Biol Res 29: 127-140 (1996)

Protein engineering as a powerful tool for the chemical modification of enzymes

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This article discusses the techniques of site-specific mutagenesis and protein engineering and their application in the study of enzyme active sites and the mechanism of enzyme action. Particular emphasis is given to beta-lactamase.

Key terms: *beta-lactamase; non-natural amino acids; polymerase chain reaction (PCR); protein engineering; site-specific mutagenesis.*

INTRODUCTION

Other papers of this issue illustrate the use of controlled chemical modification of amino acid side-chains within proteins, and show the insight that can be gained from it. Unfortunately, proteins frequently have more than one exposed residue of a particular amino acid type, and the researcher has to rely on their differential chemical reactivity, conferred by the protein environment, and hope to find conditions to single out the residue of interest.

The development of recombinant DNA methods has opened many new avenues to bypass this problem and, in general, for the study of proteins. Specific alteration of DNA molecules (site-specific mutagenesis) is technically easy and more precise than residue-specific alteration of proteins. Thus, a suitable expression system allows the researcher to alter the gene coding for the protein of interest at a specific codon, and study the effects of the alteration on the product of the expressed gene. This simple concept constitutes what is called today "protein engineering".

In this article, we will present developments in protein engineering for the study of enzyme active sites. We will review the current state of mutagenesis approaches and briefly consider alternative expression systems. We will also discuss some of the diverse ways in which protein engineering could be used, directly or indirectly, to enhance the battery of methods available to study enzyme catalysis. At the end of the paper we illustrate the use of combinatorial mutagenesis with active-site studies of beta-lactamase, performed in our laboratory.

CURRENT ADVANCES IN SITE-DIRECTED MUTAGENESIS

It has been well over a decade since the first successful experiments proved that the "ultimate mutagen", the oligonucleotide, can be used to introduce predetermined alterations in a gene (Dabalie-McFarland *et al*, 1982; Shortle *et al*, 1981; Smith, 1982). The techniques used to accomplish such a goal, which won a Nobel prize to Michael Smith in 1993 for his pioneering work, have been developed and refined continuously and abundantly applied to many problems since then.

Today, any laboratory equipped for recombinant DNA work can easily set up site-directed mutagenesis techniques. The many protocols currently available aim mainly at achieving good efficiency and reproducibility. Most of them are variations of

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essentially the same procedure, which is shown in Figure 1.

Several clever manipulations have been developed to improve the mutant yield and to simplify the procedures, and some of them are commercially available in kit form. Table I compares the features of some of the most popular methods.

In the most recent years, the appearance of the Polymerase Chain Reaction (PCR (Saiki *et al*, 1988)), which also won a 1993 Nobel price to its inventor, Kari Mullis, has had an impact in mutagenesis as well. Basically, the PCR consists on DNA segment amplification using a couple of flanking oligonucleotide primers and a thermostable polymerase, initially Taq DNA polymerase isolated from Thermus aquaticus. This kind of polymerase allows exponential DNA amplification using temperature cycles of DNA denaturation, primer annealing, and primer extension. Just as with previous methods, a good deal of variations of PCR mutagenesis can be found in the literature (Reikofski & Tao, 1992). Some of them are presented in Table II and examples given in Figure 2.

Due to the diversity of methods currently in use in many laboratories it is rather difficult to be aware of every new improvement introduced. For the same reason, it is not simple to favor a particular procedure; several methods have complementary

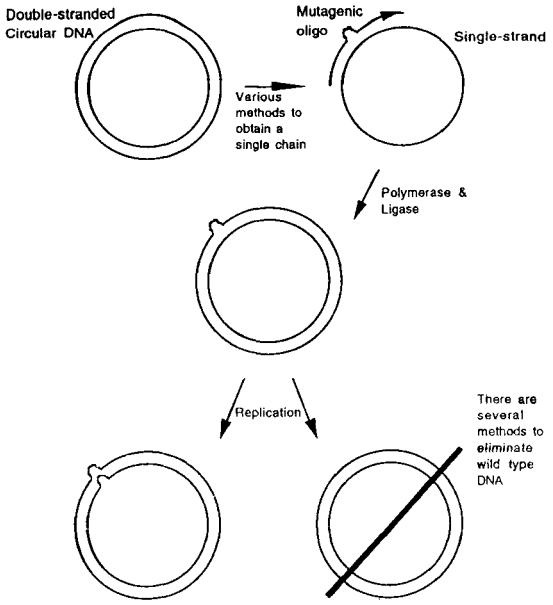

Fig. 1. General scheme for oligonucleotide directed mutagenesis. See also Table I.

virtues. Nonetheless, we tend to prefer methods based on PCR, mainly because of their simplicity. Nowadays PCR is a widespread, very robust technique, which is easily implemented even in laboratories with limited experience in recombinant DNA work.

Inherent to PCR methods is the possibility of introducing non desirable extra mutations. Taq DNA polymerase lacks proofreading

Table I

Schemes for site-directed mutagenesis

SCHEME	DESCRIPTION / NOTES	REFERENCES
Basic method with M13 vectors	M13 phage vectors are used to afford the single-stranded template. The procedures are simple, but the yield of mutants is frequently low (a few percent).	Zoller & Smith, 1982
Gapped duplex/ second primer	The introduction of a second, upstream primer enhances the efficiency of incorporation of the mutagenic primer. Similarly, the generation of a gapped segment enhances the mutant yield. Protocols have been developed for single stranded and double stranded templates.	Zoller & Smith, 1984; Schold *et al*, 1984; Kramer *et al*, 1984
Coupled priming methods	In these methods, a second primer introduces a mutation in a selectable marker gene within the vector. This event is highly linked to the incorporation, in the same strand, of the relevant mutagenic primer.	Carter *et al*, 1985; Stanssens *et al*, 1989
Modified template strand	Selective elimination of the template strand enhances the mutant yield. This is due to the fact that unmodified, in vitro synthesized DNA is preferentially repaired in vivo. Two popular methods, utilizing uracil or phosporothioates as markers of the template strand, belong to this category. Adaptation of one of the methods to plasmid DNA increases its general applicability.	Olsen & Eckstein, 1990; Kunkel, 1985; Taylor *et al*, 1985

Table II

Schemes for PCR-based mutagenesis

SCHEME	DESCRIPTION / NOTES	REFERENCES
Basic (Near the end)	Use a mismatched oligonucleotide at the target place, which must be located near a unique restriction site; amplify with another oligonucleotide beyond the next unique restriction site. Digest and clone the product. Pros:simple; requires only two oligonucleotides. Cons: appropriately located restriction sites are needed.	Kadowaki *et al*, 1989
Overlap extension	See Fig 2a. Pros: efficient, general. Cons: requires four oligonucleotide primers.	Ho *et al*, 1989
Mega-primer	See Fig 2b. Pros: simple. Cons: low yield of mega-primer may limit applicability (in our hands, megaprimer size should be kept to < 500 bp for best results). Spurious addition of A at the 3' end of the primer by Taq polymerase.	Sarkar & Sommer, 1990
Non-Template hybrid primer	See Fig 6. Pros: General Requires only one primer if adapted to universal vectors (Merino *et al*, 1992). Cons: Requires three primers if new template is employed. Abortive extension may lower yield of mutants.	Nelson & Long, 1989
Restriction site inactivation	A restriction site is inactivated and recreated at one end of the amplified product by the appropriate primers. This, in turn, eliminates the wild type sequence at the digestion step before cloning. Pros: General. Cons: Requires four primers for the first mutation.	Ito *et al*, 1991
Whole plasmid protocols	Divergent PCR is performed on the circular plasmid. Subsequent recircularization of the product, by ligation or recombination, leads to a replicative molecule containing the mutation carried by one or two of the primers. Pros: Simple. Only two primers needed. Cons: Efficiency may drop as the plasmid gets larger. Higher probability of additional mutations Some protocols require up to four primers.	Hemsley *et al*, 1989; Jones & Winistorfer, 1992

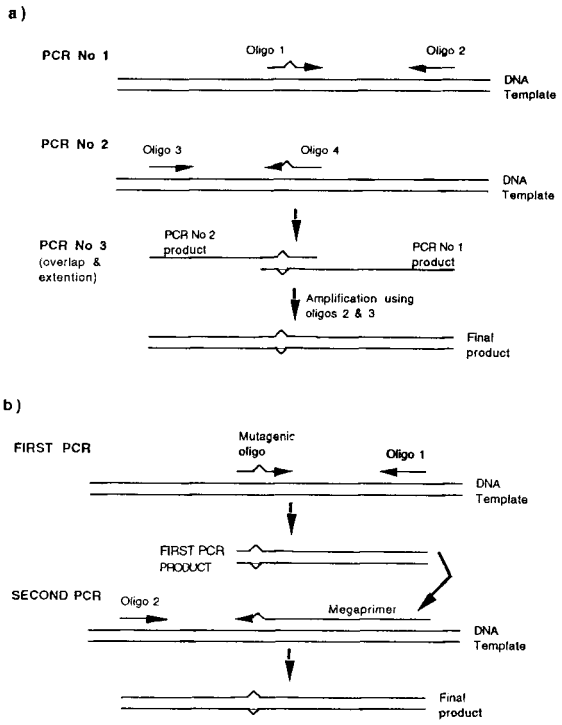

Fig. 2. Two examples of PCR-based oligonucleotide directed mutagenesis. a) Overlap extension method; b) Megaprimer method. See Table II and also Figure 6.

capabilities, and errors have been calculated to appear as frequently as one in 400 base pairs (Clackson *et al*,1991). However, in practice these could be kept to about one in 1.000 base pairs. Using other thermostable polymerases with proofreading activity might improve the numbers. At any rate, it is normally required that any piece of DNA subjected to a mutagenesis procedure is sequenced entirely, regardless of the method employed. Although it is increasingly possible to successfully and accurately amplify fragments up to ten thousand or more base pairs (Cheng *et al*,1994), it is always a good practice to keep the amplification or subcloning fragment below 500 base pairs in order to minimize the problems. Some methods do not require subcloning of a mutagenized fragment but, in our view, this marginal advantage is offset by the need to use special vectors or bacterial strains.

When the target region is flanked by restriction sites, or appropriate ones have been introduced, the so-called cassette mutagenesis may be employed. This simply

involves the synthesis of oligonucleotides comprising the entire region between the restriction sites incorporating the corresponding changes, and its cloning in replacement of the original fragment (Wells *et al*, 1985). Cassette mutagenesis is an efficient method for the generation of multiple mutations at defined sites (Matteucci & Heyneker, 1983). Similarly, total synthesis of genes is clearly a feasible endeavor (Jayaraman & Puccini, 1992; Prodromow & Pearl, 1992), which may even be required in some cases (Makoff *et al*, 1989). In a totally synthetic approach, convenient restriction sites can be introduced all over the gene, thus facilitating subsequent replacement of chosen segments with altered versions.

The reader is referred to several reviews (Zoller, 1991; Zoller, 1992) and to the book edited by McPherson (1991), for detailed descriptions of several mutagenesis approaches, both directed and random.

THE NEED FOR AN EXPRESSION SYSTEM

Obviously, the mutated gene is inert until it is introduced into a system that is capable of its transcription and translation. Depending on the protein of interest, this can constitute a serious bottleneck. In cases where the protein requires post-translational modifications or disulfide bridge formation, the simplest expression system, *E. coli*, would probably be unsuitable. Fortunately, nowadays a number of alternative expression systems are available, both of prokaryotic and eukaryotic origin. A few of the most popular expression systems are summarized in Table III. In addition, the possibility of expressing the proteins in vitro is a very real one. Recent developments permit the production of proteins, biosynthesized in the test tube, in sufficient quantities for their biochemical and biophysical characterization. Further, manufacturing proteins in vitro has opened other new, exciting possibilities described later in this article.

USING NATURE'S AMINO ACID REPERTOIRE

In contrast with the chemical derivatization approach to active site alterations, which normally results in chemical moieties not present in natural proteins, the immediate result of a protein engineering experiment is the substitution of the side chain of one residue by another, out of the 19 remaining natural amino acids. Despite such limited repertoire, the use of this basic technique has helped shed light on the mechanisms of many enzymes, a few of which are mentioned below.

Catalytic mechanism studies

Soon after its introduction, site-directed mutagenesis revealed its power for the analysis of catalytic mechanisms (Clarke *et al*, 1986). Serine proteases constitute a "textbook" case. The well known catalytic triad has been extensively studied in the subtilisin family. In a very revealing study, the three residues which comprise the principal component of the catalytic machinery (Ser221, Asp32 and His64), were replaced systematically by alanines in subtilisin BPN' (Carter & Wells, 1988). The authors found that eliminating the OH group of serine lowers the catalytic activity; while the activity of the native enzyme is about 4×10^9 fold over the non enzymatic rate, the mutant still catalyzes the reaction 3000 times faster than the spontaneous reaction. However, replacement of one or two of the other members of the catalytic triad does not reduce the residual activity of the S221A mutation any further. The most likely source for the residual activity is transition state stabilization by other residues, notably the oxyanion site, one of whose components is Asn155. In an extension of their work, the same authors determined the interaction between members of two components of the catalytic machinery (Carter & Wells, 1990). The surprising result is that although individual mutations at Asn155 also lower the catalytic activity of the enzyme, the combination of N155G with S221A results in a better enzyme (about 10 fold). The authors explain these results by invoking a different catalytic mechanism in the serine-lacking mutant. With this mechanism, Asn155 would get in the way, rather than help catalysis. Thus, transition state stabilization must come from other sources, in addition to the oxyanion site.

Replacement of Ser221 by Cys makes subtilisin BPN' more efficient to ligate esterified peptides specifically onto the N-termini of proteins or peptides, due to the inherently better reactivity of thioesters towards aminolysis, as opposed to hydrolysis (Abrahmsén *et al*, 1991). For this effect to manifest itself efficiently, it must be accompanied by mutations down the alpha-helix to which residue 221 is attached, so as to make slightly more room for the bulkier sulfur atom. Moreover, the resulting peptide ligase could be endowed with altered specificity, if mutations previously shown to change the specificity of the wild type enzyme are added.

Another interesting example is provided by studies with lactate dehydrogenase (LDH). The authors (Clarke *et al*, 1986), replaced a conserved arginine (R109) by glutamine, thus roughly preserving bulk and hydrophilicity, but removing the positive charge. This arginine moves toward the substrate during binding. The mutant enzyme exhibits severely reduced reactivity for the bound substrates (NADH and pyruvate). Pyruvate affinity is reduced to about 5%, while NADH affinity is not affected. In the authors' words, the gross active-site structure is not altered by the mutation since an alternative catalytic function of the enzyme (the rate of addition of sulfite to NAD^+), is insensitive to the substitution. They finally show that a previously proposed role for this arginine during catalysis is erroneous. It was suggested that R109 destabilized the enzyme-NADH-pyruvate complex by charge repulsion against a protonated histidine that participates in catalysis. This model predicts that the binding of oxamate (a competitive inhibitor) to the mutant enzyme should be tighter, but it was found to be weaker. After a careful examination of their data, they propose an alternative catalytic mechanism, including the arginine as a strong transition state stabilizer.

Enzyme specificity

Dissecting the elements involved in specificity has also proved fruitful in serine proteases. The specificity of subtilisin BPN' has been engineered (Carter *et al*, 1989) by removing His64 to make it an attractive site-specific protease (effectively narrowing its specificity). Conversely, a protein engineering and crystallographic study (Bone *et al*, 1989) demonstrated that alpha-lytic protease adapts to some amino acid replacements with structural plasticity, consequently broadening its specificity. Many other examples of changes in substrate specificity have been described (Rheinnecker *et al*,1994; Bocanegra *et al*, 1993).

The examples above, illustrate the power of protein engineering for the dissection of diverse components of the catalytic machinery and the determinants of substrate specificity .

Amino-acid probes

Engineering an enzyme to have a single amino acid probe is also possible. A pioneering work made use of such an approach to prove that the limiting step in LDH catalysis is a major protein rearrangement. The authors (Waldman *et al*, 1988), replaced all natural LDH tryptophan residues with tyrosine, and inserted a tryptophan at a specific site where motion was detected by changes in fluorescence.

Another relevant work (Smith *et al*, 1991), while not about active site studies, is worth mentioning here. It involves the construction of a series of nine LDH mutant enzymes carrying a single tryptophan, either leaving a natural one or substituting a tyrosine. Tryptophans served as probes to follow, by time-resolved fluorescence anisotropy, the loss of different structural levels at specific zones in LDH folding intermediates. This approach has also been used to probe substrate binding (Chung *et al*,1993b; Chung *et al*, 1993a), noncatalytic-site binding (Weber *et al*, 1994), domain motions (Ibba & Hennecke, 1994), and for side-chain motions and general fluorescence studies (Cornish & Schultz, 1994; Felter *et al*, 1992).

A natural extension of this idea is to make use of the selective reactivity of natural amino acid side chains. As just described for tryptophan, site-directed mutagenesis allows the introduction of any reactive residues at predefined places in the three-dimensional structure of the protein, for instance, near the

active site. Complementary to this, selective elimination of reactive side chains can be used to direct chemical derivatization only to the reactive residue(s) that remain in the mutated protein. Such approach has been used, mainly altering the number and location of Cys residues. Thus, for instance, extrinsic probes, such as fluorescence and spin-labels, have been added to test several protein structural and dynamic features, such as the membrane insertion of Colicin A (Lakey *et al*, 1991), immersion depth of bacteriorhodopsin in lipid bilayers (Altenbach *et al*, 1994), and side chain mobility of beta-lactamase A (Calciano *et al*, 1993).

Another interesting report (Persson *et al*, 1991) describes the introduction, by genetic engineering, of a cysteine residue near the active site of glucose dehydrogenase. Subsequent covalent attachment of NAD cofactor to the cysteine allows regeneration of the cofactor without loss from the bioreactor.

Similar approaches to those mentioned above shall prove useful on active-site studies.

EXPANDING THE GENETIC CODE: INTRODUCTION OF NON-NATURAL AMINO ACIDS DIRECTLY INTO THE POLYPEPTIDE CHAIN

The ultimate goal in the alteration of an active site would be to introduce exactly the desired change, exclusively at the desired place. Interesting changes may include bulkiness, chemical reactivity, hydrogen bonding capabilities, nucleophilicity, pK, spectroscopic properties, etc. The natural repertoire of 20 amino acids offers limited possibilities, so that introduction of non-natural versions of the building blocks would be highly desirable. Such goal has been possible, in principle, by *de novo* chemical synthesis of the polypeptide but, unfortunately, with this approach size limitations are severe, effectively precluding its general application. Recently, relying on recombinant DNA techniques and organic chemistry, methods have been developed that allow the versatile introduction of non-natural amino acids in ribosome-synthesized proteins.

For many years it had been known that termination codons could be used by special tRNA molecules (called suppressors) which were able to introduce the amino acid they were charged with and support the continuation of the translation process (Lewin, 1994). Several different suppressor tRNAs could be obtained by geneticists, thus allowing J. Miller and coworkers to develop a method that permitted the introduction of a different amino acid at a predetermined stop codon (introduced by site-directed mutagenesis), merely by changing the bacterial strain (Miller *et al*, 1979). However, to expand the repertoire, the artificial charging of a suppressor tRNA molecule with a non-natural amino acid was required, coupled with an in vitro translation system to allow its incorporation into the protein. This strategy, pioneered by Hecht and coworkers (Hecht *et al*, 1978), and based on the in vitro transcription/translation system of Zubay (Zubay, 1973) has been recently developed and widely utilized by P. Schultz and his collaborators (Noren *et al*, 1989; Mendel *et al*, 1993; Chung *et al*, 1993), as well as by Benner and his group (Smith *et al*, 1991).

Catalytic mechanisms

It can be easily recognized that incorporating unnatural amino acids should lead to a better understanding of catalytic mechanisms by precise dissection of each side chain s role. Judice *et al* (1993) designed non natural amino acid substitutions to assess the catalytic mechanism of staphylococcal nuclease. They interchanged two arginine residues (R35 and R87) by groups that helped dissect relative contributions of electrostatic interactions and hydrogen bonding ability to catalysis. Also, they substituted glutamic acid 43 (E43) by its nitro analog, S-4-nitro-2-aminobutyric acid (NABA), to assess this amino acid's possible role as a general base (NABA is a poorer base), and some changes to assess geometric restrictions. Their results show that E43 might play a complex structural role during catalysis, and that both arginines act by electrostatic stabilization of, but also by bidentate hydrogen-bonding interactions with the transition state. Two other studies, performed

at the same laboratory (Ellis *et al*, 1991; Weber *et al*, 1993), utilize similar approaches to assess the structural and catalytic roles of amino acids at several loops of Ras protein.

A very interesting feature of the method described above is that it permits the incorporation of variation at the level of the main chain. In a study with T4 lysozyme (Ellman *et al*, 1992), it is demonstrated that α,α-bisubstituted amino acids, N-alkyl amino acids, and lactic acid could be used for charging the tRNA and are incorporated into the protein. The authors conclude that the *E. coli* biosynthetic machinery should allow the introduction of a wide range of backbone substitutions.

Non-natural amino-acids as biophysical probes

Incorporating a site-specific biophysically-enhanced probe should also be very useful, and one would not need to eliminate noisy amino acids at other sites. Recently (Weber *et al*, 1994), a paper was published describing the introduction of various biophysical probes into several sites of the T4 lysozyme structure. The authors report the successful incorporation of a spin-labeled, a fluorescent, and a photoactivable amino acid; and discuss several aspects on the efficiency of non natural amino acid incorporation by the *E. coli* protein biosynthetic machinery.

Recent reviews on this subject (Haran *et al*, 1992; Elofsson *et al*, 1991; Felter *et al*, 1992), show that this methodology will help answer several important questions about proteins. A very important one being catalytic mechanisms including substrate binding and protein dynamics, and much more. In our view, this constitutes a more powerful and versatile approach than any other chemical modification scheme.

THE VARIATION-SELECTION SCHEME AND ITS USE IN THE INVESTIGATION OF ENZYME ACTIVE SITES

Mutagenesis schemes need not be limited to the predetermined introduction of a specific amino acid at a specific place. The in vitro manipulation of DNA molecules is uniquely suited for the creation of variability, which may in turn be exploited as a useful source of information about the molecules involved. In its simplest form, one can create, in a single experiment, a collection of genes coding for each of the 20 amino acids at a particular site within the protein. This site-saturation mutagenesis experiment is easily done by utilizing a collection of oligonucleotides, synthesized in batch form, incorporating a mixture of the four bases at each of the three positions constituting the corresponding codon. The complexity of the resulting library of clones (64 possible triplets coding for 20 amino acids or a stop signal) can be easily dealt with by direct physical characterization (sequencing) and/or functional assays. Frequently, site-saturation mutagenesis experiments provide unanticipated results, highlighting our limited knowledge about systems as complex as proteins. Our experiments with beta-lactamase have shown this repeatedly, as exemplified below.

Since its introduction, the approach has been improved in several ways and, more notably, applied to create much more extensive variability (Merino *et al*, 1992). The creation of collections of molecules with astronomically high numbers of variants is a relatively easy task (consider introducing 10 randomized consecutive codons, which is readily achieved with today's oligonucleotide synthesis methods, and obtaining 4^{30} different DNA sequences, coding for 20^{10} different amino acid sequences) but, in order to be useful, a selection or screening system must be employed, so as to sort out interesting mutants. In recent years, the variation-selection system has received tremendous impulse, particularly due to the development of powerful screening systems such as phage display (Scott & Smith, 1990), in which proteins expressed at the surface of viral particles are panned by immobilized target molecules. Such *in vitro* evolution systems have proven very useful in evolving proteins and peptides with novel binding (Haynes *et al*, 1994) and catalytic activities (Barbas, 1993).

This approach has also the potential to convey useful information for the basic understanding of protein structure and function, including catalysis (Dunn *et al*, 1991).

In our laboratory we have set out to try to derive information about the catalytic machinery of various enzymes through the generation and screening of large collections of variants, with amino acid replacements clustered at the active site. We will describe now some of the results we have obtained with beta-lactamase.

Beta-lactamase active-site studies using combinatorial mutagenesis

Beta-lactamases are enzymes of considerable clinical interest, for they are the main cause of bacterial resistance to antibiotics of the penicillin family (Ambler, 1980; Bush, 1989). Among the beta-lactamases, the class A family is particularly well characterized and has attracted our interest for several reasons. Knowledge about these enzymes includes sequence of more than 20 variants (Couture *et al*, 1992), from various bacterial sources; several crystallographic structures (Strynadka *et al*, 1994; Jelsch *et al*, 1993; Herzberg, 1991; Moews *et al*, 1990); and a catalytic mechanism (serine hydrolase), which is related to other well characterized enzymes. Central to the employment of a variation-selection approach is the fact that many related compounds are available (the beta-lactam antibiotics) which may or may not be hydrolyzed by class A beta-lactamases (see Fig 3). Thus, selection for bacterial growth in the presence of such antibiotics constitutes a powerful screening system for variant enzymes active against them.

We have focused part of our efforts in trying to understand the structural basis of specificity, utilizing the RTEM beta-lactamase from *E. coli*. By using the approach presented in Figure 4, our aim is to construct a sequence-activity database, which should help identify the key residues involved in substrate recognition. This information could in turn be used to infer the likelihood that mutants against particular beta-lactam antibiotics arise, and as a basis to design better drugs and inhibitors of these enzymes.

Based on the crystal structures and on the sequence of clinically-isolated mutants that display activity against third generation cephalosporins, we defined a mutagenesis "window", comprising the residues most likely involved in determining the specificity (see Fig 5). Note that the targeted amino acids form a group that is not contiguous in sequence. Moreover, we hypothesize that changing the specificity for some substrates may require more than one amino acid replacement. Therefore, we set out to introduce controlled variability in the vicinity of the active site, creating a combinatorial library containing single and

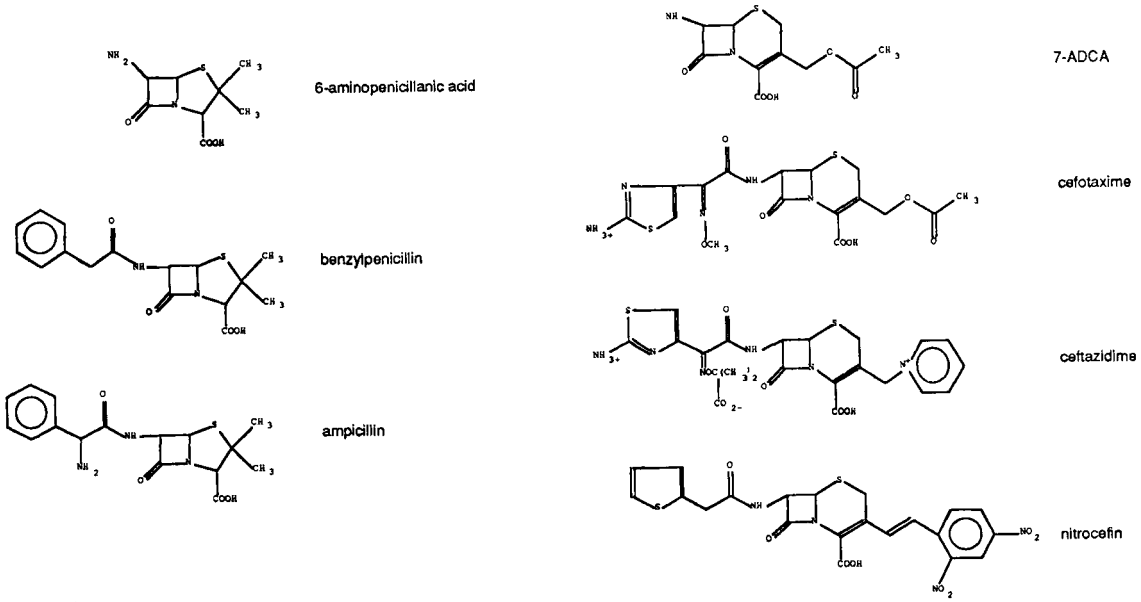

Fig. 3. Structures of several b-lactam antibiotics of the penam (penicillin) and cepham (cephalosporin) families.

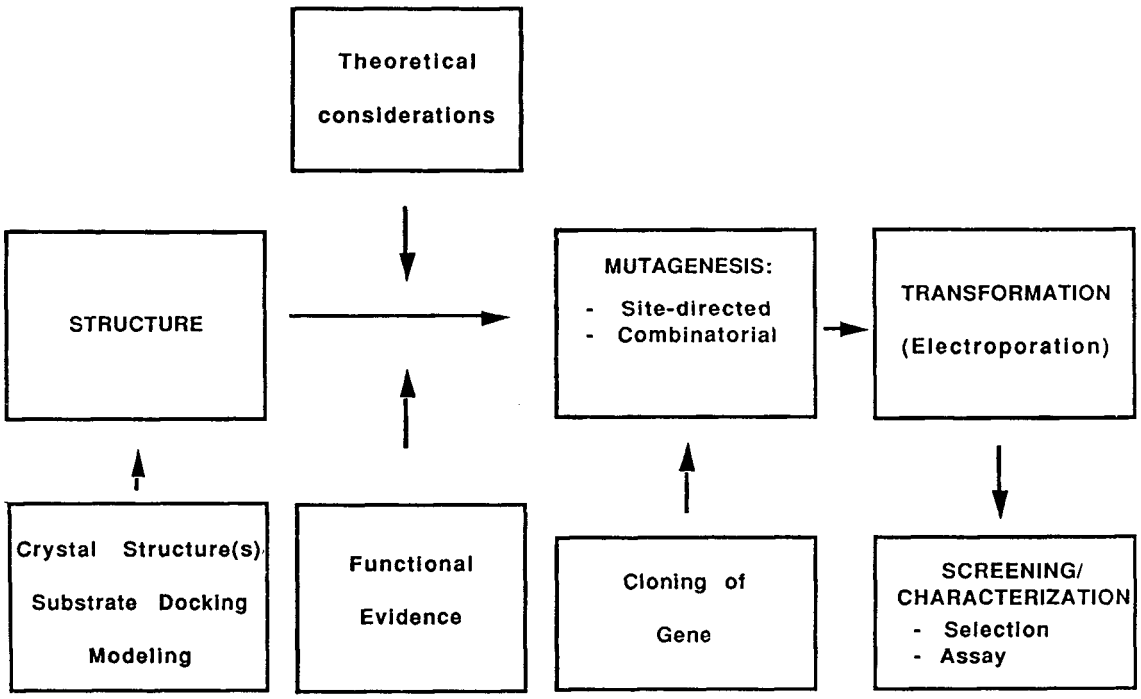

Fig. 4. General scheme used in our laboratory for the generation of a sequence-structure-activity database of an enzyme active site.

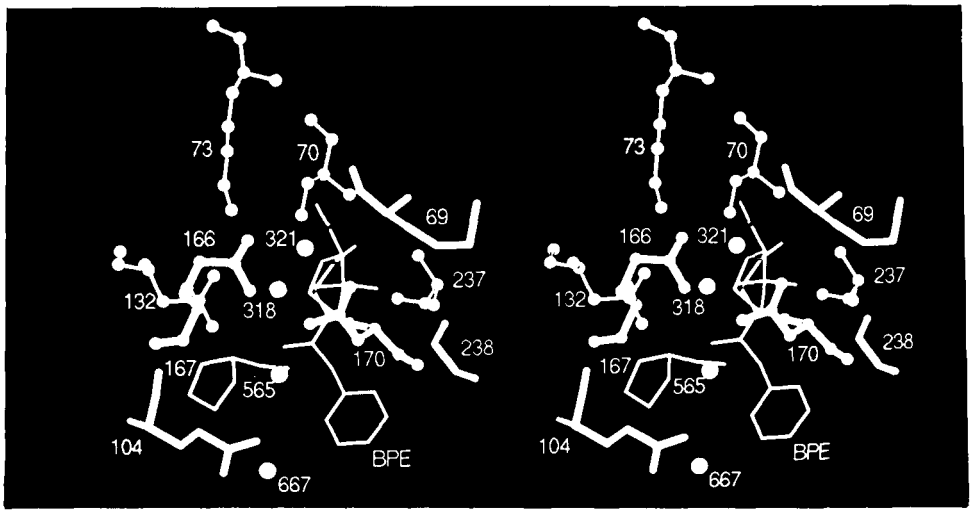

Fig. 5. Stereo view of the beta-lactamase active site. The substrate, bencyl-penicillin, docked into the active site cleft, is depicted in thin bars. Residues, shown as thin bars and balls, play a direct role in catalysis. Those with thick bars are hypothesized to be mainly involved in determining specificity. Amino acids depicted as thick bars with balls are likely to play a role in both. Numbered balls correspond to crystallographic water molecules. [Based on coordinates of RTEM ß-lactamase, kindly provided by MNG James (Strynadka *et al*, 1994)].

multiple amino acid replacements within that non contiguous area. Control over the mutagenesis rate is exerted at the level of oligonucleotide synthesis, by virtue of the introduction of limited amounts of contaminating bases at the selected positions (del Río *et al*, 1995). These oligonucleotides are incorporated into the gene and combined with each other by means of a PCR-based scheme, illustrated in Figure 6. Upon cloning of the PCR fragments, the resulting library is comprised of beta-lactamase variants containing one or more amino acid replacements, within the targeted region. The

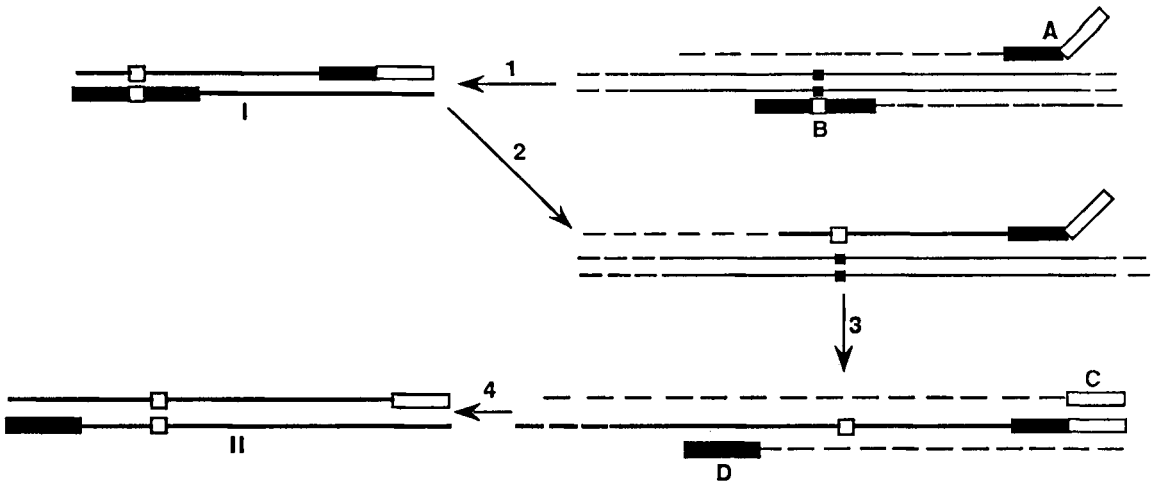

Fig. 6. Outline of the combinatorial mutagenesis scheme (Merino *et al.*, 1992). Oligonucleotide zones complementary to the gene are drawn in black; non-complementary zones are empty. Oligonucleotide A has a 31 end complementary to the gene, and a non-complementary "tail"; oligonucleotide B introduces the desired mutation. The product from oligonucleotides A and B is selectively amplified during subsequent PCR reactions using oligonucleotide C, identical to the tail of oligonucleotide A. Oligonucleotide D can introduce further mutations, or complete the mutant piece of gene necessary for subcloning.

Table III

Examples of expression systems

ORGANISM	NOTES	REFERENCES
BACTERIA		
Escherichia coli	The most widely used organism; several vectors and strains for almost any purpose. As expected, posttranscriptional mamalian-like modifications do not occur, making it inadequate for some purposes, but in general the best choice for protein active-site studies.	Sambrook *et al*, 1989; Blight & Holland, 1994; and almost any molecular biology product catalog
Streptomyces	Useful for production of secreted proteins. Extensive experience in fermentation conditions make *Streptomyces* an attractive host.	Brawner, 1994
YEAST		
Saccharomyces cerevisiae	The first eykaryotic expression system, well characterized. Posttranslational modifications occur, but it glycosylates protein in excess.	Romanos *et al*, 1992
Pichia pastoris Hansenula-polymorpha, Kluyveronmyces lactis	Options which seem to work better than *Saccharomyces* at expressing and glycosylating proteins.	Hodgson, 1993
ANIMAL CELLS		
CHO cells	Chinese hamster ovary cells, the first mammalian expression system. Solves the problem of posttranslational modifications.	Hodgson, 1993
Baculo-virus/ Insect cells	Posttranslational modifications are appropriate, and yield are higher than in mammalian cells.	Luckow & Summer, 1988; Luckow *et al*, 1993

mutagenesis rate is controlled so as to obtain the desired average multiplicity of replacements.

After selecting for bacterial growth in the presence of an antibiotic that is not normally hydrolyzed by RTEM beta-lactamase, we have isolated a large number of mutants with altered substrate specificity. The study of the catalytic parameters of some of these mutants is starting to unveil some aspects of the underlying molecular recognition mechanism.

One of the first notable examples is provided by the E104M/G238S double mutant, which displays an 800 fold increase in activity against cefotaxime (Viadiu *et al*, 1995). Construction of enzymes containing the isolated single replacements revealed an activity increase of about 20 fold each and, furthermore, revealed that the E104M mutant is, by itself, a better enzyme against several substrates, including penicillin G.

Another unexpected observation is that it is much easier to obtain enzymes active towards cefotaxime than it is to other cephalosporins. Many of the amino acid replacements that gained such specificity also became much less active towards penicillins.

A complete rationalization of these results must await crystallographic studies of the mutant enzymes and their complexes with the substrates, but computer modeling suggests that an increased hydrophobic interaction with the substrate side-chain, coupled with subtle movements of the structural elements (Viadiu *et al*, 1995), are responsible for the increased activity. Similar to the case of subtilisin, it is likely that alternative catalytic mechanisms arise in the case of some mutants, allowing water to diffuse to the active site when sufficient room is made for it. Such an alternative mechanism seems to arise only in certain contexts (Osuna *et al*, 1995) and, as mentioned above, more frequently only with some substrates (Palzkill & Botstein, 1992; Palzkill *et al*, 1994). From this kind of data, together with those derived from several other laboratories (Herzberg & Moult, 1991) a clearer picture of the catalytic mechanism and specificity of class A beta lactamases is emerging.

We believe that variation-selection schemes will continue to be a valuable alternative for the study of enzyme active sites, especially due to the increasing sophistication of these methods. Notably, beta lactamase has been expressed in the phage display system (Soumillion *et al*, 1994). It was also used to demonstrate the capabilities of an exciting, newly developed method for in vitro recombination of genes (Stemmer, 1994), which illustrates the power and maturity that the combinatorial approach has reached.

CONCLUDING REMARKS

After mentioning all these examples on the use of molecular biological methods to study protein function in general, and active sites in particular, we have discussed two main approaches: a directed (or "rational") approach and an evolutionary (or variation-selection) approach. The most promising work on the rational side seems to be the use of non-natural side chains to dissect particular properties functioning during catalysis. The second approach has been quite successful and it will surely constitute an important way to look out for new activities or specificities. A clever analysis of the experimental results will surely contribute to the database which will finally lead us to a better understanding and manipulation of enzyme catalysis.

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