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Phage Display of Enzymes and In Vitro Selection for Catalytic Activity

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ABSTRACT

Despite recent progress, our understanding of enzymes remains limited: the prediction of the changes that should be introduced to alter their properties or catalytic activities in an expected direction remains difficult. An alternative to rational design is selection of mutants endowed with the anticipated properties from a large collection of possible solutions generated by random mutagenesis. We describe here a new technique of in vitro selection of genes on the basis of the catalytic activity of the encoded enzymes.

The gene coding for the enzyme to be engineered is cloned into the genome of a filamentous phage, whereas the enzyme itself is displayed on its surface, creating a phage enzyme. A bifunctional organic label containing a suicide inhibitor of the enzyme and a ligand with high affinity for an immobilized receptor are constructed. On incubation of a mixture of phage enzymes, those phages showing an activity on the inhibitor under the conditions of the experiment are labeled. These phages can be recovered by affinity chromatography.

The design of the label and the factors controlling the selectivity of the selection are analyzed. The advantages of the technique and its scope in terms of the enzymes that can be engineered are discussed.

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Index Entries: Bacteriophage, in vitro selection; phage enzymes; phage display; protein engineering; suicide inhibitors.

Abbreviations: DTT: dithiothreitol; fd-bla(+): fd phage displaying active β-lactamase; fd-bla(−): fd phage displaying a β-lactamase inactivated by site-directed mutagenesis; g3p: product of gene 3 of fd phage; PCR: polymerase chain reaction; PEG: polyethylene glycol.

INTRODUCTION

The discovery of enzymes with new properties (new catalytic activity, increased stability, altered specificity, displaced pH profile and so on) is of great interest. Although very substantial progress has been made in the understanding of the structure–activity relationship of proteins (*1,2*), the prediction of the modifications to be introduced in a protein to alter its properties in an expected direction remains difficult. Consequently, one frequently resorts to a strategy where, starting from the gene of an existing enzyme, a large library of random mutants is created (for a review, *see* ref. *3*). It is hoped that some of these genes will code for proteins with the anticipated properties. Proteins having new enzymatic properties can also be obtained by resorting to the technique of monoclonal antibodies. In both cases, it is necessary to test a very large number of mutants and to have an efficient screening or selection process.

Efficient screening techniques are based on the elaboration of sensitive tests for the enzymatic activity. A population of mutants on a Petri dish is searched for those producing the expected enzyme (*see,* for instance, ref. *4*); this technique, however, is potentially quite painstaking.

An advantageous alternative arises when the expected catalytic activity can be profitable to the survival or the development of the microorganism expressing the corresponding gene. Then exerting a selection pressure on the culture medium where the population of mutants producing the enzyme is grown allows the isolation of the strains producing the expected enzyme (*see,* for instance, refs. *5* and *6*). This efficient in vivo selection technique, however, is applicable only for the discovery of enzymes affording a selective advantage.

Recently, it has been shown that it is possible to display foreign peptide sequences and proteins on the surface of "filamentous" phages whose genomes have been manipulated to contain the genetic information encoding the displayed polypeptide (for reviews of the biology of these phages, *see* refs. *7* and *8*). Oligopeptides (*9–14*), antibodies with variable regions or full antibodies (*15–18*), hormones (*19,20*), proteases inhibitors (*21,22*), and receptors (*23,24*) have been displayed by creating fusions with a minor coat protein, the product of gene III, or the main coat protein, the product of gene VIII. By affinity chromatography on a support immobilizing a suitable receptor, the phages exposing the peptides with the expected properties can be isolated (Fig. 1). Then their

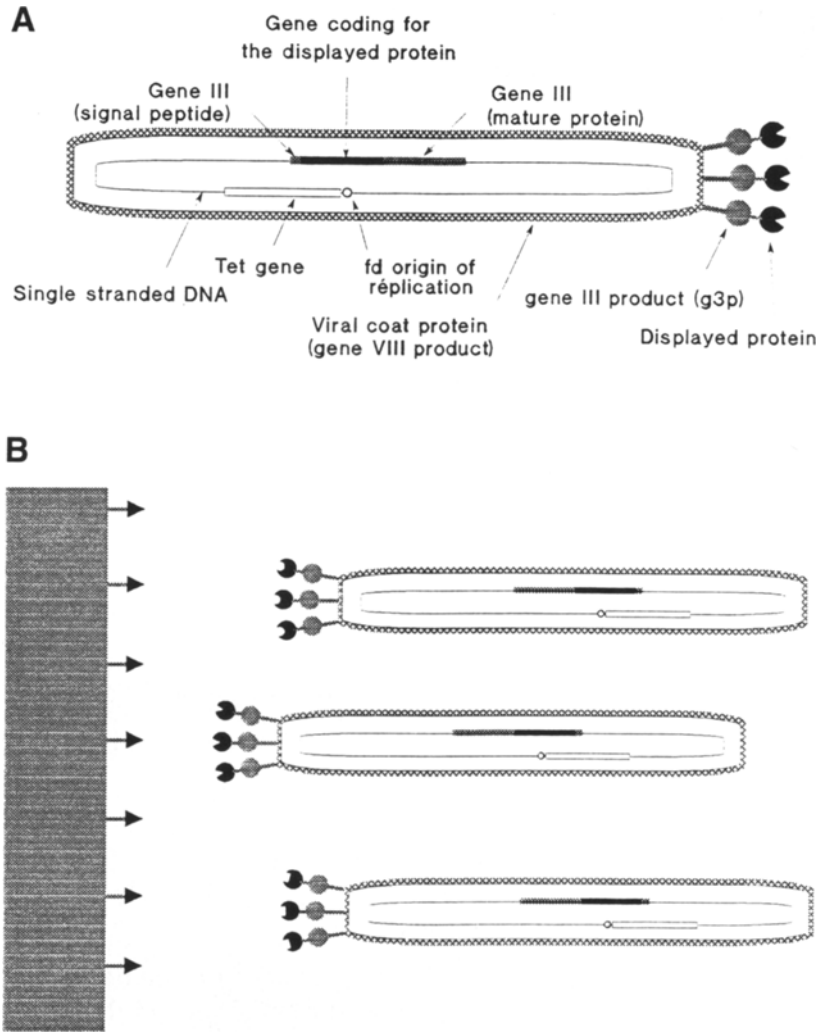

Fig. 1. (**A**) Schematic representation of a phage displaying a foreign peptide or protein: the gene coding for the displayed polypeptide is inserted in the phage's gene III between the sequence coding for its signal peptide and the mature g3p; a tetracycline-resistance gene has also been inserted in the phage's genome for selection purposes. (**B**) Chromatographic selection of phages with affinity for an immobilized ligand.

genomes can be amplified very efficiently by infection of an *E. coli* culture for recloning and characterization. The linkage between recognition and replication affords a very efficient selection process.

Enzymes have also been displayed on pages (*25,26*). They appear to retain their catalytic activity. To search for a peptide with catalytic activity, however, the in vitro selection process should not be based on binding capacity, but on catalytic efficiency. The purpose of the present article is to describe a rather general method that allows such a selection in

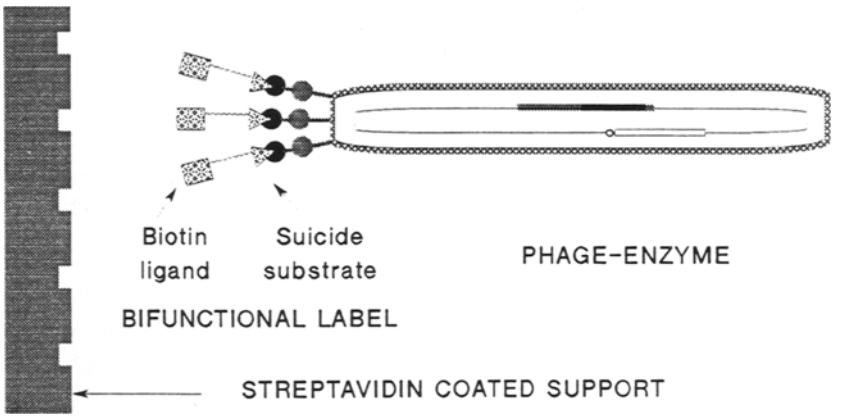

Fig. 2. Chromatographic selection of a phage labeled by reaction with a bifunctional organic compound containing a biotin ligand and a suicide inhibitor.

a two-step process. In the first step, the phages bearing catalytically active enzymes are labeled by reaction with a bifunctional organic compound incorporating an irreversible inhibitor and a biotin ligand. These labeled phages are later isolated by affinity chromatography (Fig. 2). The labeling is designed to select for catalytic efficiency, since it uses mechanism-based inhibitors (for a review, *see* ref. *27*) of the displayed enzyme.

RESULTS AND DISCUSSION

Phages Displaying a β-lactamase

To illustrate the possibility of selecting phages on the basis of the enzymatic activity rather than on the basis of the substrate or inhibitor affinity of displayed enzymes, we have first constructed two recombinant phages differing only in the presence or absence in the active site of a nucleophile essential for activity. The RTEM β-lactamase was chosen as the first system to investigate on several grounds. Although no construction in which it is fused as amino terminal to another protein has been described, several carboxyl terminal fusions in which this enzyme is active and used as a reporter have been constructed. The tertiary structure of that enzyme has been determined (*28*). Its carboxyl terminal appears to be quite accessible at the end of a long α-helix, so that it was expected that a hybrid created by fusion of a coat protein and β-lactamase could fold properly and would be fully functional. Because of its usefulness in biology and medicine, the mechanism of this enzyme has been intensively investigated (*29*), and many mutants have been produced and analyzed; particularly, it has been shown that the mutation of an essential serine in the active site inactivates β-lactamases without impairing substrate bind-

ing (*30–33*). Several mechanism-based inhibitors have been designed and their mode of action investigated (for reviews (*see* refs. *27* and *34*). Finally, ampicilin resistance could be conferred to colonies affected by phages displaying an active β-lactamase; this in vivo selection property could facilitate the development of the in vitro selection method.

Construction of the fd-bla Phages

We have constructed two recombinant filamentous phages displaying, respectively, an active β-lactamase and a β-lactamase inactivated by mutation of the essential serine 70 (*see* ref. *35* for numbering; these are designated below active and inactive phages or fd-bla[+] and fd-bla[−] phages, respectively). These phages were constructed from the phage fd-DOG1 (*17*), a derivative of fd-tet containing a tetracycline gene inserted in the intergenic region close to the origin of replication (to allow it to be propagated like a plasmid [*36*]). In fd phages, gene III codes for a coat protein displayed at the tip of the filament. In fd-DOG1, a polylinker (containing among others the *Apa*L1 and *Not*1 restriction sites) has been introduced in gene III between the sequence coding for the signal peptide and that coding for mature g3p. This allows the insertion of foreign DNA sequences coding for peptides or proteins to be displayed.

The phages were constructed as follows. A bothersome *Apa*L1 site in the nucleotide sequence coding for RTEM β-lactamase (*37*) cloned in phagemid p-Bluescript S/K$^+$ (from Stratagene) was first removed in a mutation that keeps the protein sequence unchanged and generates phagemid p-Bluescript-M$^+$. In this phagemid, the codon corresponding to Ser 70 is changed to one encoding alanine (p-Bluescript-M$^-$). The nucleotide sequences coding either for mature wild-type or for the inactive β-lactamases were then isolated from the p-Bluescript-M phagemids by PCR amplification with the introduction of *Apa*L1 and *Not*1 sites. The 3′ primer used for amplification is a 47-mer complementary on 21 bases to the end of the β-lactamase gene; it contains an extension that replaces the stop codon by a glycine and introduces four codons between the gene coding for β-lactamase and the gene coding for the mature g3p protein, as well as the restriction site *Not*1 for cloning. The four codons encode an Ile-Glu-Gly-Arg sequence recognized and cleaved by factor Xa (*38*). The fusion proteins constructed are presented in Fig. 3. They will be displayed by the active and inactive phages, respectively.

Protein g3p ensures the infectivity of the phages (*39*). If the presence of a protein as large as β-lactamase should affect the infection capacity, an incubation with factor Xa could restore it. More importantly, this proteolysis site offers a possibility of eluting the phages from an affinity column on which they have been immobilized (*see* Enrichment Protocol).

Characterization of the fd-bla Phages

The infection by fd-bla(+) phages confers ampicillin resistance to *E. coli* cells as shown by the fact that they grow when plated on Amp

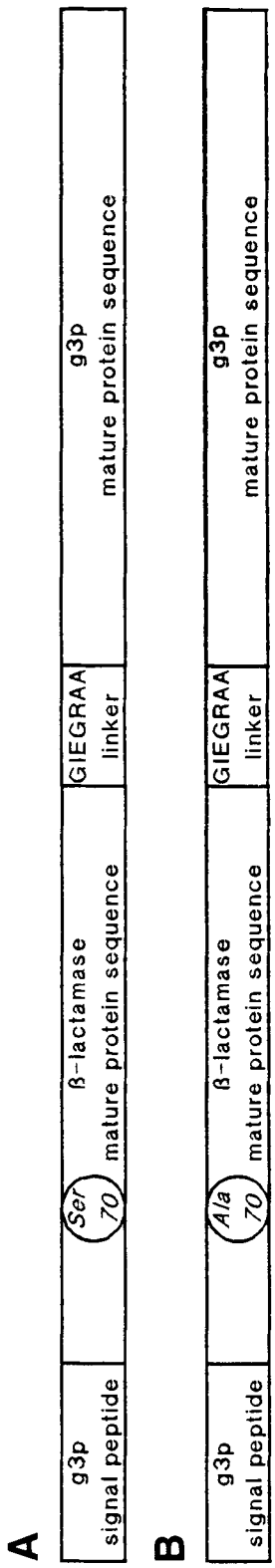

Fig. 3. Construction of the fusion protein between active (**A**) and inactive (**B**) β-lactamase and g3p: the linker contains the Ile-Glu-Gly-Arg (IEGR) sequence forming the factor Xa cleavage site.

medium. Concentrated phages solutions were prepared by precipitating the supernatant of a centrifuged culture with PEG and resuspending in buffer. The β-lactamase activity of these phages could be measured directly with nitrocefin as substrate; the transducing units' titer of these solutions has also been determined; from these data, the β-lactamase specific activity of the phages could be obtained and compared with that of the soluble enzyme. The displayed enzyme appears to be fully active. To show that the enzymatic activity is physically associated with the phages and not coprecipitated with them, the solutions were ultrafiltrated on a membrane allowing a soluble β-lactamase to go through; no enzymatic activity was detected in the ultrafiltrate. No activity was detected in a similar experiment with the fd-bla(−) phages.

The proteins of the coat of the phage fd-DOG1, fd-bla(+), and fd-bla(−) were analyzed by SDS-Page electrophoresis, "Western blot," and immunological detection with antibodies against g3p and β-lactamase. The horseradish peroxidase chromophoric substrate revealed a g3p band at apparent mol wt of 70 kDa with fd-DOG1, an abnormal position already observed previously (*15*), and bands detected as g3p and β-lactamase at 105 kDa for fd-bla(+) and fd-bla(−). This confirms that the construction was correct and that, during the virus morphogenesis, there was no significant proteolytic cleavage that would lead to β-lactamase disconnection from the phage.

Design and Synthesis of the Activity Labels

We have designed and prepared a bifunctional activity label that by incubation with a mixture of phages, would selectively react with the active ones and allow a subsequent separation by affinity chromatography. The label consists of a bifunctional organic compound in which two entities are connected through a linker, including an easily cleaved function, a disulfide bond (Fig. 4). The first entity is a ligand, in this case biotin, extensively used in biological analyses because it forms very tight complexes with avidin or streptavidin in their free form or on immobilized support (*40*). The second one is a suicide substrate (also called mechanism-based inhibitor or suicide inhibitor) of β-lactamase. This inhibitor will form a covalent bond with the enzyme and remain irreversibly attached to the active site. The properties of the inhibitor are crucial for the selection process. Mechanism-based inhibitors are, ideally, relatively unreactive organic compounds that become activated by the target enzyme itself using its specific mechanism. Accordingly, a β-lactamase suicide inhibitor will be a modified β-lactam substrate, which, after the normal ring cleavage as a triggering event, will evolve to a highly reactive electrophile that will efficiently react with any nucleophilic center present in the active site and block the enzyme. The suicide moiety of the label is a penicillin derivative modified to decrease the stability of the five-membered ring vs

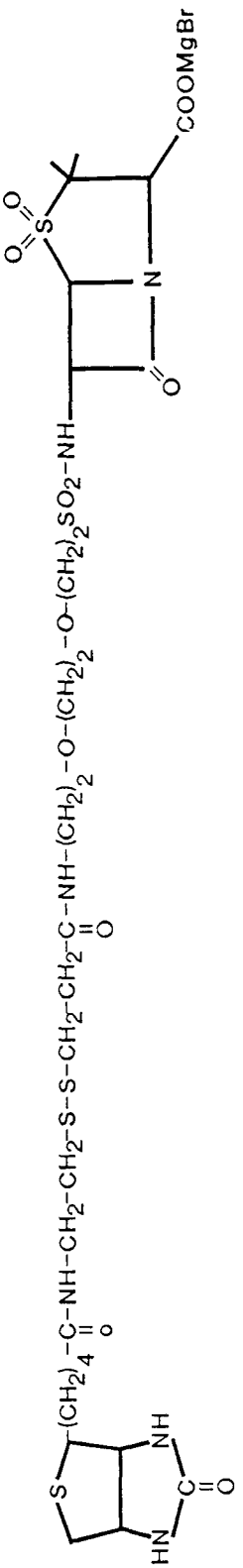

Fig. 4. Bifunctional label used for the selection of fd-bla(+) phages.

β-elimination. The acidity of the C_6-H function is increased by transformation of the exocyclic amino to a strongly electron-withdrawing sulfonamide. The leaving group ability of the thiazoline sulfur is increased by oxidation to the sulfone level. These transformations have been shown to transform a β-lactamase substrate into a mechanism-based inhibitor (*41–43*) following a mechanism analyzed by Knowles and collaborators and summarized in Fig. 5 (for a review, *see* ref. *34*).

Enrichment of Mixtures of Active and Inactive Phages

The phages whose DNA includes the active β-lactamase gene and that are displaying the corresponding enzyme attached to the g3p coat protein can be selected through a reaction with the label followed by affinity chromatography on a streptavidin-bearing support.

Mixtures of active and inactive phages are incubated with the labeling inhibitor, precipitated, and resuspended to remove the excess inhibitor. Then streptavidin-coated beads are added to the solution left under gentle stirring to favor the attachment of the labeled phages. After extensive washing of the unbound phages, the bound phages are released either by chemical cleavage of the disulfide bridge in the linker with DTT or proteolytic cleavage of the factor Xa recognized peptide connecting the enzyme and the g3p coat protein.

Depending on the initial ratios of active vs inactive phages and of the conditions of the labeling, from 15 to 20% of the loaded active phages appear to have been immobilized and to be chemically or proteolytically released from the streptavidin-coated support. Surprisingly, a significant percentage of the inactive phages have also been immobilized: about 0.5% of these phages are released with factor Xa and up to 3.5% with DTT. From these numbers, an enrichment ratio is calculated: the ratio of the percentage of the loaded active phages released vs that of the inactive phages. This ratio varies between 20 and 50 for proteolytic elution and between 6 and 12 for chemical elution.

After incubation with a biotin-bearing suicide inhibitor of β-lactamases, phages displaying an active enzyme are immobilized on streptavidin-coated beads more efficiently than phages displaying an enzyme that is inactivated by site-directed mutagenesis of an essential serine residue. In this protocol, we observe a selection for catalytic activity vs binding affinity. It has been shown with the RTEM or the *Streptomyces* R61β-lactamases that mutation of the essential serine does not impair binding or lead to an unfolded enzyme (*30–33*). On the basis of these established observations, we think that it is quite unlikely that, although the active enzyme would be properly folded and fully active at the surface of the phage, the inactive one would be displayed in an incorrect folding state. In the biochemical characterization of the phages, a fusion protein was detected both with fd-bla(+) and fd-bla(−) with anti-g3p and anti-β-lactamase antibodies on Western blot. The display of an unfolded inactive protein on fd-bla(−) would have favored its proteolysis during morphogenesis of the phage, this is not observed.

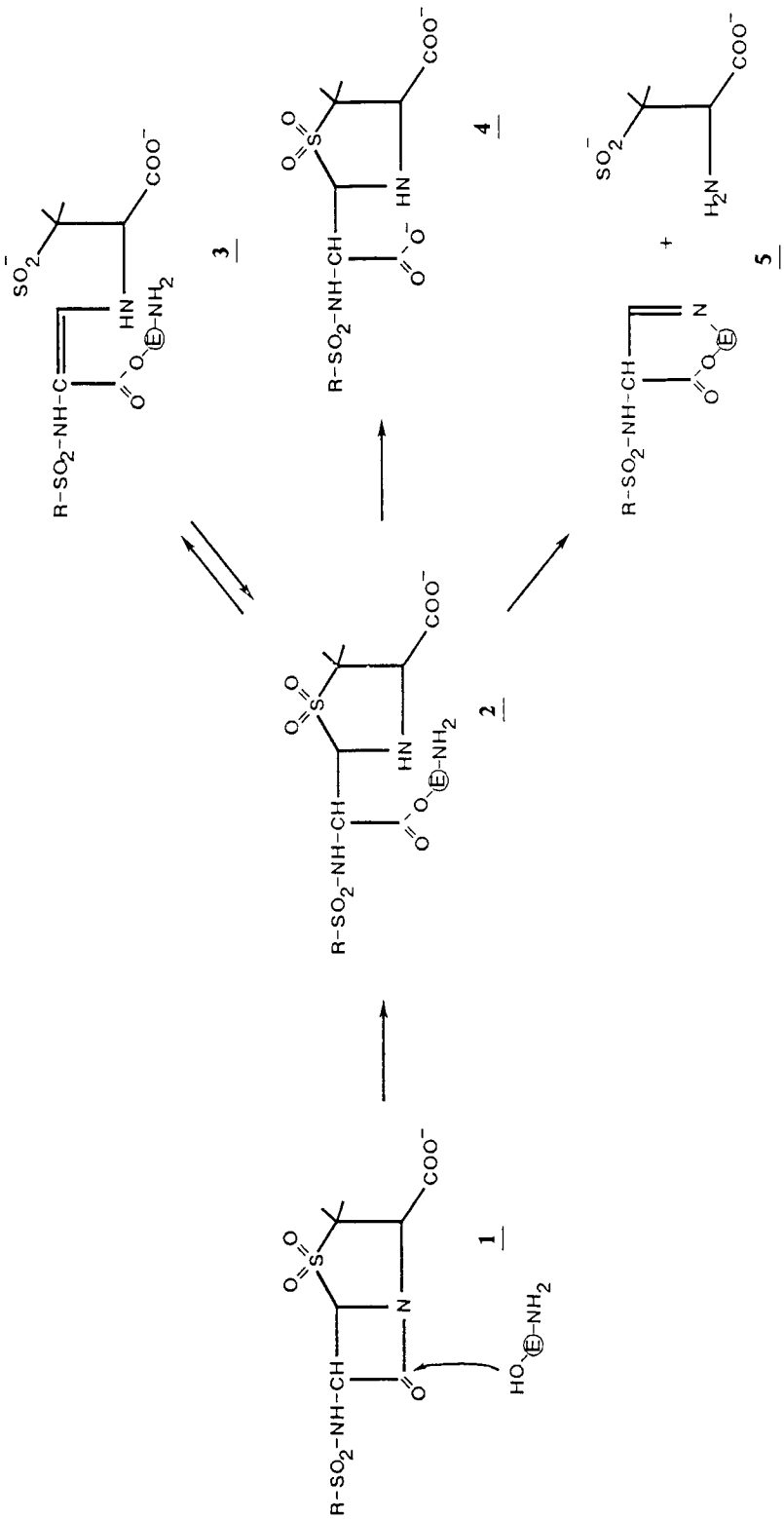

Fig. 5. Mechanism of the reaction of the suicide substrate with a β-lactamase containing in its active site an essential serine and a lysine nucleophile: the acyl-enzyme (2) formed by attack of Ser70 on the β-lactam substrate (1) can hydrolyze quickly to give an inactive product (4), or undergo a reversible β-elimination reaction to afford a more stable acyl-enzyme (3) or a transimination with the lysine to afford an irreversibly blocked enzyme.

The reaction of the phages with the inhibitor seems to involve some nonspecific labeling. This is presently limiting the selection factor. This side reaction might reflect the high affinity of the β-lactam function particularly in the oxidized form of penicillin. Unspecific protein labeling by penicillin derivatives is indeed a likely origin for the immunological problems associated with these drugs (*44*).

In principle, incubation of the beads with factor Xa should only elute the phages connected through β-lactamase. Accordingly, this elution should be more specific, phages attached as a consequence of nonspecific reactions with the major coat protein should not be released by the enzyme. We indeed observe a difference between the number of phages eluted by cleavage of the disulfide bridge in the label with DTT or by factor Xa, in favor of the former as expected. The difference is, however, quite small. Two tentative explanations can be given for this: factor Xa releases the nonspecifically labeled phages through a nonspecific proteolysis presumably because of the presence of other proteases as impurities, or the nonspecific labeling occurs preferentially on the β-lactamase protein itself. Work is in progress to clarify these points and control these reaction better.

Phages Displaying a Subtilisin

Subtilisins are proteases of industrial interest with a broad specificity. They have been engineered in many ways, for instance, to change their specificity or to increase their stability (*45,46*). These enzymes are biosynthesized as pre-pro-subtilisins (*47*); a pro-region located immediately after the signal sequence (pre) and N terminal to the mature subtilisin is indispensable for guiding the proper folding of the enzyme into its correct tertiary structure (*48*). This sequence is cleaved off autocatalytically after folding. The mechanism of the autocatalytic cleavage is not yet completely understood. It has been proposed to occur intermolecularly (*49,50*), but other data suggest that the maturation is truly intramolecular (*51,52*). The presence of the pro-region has also been shown to favor association with membranes (*49,53,54*). Mutant subtilisins expressed in *B. subtilis* and unable to mature and cleave the propeptide remain associated with membranes (*54*) possibly in the pre-pro-subtilisin form (*55*). Release of the immature protein can occur, however, very slowly on prolonged incubation (*54*).

In order to investigate whether the complex maturation process would also occur on phages to afford viable viruses displaying active subtilisin, we have cloned the gene of subtilisin E (*56*) in fd-DOG1 by inserting the sequence coding for the pro-peptide and the mature enzyme downstream from the signal sequence of gene III between the *Apa*L1 and *Xho*I restriction sites (*17*). This construction is similar to that described for β-lactamase, but without introduction of a factor Xa proteolytic site.

After transformation, *E. coli* strain JM109 produces fd viruses containing in their genome the inserted pro-subtilisin coding sequence. These viruses are infective; however, on standing in concentrated solution, they slowly loose their infectivity. When infected cultures are grown at low temperature, after centrifugation of the cells and precipitation of the supermutant, subtilisin activity on the *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide substrate (Sigma) is detected in buffer solutions (0.1*M* TRIS pH 8.6) where the phages have been resuspended. However, on ultrafiltration on a membrane with a 300,000-dalton cutoff that retains the phages (ultrafree MC filters from Millipore), about 80% of the subtilisin activity appears to be ultrafiltrable, whereas the remaining 20% is not. Subtilisin molecules are thus displayed on the phages, but the connection between the enzymes and the phages is not stable.

The fact that fd-sub phages are produced at about the some rate as the fd-Bla(+) phages indicates that the fusion protein subtilisin-g3p does not remain strongly associated with the membrane and suggests that maturation occurs. The detection of the activity in solution supports this conclusion.

It is known that on incubation with soluble subtilisin, fd phages loose their infectivity, whereas the g3p is proteolytically cleaved to release a 36-kDa fragment (*57,58*). A large portion of the subtilisin activity detected with the redissolved phages is not physically linked to the phages, but is probably coprecipitated with them on PEG addition. Proteolytic disconnection between a phage-displayed protein and g3p has been reported by other authors (*15*). With the fd-sub phage this problem appears to be rather severe. Since the phages are initially infective, the initial proteolysis is not a subtilisin cleavage in the g3p sequence. The slow loss of infectivity, however, is probably related to that reaction. Further experiments are in progress to clarify these points and eventually stabilize the construction.

CONCLUSIONS

Up to now, four enzymes have been displayed and shown to be active on filamentous phages. Trypsin and β-lactamase are monomeric enzymes; alkaline phosphatase is active as a dimer; subtilisin requires an autocatalytic maturation after folding. It appears that dimerization (*25*) and maturation can occur on phages or during their morphogenesis. This indicates that these enzymes retain their essential properties on the phages. These four enzymes are normally excreted proteins; it will be important now to test whether intracellular enzymes can also be displayed in the same way.

The design of the inhibitors used to label the enzymes is critical to the in vitro selection technique. In this article, we have used the suicide inhibitor concept to ensure selection for catalytic efficiency. The work that has been done by enzymologists and by scientists interested in rational drug design has led to the introduction of suicide inhibitors for many

classes of enzymes: dehydrases and isomerases, pyridoxal-phosphate-dependent enzymes, flavin-dependent enzymes, hemoproteins, non-heme metalloenzymes, enzymes involved in nucleotides modifications, hydrolytic enzymes like proteases, glycosidases, and β-lactamases (*27*). All that work will be very helpful for the application of the technique discussed here to other enzymes or to abzymes.

The possibility of labeling a phage-displayed enzyme on the basis of its activity opens the way to several new applications in enzyme engineering and mechanistic investigations in which we are presently involved.

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DISCUSSION

J. Fastrez

Tramontano: Is there an intrinsic problem in removing the unreacted bifunctional linker before applying the phage to the column? If you do not remove it, would you saturate the column with the linker?

Fastrez: No, it is easy. You just precipitate the phages.

Tramontano: Is that sufficient?

Fastrez: Yes. Two precipitations and that is it.

Hansen: Could you follow up on your comment that engineering a new catalytic activity would be very difficult? It seems to me that if you just use a different suicide inhibitor and then look for this process to work, you might indeed change the activity.

Fastrez: If you do that with a catalytic antibody, the selection method may work, but this would be extremely difficult to do with an enzyme. The technique may work if you are working with a protein fold that can support different catalytic activities. The most recent example of such a protein fold is the α-β hydrolase fold, which expresses esterase, protease, and dehalogenase activities (*1*). With this type of structure, we

would be able to go from one catalytic activity to another. Of course, this is not a *de novo* creation of activity.

Green: I think your approach is very elegant. My question is whether you are restricted to specific mechanisms that you can select if you use suicide inhibitors. If the catalyst expressed on phage particles is mutagenized, for example, the specificity and mechanism of the reaction may change and you may fail to pick up these molecules because of the initial design of the suicide inhibitor.

Fastrez: Yes, we are working on developing a suicide inhibitor that would work for selection of protease activity based only on the release of the amine independently of the mechanism. I think it will be possible to do this.

Tramontano: Why did you not choose to use the existing transition-state analog inhibitors for proteases or other enzymes?

Fastrez: I think that the two approaches are complementary. If you have a very good suicide inhibitor, you are selecting for catalytic efficiency of the system. With a transition-state analog, you would hope that the difference in binding constants between the transition-state analog and the substrate reflects most of the features of the catalysis. We know, for many transition-state analogs, that the difference in binding constant is not very large. This means that the ability to recruit a very efficient catalyst using a transition-state analog could be limited, but both approaches are of interest and a comparison of these approaches will be useful.

Paul: Do you know if the K_m value of the enzyme displayed on the phage particle is different from that in solution?

Fastrez: We have not measured that. We simply measured the k_{cat}/K_m with the nitrocefin substrate. It is first-order kinetics and the k_{cat}/K_m ratios for the phage-displayed and free enzymes are very similar.

DISCUSSION REFERENCES

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