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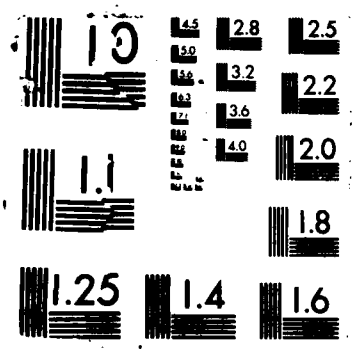
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This report discusses how proteins function as molecular machines, how to introduce novel activities into proteins, how the function of a protein is related to its three-dimensional structure and in turn to its sequence of amino acids, how the various elements of a protein contribute to its structural stability. these questions form the general focus of this work. (beta) (alpha)

To gain the appropriate insights into the general rules that govern these relationships, we are developing and using various techniques of mutagenesis to alter the amino acid sequence in enzymes such as (β) lactamase (responsible for resistance to penicillin therapy in many strains of infectious bacteria) and the serine protease, α-lytic protease. We also employ novel chemical modifications of mutant proteins to achieve structures that cannot be obtained by purely biochemical approaches. The eventual objective is to be able to develop new enzymatic catalysts that, for example, will accelerate the hydrolysis of toxic organophosphates, some of which are potent nerve bases. Keywords:

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ANNUAL REPORT

June 15, 1987

Mutant Proteins--Enzymes to Hydrolyze Toxic Organophosphates

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During the past year work has continued on studies of the relationship between the amino acid sequence of a protein, its three-dimensional structure, the stability of the folded protein (for example, its resistance to stressful environments such as high temperature and acidic or basic pH), and catalytic activity. We have also explored approaches that change the types of reactions catalyzed by a particular enzyme. These studies have focused principally on two amidases, β-lactamase and the serine proteinase, α-lytic protease.

1. β-Lactamase

a. We have investigated the role of the disulfide bond in stabilizing wild-type and mutant RTEM-1 β-lactamase.

We have previously generated and characterized mutants of RTEM-1 β-lactamase with all possible amino acid substitutions (site-saturation) at Thr 71; this residue is part of the conserved triad (-Ser-Thr-Xaa-Lys-) in class A β-lactamases that contains the active site serine residue. Surprisingly, fourteen of the mutant proteins retain considerable catalytic activity but all 19 are less stable to proteolysis and thermal denaturation than wild-type β-lactamase. Uniquely among class A β-lactamases, the RTEM-1 (and RTEM-2) enzymes contain a single disulfide bond between Cys 77 and Cys 123. To study the possible role of this naturally occurring disulfide in stabilizing β-lactamases with mutations at residue 71, this bond was removed by introducing a second mutation Cys 77 → Ser. We first generated and characterized a singly mutant Cys 77 → Ser β-lactamase. Both the wild-type and the Cys 77 → Ser enzyme confer the same high levels of resistance to ampicillin *in vivo* to *E. coli*; at 30°C, the specific activity of purified Cys 77 → Ser enzyme is also the same as that of the wild-type enzyme. However, above 40°C or above pH 8 the mutant enzyme is significantly less stable than wild-type enzyme. We then introduced the Cys 77 → Ser mutation into all 19 of the β-lactamases containing mutations at residue 71 and analyzed the ability of the 19 resulting doubly mutant proteins to confer ampicillin resistance to *E. coli*. None of the doubly mutant proteins conferred resistance to ampicillin *in vivo* at 37°C; proteins with Ala, Val, Leu, Ile, Met, Pro, His, Cys and Ser at residue 71 conferred low levels of resistance to ampicillin *in vivo* at 30°C. We also estimated the relative quantities of mutant proteins in whole cell extracts of *E. coli* by use of electrophoretic blots stained with antibodies against β-lactamase. We observed that all 19 of the doubly mutant enzymes seem to be proteolyzed much more readily than their singly mutant analogues that contain a disulfide bond. We interpret these results to suggest the disulfide bond of the RTEM-1 β-lactamase can significantly reduce the destabilizing effect of mutations at Thr 71. These results also emphasize the unique and essential role Thr 71 performs in the stable folding of RTEM-1 β-lactamase.

b. Regeneration of essentially complete catalytic activity by chemical modification has been achieved by treatment with ethylene imine of a mutant of β-lactamase (Lys 73 Cys). This mutant affects a lysine residue in the catalytic site of the enzyme; this lysine forms a hydrogen bond to the hydroxyl group of the catalytic serine. The loss of the basic amino group in the Lys 73 Cys mutant reduces the activity by a factor of ~10^4. Can one recover a high level of catalytic activity by reintroducing an amino group with the proper relationship to the polypeptide backbone, as by extension from the sulfur atom of the cysteine residue by a dimethylene amino group? Indeed, we have shown that such chemical modification can accomplish this result though the reactivation requires reaction with ethylene imine under mildly denaturing conditions (4 M urea) necessary to partially unfold the protein and thereby expose the sulfhydryl group of the cysteine residue. The resulting aminoethylcysteine is essentially isosteric with lysine.

$$\text{E-CH}_2\text{SH} + \underset{\triangle}{\overset{\text{NH}}{}} \longrightarrow \text{E-CH}_2\text{SCH}_2\text{CH}_2\overset{\oplus}{\text{N}}\text{H}_3$$

The enzyme reactivated in this way and purified has regained essentially complete activity, i.e., increased by 10^4 its catalytic effectiveness over its mutant precursor. Such chemical modification of specifically generated mutants, a combination of biological and chemical techniques, opens new, generally useful approaches to structure-function studies and to the creation of new catalytic agents not otherwise accessible.

c. D,D-carboxypeptidase activity has been introduced into β-lactamase as pointed out in last year's report, by creating the following chimeric protein;

	50 * 78
β-Lactamase:	-DLNSGKiLes-frpeeRFpmmStfKvllcG-
PBP5 Sequence:	-DLNSGKvLaeeqnadvRrdpaSltKmmtsG-

this protein differs by about 8% overall from β-lactamase itself and shows about 3% of the activity on L-Lys-D-Ala-D-Ala derivatives of the D,D-carboxypeptidase PBP5 (the wild-type β-lactamase completely lacks such activity). We are now using a synthetic approach to introduce a number of amino acid substitutions into this region of the enzyme and screening the resulting mutants on a chromogenic depsipeptide system to drive mutation to increase carboxypeptidase activity.

d. A mutation (Ala 172 → Thr) has been reported (1) to increase the substrate specificity of RTEM-2 β-lactamase for cephems relative to penams. We are studying the role of other residues at this position by a site-saturation experiment of the type previously employed at residues 71 and 73. To this end we have introduced restriction sites at positions near residue 172 to allow use of cassette mutagenesis to obtain the desired variants which are now in hand. DNA sequencing of colonies is underway to identify mutants containing each of the 19 possible variations at 172 before assaying the effect of these changes on substrate specificity.

e. The recent structural work on the *Staphyllococcus aureus* β-lactamase (2) (of the same family as the RTEM enzyme) calls attention to two residues conserved in all class A lactamases: Glu 266 and Lys 234. We have introduced unique

restriction sites preparatory to using cassette mutagenesis to carry out site-saturation studies at these positions.

1. Hall, A. and Knowles, J. R. (1976) *Nature* 264, 803-804.
2. Herzberg, O. and Moult, J. (1987) *Science* 236, 694-701.

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