the DNA helix, followed by synthesis of an RNA primer. The replication extends from the primer in the 3' direction (of the growing strand) copied from the leading strand and as the helix is unwound the lagging strand is copied discontinuously (1). Fidelity is believed to be maintained by a continuous 3'-5' exonuclease activity of the polymerase which acts to edit out incorrectly placed bases. All of the components necessary for the replication of bacteriophage T4 DNA have been identified.

The products of seven genes of the bacteriophage T4 are necessary for <u>in vivo</u> replication of its DNA; genes 32, 41, 44, 45, 61 and 62 (2,3). Gene 41 and 61 proteins are both involved in the synthesis of the pentanucleotide primer, $pppACN_3$ (4). The sigmoidal dependence of the activity of protein 41 on protein concentration suggests it forms oligomers (5). Gene 32 protein is a single-strand DNA-binding protein that serves to destabilize secondary structures of self-complementary single stranded DNA and to prevent renaturation of melted duplex DNA. Gene 43 protein is the 110 kdalton T4 DNA polymerase. It has a 3'-5' exonuclease act-

ivity and can by itself catalyze the elongation of a primed template. However, the T4 polymerase will incorporate only a few nucleotides before dissociating from the template (6). The products of genes 44 and 62 copurify as an ATPase (7). Addition of the 44/62 and the gene 45 protein to the T4 polymerase caused an increase in the processivity of the reaction in a manner dependent on the hydrolysis of ATP (8). The addition of the 44/62 protein and the gene 45 protein also stimulated the 3'-5' exonuclease activity of the T4 polymerase (9). These results have been interpreted as indicating the accessory proteins (gene 44/62 and 45 proteins) serve to hold the polymerase on the DNA template. A quantitative examination of the on and off rates of the T4 polymerase binding to its DNA template in the presence and absence of the accessory proteins can be made.

The experimental design will follow an elegant series of experiments carried out on DNA polymerase I (Pol I) (10). The system used included a homopolymer of poly(dA) as template, an oligomer of dT as the primer, and dTTP as substrate. A preferential order of binding was established by isotope partitioning experiments. Pol I was incubated with [^{3}H]dTTP to form the bound complex and then the template-primer system and unlabeled TTP were added. The failure to detect incorporation of high specific activity dTTP into the template at short reaction times indicated the [^{3}H]dTTP was released before the DNA bound to the enzyme. In a complemen-

tary experiment, Pol I was preincubated with template-primer and then [^{3}H]dTTP and an excess of denatured challenger DNA was added. The incorporation of [^{3}H]dTTP into template DNA was indicative of a productive Pol I-template DNA complex, and analysis of the curvature of the incorporation of counts into DNA vs. reaction time allowed a direct measurement of the off rate of Pol I-template binding. Scatchard analysis of the same experiment at varying conentrations of template DNA allowed measurement of the intrinsic dissociation constant of the Pol I-template DNA complex. These results demonstrated a preferred order of binding of Pol I to template and then to dTTP, consistent with the role of Pol I as an editing polymerase. The rate of association of the Pol I-template complex was determined by calculation from the known dissociation rate and the known equlibrium constant of dissociation and was also measured directly by the rate of increase of light scattering. Similar experiments will be carried out using the T4 replication system. Rates of association and dissociation of T4 polymerase and template DNA will be measured in the presence and absence of the gene 44/62 protein, the gene 45 protein, and ATP. If the accessory proteins serve to anchor the T4 polymerase to its template, then the rate of dissociation the polymerase in the presence of the accessory proteins should be less than in their absence.

The increase in the processivity of T4 polymerase in the

presence of the accessory proteins could be due an increase in the rate of addition of bases to the template. Further experiments will determine if addition of the accessory proteins to T4 polymerase causes any change in the kinetics of the polymerization reaction itself. An examination of the kinetics of incorporation of [^{3}H]dTTP during the first turnover by Pol I was made using stopped-flow instrumentation and chemical quenching of the reaction by addition of EDTA. Biphasic kinetics were observed and the curve shape was determined to be consistent with a mechanism containing two partially rate-determining steps. These two steps were suggested to be conformational changes of the enzyme that first allow hydrolysis of the bound dTTP and second cause the translocation of the enzyme to the next base on the DNA template. Similar rapid kinetics experiments on the incorporation of the first dTTP into prebound DNA template and T4 polymerase will be carried out in the presence and absence of accessory proteins and ATP.

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Proposition 5

Protein-Protein Interactions in the *hin* Recombinase System

Site-specific recombination systems have been isolated from several prokaryotes. These systems mediate specific DNA rearrangements that are involved in several important functions, such as separation of chromosomes, regulation of gene expression and transposition. Four systems of varying complexity have been characterized. The lox-cre system of bacteriophage P1 requires only two 34 bp recognition sequences and the cre gene product, a 35 kdalton protein (1). The lox-cre system is important in circularizing linear duplex P1 DNA on entering the host cell and in resolving dimers of P1 DNA formed during replication (2,3). The resolvase system operates on the Tn3 family of transposons. It requires a supercoiled DNA substrate containing three resolvase binding sites in a defined orientation (4,5). The att-Int system requires the involvement of a host protein in addition to a complex of four binding sites and the integrase, IHF, which is composed of two polypeptides of molecular weights 11 and 9.5 kdaltons (6).

The control of expression of the flagellin genes of Salmonella typhimurium is determined by hin recombinase, representative of the fourth type of system. The inversion of a 996 bp sequence of chromosomal DNA switches the position of a promoter for one flagellin gene and the repressor of a second flagellin gene. The DNA requirements for the inversion are two 26 bp segments in an inverted configuration and

a 60 bp enhancer sequence that can increase the rate of inversion 150-fold (7). The proteins required for recombination include the hin protein, a 21 kdalton DNA binding protein and Factor II, a 12 kdalton protein which has been shown to bind the enhancer sequence. The presence of E. coli histone-like protein HU also increases the rate of inversion, probably by binding nonspecifically to the DNA and promoting bending of the DNA that helps bring together the two recombinational sites (8). Interestingly, the gene for the hin protein resides within the 996 bp inversion segment and shows 40% homology to tnpR, the gene for Tn3 resolvase (9). The distance between the enhancer sequence and the proximal hin recognition site was varied and efficient enhancement of the inversion rate was found to be invariant with distance beyond a minimum of about 100 bp (7). Hin and Factor II have been shown to bind independently to their respective binding sites (10). The orientation between the enhancer sequence and the proximal hin binding site, either between the two hin sites or on the outside, did not effect the enhancement of the inversion rate (7).

The role of the host protein, Factor II, in increasing the rate of inversion is not well understood. One possibility that has been suggested (8) is that specific protein-protein interactions between Factor II bound to the enhancer sequence and hin recombinase bound to the distal hin site help to increase the rate of formation of the synaptic

complex which then undergoes inversion. Experiments designed to test this possibility and to detect Factor II-hin recombinase interactions will be carried out.

To determine if tracking, the binding of one hin subunit to a hin site then diffusion of a second subunit along the DNA until the second site is found, is the basis of the baseline inversion rate in the absence of Factor II, the following experiment will be carried out. Catenanes will be made of pBR322 containing one hin site constructed by ligating into the SalI site synthetic oligonucleotides encoding the 26 bp hin site with SalI compatible linkers on either end. The 60 bp enhancer sequence may be incorporated into any other single-cut restriction site in a similar fashion. The construction of catenanes by DNA gyrase has been described as has the importance of tracking in the resolvase system (5). This technique of construction will allow the variation of the distance between the hin site and the enhancer sequence. If hin recombinase tracks along its DNA substrate, it will not jump to the sister plasmid and recognize the second hin site so no inversion will take place. If inversion does take place it will lead to the formation of a single circular DNA molecule. Detection of inversion can be accomplished by digestion with any single cutting restriction endonuclease and subsequent agarose gel electrophoresis. Inversion and endonuclease digestion will give three DNA fragments. Absence of inversion and endonuclease digestion will

give linearized plasmid. The effect of adding Factor II will be determined. If specific Factor II-hin recombinase interactions exist they may cause hin recombinase to jump strands and catalyze inversion.

The possibility of specific interactions between Factor II and hin recombinase will also be tested by crosslinking experiments, using the bifunctional crosslinking reagent dimethyl suberimidate. To determine the conditions under which the hin protein can form oligomers in the absence of DNA the concentration of hin protein will be varied using a constant concentration of crosslinker and vice versa. Analysis of crosslinked products will be carried out by SDS-PAGE and silver staining. When the conditions for hin protein oligomerization have been established the same experiment will be performed in the presence of plasmid DNA containing one or two hin sites. The same experiments will be carried out using Factor II. These experiments will determine if a limited number of protein molecules interact with each hin or enhancer site.

When the conditions for hin-hin and Factor II-Factor II crosslinking have been established, the possibility of hin-Factor II crosslinking will be explored. To determine the conditions under which hin and Factor II associate in the absence of DNA crosslinking will be attempted with various concentrations of hin holding the Factor II concentration conconstant and vice versa. The effect of adding a DNA sub-

strate containing binding sites for both hin and Factor II will be determined. Presumably, if direct association between hin and Factor II occurs, it should be possible to crosslink them in the presence of DNA containing both binding sites under conditions where they are not crosslinked in the absence of DNA substrate. The control reactions which include plasmid DNA which alternately has no hin or enhancer site will also be carried out to show that the detected interactions between hin protein and Factor II depend on the presence of specific binding sites. Another control reaction would include plasmid DNA that has one hin site and the enhancer sequence separated by various distances. Since enhancement is prevented by placing the enhancer and the hin site at a distance of 48 bp it will be interesting to see if crosslinking is prevented by this arrangement. If the amount of hin-hin and Factor II-Factor II crosslinking is too great it may be possible to reduce it by binding Factor II to its enhancer site and then adding the crosslinker, followed by dialysis to remove excess crosslinker and addition of hin protein. To show that any detected interaction between hin and Factor II is specific and not induced by the crosslinking reagent, crosslinking between Factor II and E. coli histone-like protein HU will be attempted. If Factor II-hin protein interactions are specific, no crosslinking between Factor II and HU should be detected.

If large numbers of hin subunits and Factor II are

crosslinked it may not be possible to distinguish complexes by SDS-PAGE gels since resolution of higher molecular weight complexes may be poor. However, useful information about the structure of crosslinked complexes of Factor II, hin protein, and DNA may come from electron microscopic studies. Observation of complexes between E. coli DNA gyrase and its DNA substrate have led to the development of models for gyrase interaction with DNA (11). The use of DNA substrates with varying distances between the hin binding sites and the enhancer sequence may allow observation of looped structures proving the existence of specific interactions between hin protein and Factor II.

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