Selection of Metalloenzymes by Catalytic Activity Using Phage Display and Catalytic Elution

Isabelle Ponsard,[a] Moreno Galleni,[b] Patrice Soumillion,[a] and Jacques Fastrez*[a]

The metallo-β-lactamase βLII from Bacillus cereus *569/H/9 was displayed on the filamentous phage fd. The phage-bound enzyme fd-βLII was shown to be active on benzylpenicillin as substrate; it could be inactivated by complexation of the essential zinc(II) ion with EDTA and reactivated by addition of a zinc(II) salt. A selection process was designed to extract active phage-bound enzymes from libraries of mutants in three steps: 1. inactivation of active phage-bound enzymes by metal ion complexation, 2. binding to substrate-coated magnetic beads, 3. release of phages capable of transforming the substrate into product upon zinc salt addition. The selection process was first successfully tested on model mixtures containing fd-βLII plus either a dummy phage, a phage displaying an inactive mutant of the serine β-lactamase TEM-1, or inactive and low-activity mutants of βLII. The selection was then applied to extract active phage-bound enzymes from a library of mutants generated by mutagenic polymerase chain reaction (PCR). The activity of the library was shown to increase 60-fold after two rounds of selection. Eleven clones from the second round were randomly picked for sequencing and to characterize their activity and stability.*

KEYWORDS:

directed evolution · enzyme catalysis · gene technology · metalloenzymes · phage display

Introduction

Enzyme engineering can be attempted either by site-directed mutagenesis or by directed evolution, that is, by creation of large combinatorial libraries of mutants, followed by selection of those mutants that show the desired new properties. When a mutation offers a biological advantage, in vivo selection of the cells expressing the mutated gene is the easiest way of isolating an interesting mutant. This approach has been thoroughly used with proteins conferring antibiotic resistance.[1, 2] However, when engineered enzymes do not provide a biological advantage, alternative strategies must be relied upon. Display of peptides or proteins fused to a coat protein of a filamentous phage has been developed to find new ligands for specific receptors: Affinity chromatography on immobilized receptor allowed to extract the best binders from combinatorial libraries of phage-displayed peptides.[3] The application of phage display to enzymology has proved more difficult as selection has to be applied not for binding but for catalysis. Several strategies have been proposed for the extraction of catalysts from libraries.[4, 5] In a first approach, affinity chromatography on immobilized transition-state analogues has been used to select catalytic antibodies[6, 7] or mutants of glutathione transferase of modified specificity;[8] it remains difficult, however, to design transition-state analogues capable of recruiting the essential features of enzymatic catalysis. Reaction with biotinylated suicide substrates followed by capture of biotinylated phages on streptavidin-coated beads has been shown to provide an efficient selection of enzymes or abzymes;[9–14] however, the lack of suitable suicide substrates for many enzymatic activities limits this strategy to a few classes of enzymes.

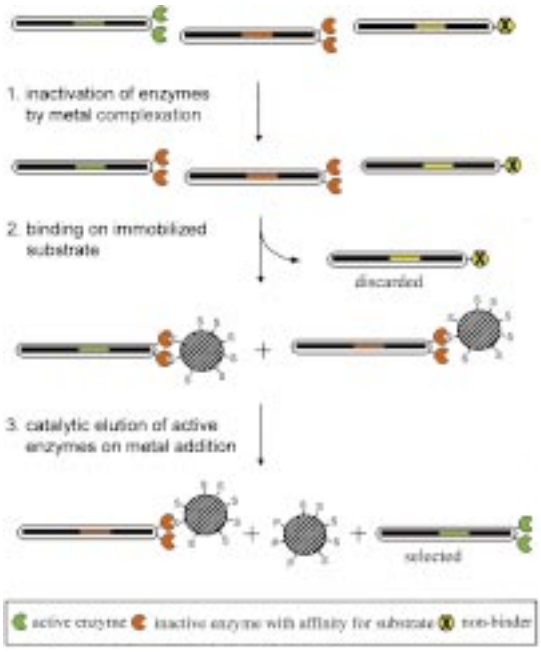
Recently, several groups have introduced new methods aiming at direct selection for catalytic activity. Pedersen et al.[15] and Demartis et al.[16] have connected both an enzyme and its substrate to a phage coat protein; after "intraphage" transformation of the substrate, phages displaying active enzymes were captured with product-specific binders. By coupling two independent reactions, the catalytic reaction leading to product and a chemical reaction connecting the substrate to the phage, Jestin et al.[17] were able to increase the fraction of active enzymes in mixtures or libraries of active and inactive phage-bound enzymes. Expression of enzymes from substrate-labelled DNA, in droplets of microemulsions in which enzymes and genes remain physically associated, has allowed Tawfik and Griffiths to extract the DNA encoding active enzymes by using also product-specific binders.[18]

In this paper we are exploring the possibility of selecting phages displaying active enzymes by affinity chromatography using catalytic elution. The method would be applicable to enzymes whose activity depends on the presence of a cofactor, particularly to metalloenzymes provided the apoenzyme is still

[a] *Prof. Dr. J. Fastrez, I. Ponsard, Dr. P. Soumillion*
Laboratoire de Biochimie Physique et des Biopolymères
Université Catholique de Louvain
Place L. Pasteur, 1, Bte 1B, 1348 Louvain-la-Neuve (Belgium)
Fax: (+32) 10-47-28-20
E-mail: fastrez@bioc.ucl.ac.be

[b] *Dr. M. Galleni*
Laboratoire d'Enzymologie et Centre d'Ingénierie des Proteines
Université de Liège
Institut de Chimie, B6, 4000 Sart-Tilman (Liège 1) (Belgium)

capable of binding its substrate. In this method, after complexation of the metal ion cofactor, the phage-bound enzymes are adsorbed on a support coated with substrate; the phages displaying active enzymes are then selectively eluted by addition of the cofactor. Active enzymes transform the substrate into a product for which they normally have a significantly lower affinity (Scheme 1).



Scheme 1. *Schematic representation of the catalytic elution protocol. Three phages displaying, respectively, an active enzyme (green) an inactive enzyme with affinity for the substrate (red), and a protein devoid of a binding site (yellow) are represented. The genes encoding the corresponding enzymes are drawn inside the phage capsid and are color-coded according to the type of enzyme that they encode. S = substrate, P = product.*

Editorial Advisory Board Member:[*]
Jacques Fastrez

was born in Belgium in 1942. He obtained his PhD in physical organic chemistry from the University of Louvain (UCL, Belgium) in 1968. In 1970 he became a member of the Belgian National Fund for Scientific Research (FNRS), working first with G. P. Hess at Cornell University, then with A. R. Fersht at the MRC Laboratory of Molecular Biology in Cambridge, and then independently at the UCL. In 1990 he joined the faculty of this university where he is now a Full Professor. His research interests are now in the field of enzyme engineering and in the development and application of new methodologies for the directed evolution of enzymes.

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Results

As model metalloenzyme for phage display, we have chosen the metallo-β-lactamase BCII from *Bacillus cereus* strain 569/H/9 (βLII)[**], an enzyme made up of 227 amino acid residues containing two zinc ions and active on penicillins and cephalosporins.[19, 20] One zinc ion, coordinated to three histidine residues, is essential for activity; the second one appears to play a structural role, but the activity on cephalosporins decreases in its absence. The structure of the protein is known: It is folded as a ββ sandwich with α helices on both faces.[21]

The nucleotide sequence coding for mature *Bacillus cereus* 569/H/9 zinc-β-lactamase was inserted into gene 3 of the filamentous phage fd-DOG1 (a derivative of fd-tet, which contains a tetracycline resistance gene).[22] In this construction, the encoded enzyme is fused to the phage coat protein g3p, between the signal sequence and the sequence coding for mature protein. On phage morphogenesis, it is displayed on the tip of the phage. The phage-bound enzyme was characterized by complete sequencing of the inserted DNA, analysis of the fusion proteins, and determination of enzymatic activity.

The phage proteins were separated by SDS-PAGE and detected on a Western blot with an anti-g3p antibody. The fusion protein was observed together with free g3p, the latter is presumably generated by proteolytic cleavage of the peptide connecting βLII to g3p.[23] The level of display depends on the temperature of phage culture: At 23, 30, and 37 °C, the percentage of g3p fused to βLII is of the order of 60, 40, and 20 %, respectively (Figure 1).

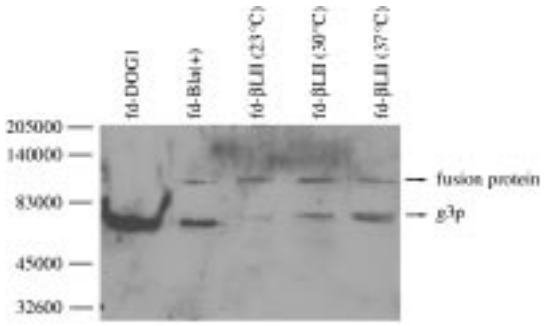


Figure 1. *Western blot analysis of phage proteins. After SDS-PAGE separation, the proteins were transferred onto a nitrocellulose membrane and detected with an anti-g3p antibody. The protein g3p is detected at a molecular weight of 65 000 Da and the fusion protein β-lactamase – g3p at 95 000 Da. The phages used and the culture temperatures are indicated above the lanes.*

The β-lactamase activity of phages purified by PEG precipitation was measured by following spectrophotometrically the disappearance of benzylpenicillin (Pen-G); curve fitting of the initial rates of hydrolysis versus substrate concentration afforded V_{max} and K_m values. Dividing V_{max} by the concentration of phage-bound enzyme determined from the absorbance of the phage solution at 265 nm gave the k_{cat} value of the phage-bound enzymes. The specific activity of the phage-bound enzymes was

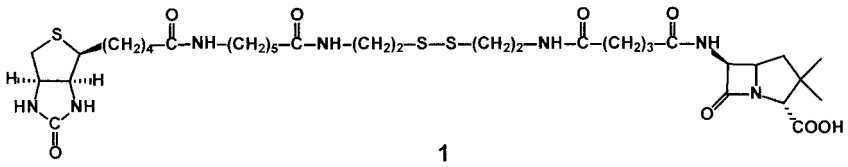
[**] *For abbreviations see ref. [32].*

observed to be quite sensitive to both the temperature of the culture and the composition of the medium used to prepare the phages. The following values were obtained for phages prepared at 23 °C in medium A: $k_{cat} = 2750\ s^{-1}$, $K_m = 0.6$ mM. The corresponding parameters have been reported for the soluble enzyme: $k_{cat} = 833\ s^{-1}$, $K_m = 0.83$ mM (at 30 °C).[24] A comparison of the values obtained with the phage-bound enzymes and the soluble enzyme allows the determination of the mean number of enzymes displayed per phage to be 3.3. The phage-bound enzymes could be inactivated by incubation with EDTA, the activity could be fully restored by addition of zinc sulfate.

Despite this level of display, phages isolated from culture at 23 °C retained their infectivity. This was monitored by determining the number of cells transduced to express tetracycline resistance by solutions of phage-bound enzymes of known concentration (based on A_{265} values): 1 – 2% of the fd-βLII phages were infective; by comparison, the infectivity of phages fd-DOG1 displaying no foreign protein amounts to 2 – 3%.

While it had been shown earlier that infection of cultures by phages displaying TEM-1 β-lactamase (fd-Bla(+) phages) transduced resistance to ampicillin at concentrations up to 400 mg L^{-1},[23] infection by fd-βLII phages did not confer resistance at 10 mg L^{-1} (below this concentration, other mechanisms allow the bacteria to survive). This difference results from the 25-fold lower k_{cat}/K_m value on this antibiotic of βLII versus the TEM-1 β-lactamase. However, as the 5/B/6 zinc-β-lactamase, which is homologous to βLII, is known to hydrolyze cephalosporins efficiently,[25] cefotaxime was tested for in vivo selection: The infected bacteria became resistant to this antibiotic at 0.1 mg L^{-1}. This selection was used in the assessment of the in vitro catalytic elution process.

The feasibility of in vitro selection of phages displaying metalloenzymes by catalytic elution was first assessed by running three series of model experiments. In the first one, mixtures of phages containing EDTA-inactivated βLII phage-bound enzymes and fd-DOG1 were incubated with streptavidin-coated magnetic beads whose biotin-binding sites had been saturated with the biotinylated penicillin derivative **1**.[26] After washing, the bound phages were eluted by addition of a zinc salt. The percentage of fd-βLII phages recovered was determined by plating on media containing both cefotaxime and tetracycline versus plating on media containing tetracycline alone.



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The results of these selection experiments are presented in Table 1. In entries 1 – 3, it is seen that the fraction of phages displaying the active β-lactamase has increased in the elution mixture: The percentage of fd-βLII is significantly higher than that of fd-DOG1, whose elution yield is typical of nonspecifically adsorbed phages. The efficiency of the selection is measured by the enrichment factor *Er* defined in Equation (1):

$$Er = \frac{E_A E_I^{-1}}{L_A L_I^{-1}} \quad (1)$$

Table 1. *Enrichment of fd-βLII phage in phage mixtures.*

Entry	Loaded phages[a]				Eluted phages[a]			Eluted versus loaded phages [%]		*Er*[c]	Experimental conditions[d]
	fd-βLII (L_A)	fd-DOG1 (L_I)	% fd-βLII	spec. act. of fd-βLII[b]	fd-βLII (E_A)	fd-DOG1 (E_I)	% fd-βLII	fd-βLII	fd-DOG1		
1	1.0×10^8	6.0×10^{10}	0.17	670 s^{-1}	6.7×10^3	2.2×10^4	23.3	6.7×10^{-3}	3.7×10^{-5}	183	
2	5.1×10^8	8.4×10^{10}	0.60	1290 s^{-1}	1.4×10^4	6.0×10^3	70.0	2.7×10^{-3}	7.1×10^{-6}	384	
3	9.6×10^7	4.6×10^{11}	0.02	2180 s^{-1}	6.0×10^4	3.5×10^5	14.5	6.2×10^{-2}	7.6×10^{-5}	821	
4	5.0×10^8	2.5×10^{11}	0.20	2180 s^{-1}	4.0×10^3	3.0×10^5	1.32	8.0×10^{-4}	$1{,}2 \times 10^{-4}$	6.7	without EDTA inactivation
5	6.9×10^8	3.9×10^{11}	0.18	2180 s^{-1}	6.4×10^3	6.1×10^5	1.04	9.3×10^{-4}	1.6×10^{-4}	5.9	elution without Zn^{II}
6	2.0×10^9	1.3×10^{12}	0.15	890 s^{-1}	3.0×10^5	2.3×10^7	1.28	1.5×10^{-2}	1.8×10^{-3}	8.5	selection for product binding
7	2.0×10^9	1.3×10^{12}	0.15	890 s^{-1}	1.0×10^7	1.8×10^7	35.7	5.0×10^{-1}	1.4×10^{-3}	360	
	fd-βLII (L_A)	fd-Bla(−) (L_I)	% fd-βLII		fd-βLII (E_A)	fd-Bla(−) (E_I)	% fd-βLII	fd-βLII	fd-Bla(−)		
8	1.0×10^8	4.0×10^{10}	0.25	1290 s^{-1}	5.0×10^4	5.5×10^5	8.33	5.0×10^{-2}	1.4×10^{-3}	36	
9	1.0×10^8	1.2×10^{11}	0.08	2180 s^{-1}	2.0×10^4	1.3×10^5	13.3	2.0×10^{-2}	1.1×10^{-4}	185	
10	5.0×10^9	1.6×10^{11}	3.03	2180 s^{-1}	3.0×10^4	1.2×10^6	2.40	6.0×10^{-4}	7.5×10^{-4}	0.8	without EDTA inactivation
11	5.0×10^9	1.6×10^{11}	3.03	2180 s^{-1}	1.4×10^4	1.5×10^6	0.92	2.8×10^{-4}	9.4×10^{-4}	0.3	elution without Zn^{II}

[a] Expressed as the numbers of tetracycline resistance transducing units for fd-DOG1 and tetracycline plus cefotaxime resistance transducing units for fd-βLII as determined by infection of an *E. coli* TG1 culture and plating on selective media. [b] Specific activity of loaded phages related to the number of copies of enzyme present per phage, dependent on the conditions of phage production and measured on PenG as a substrate at a concentration of 10^{-3} M (see text for details). [c] The enrichment factor *Er* is calculated according to Equation (1). [d] Unless stated otherwise, the elution is effected by addition of a zinc(II) salt.

L_A and E_A are the numbers of fd-βLII phages loaded and eluted, respectively, from the substrate-coated beads; L_I and E_I are the corresponding numbers of fd-DOG1 phages. The reproducibility of *Er* values in three to four independent experiments is within 50%. The enrichment factor tends to increase with the specific activity of the phage-bound enzymes engaged in the selection, that is, with the number of copies of enzyme displayed. It was also observed that it is essential to saturate the beads with the biotinylated substrate to get a good enrichment. Three control experiments were run. In the first one (entry 4 in Table 1), the phage-bound enzymes were not inactivated by EDTA before incubation with the substrate-coated beads; in the second one (entry 5), elution was run without addition of zinc salt. In both cases, a small enrichment of fd-βLII is observed, but it is at least 25-fold lower than in actual selections (entries 1 – 3). In the third control experiment, comparative selection experiments were run on magnetic beads coated with biotinylated product (entry 6) or biotinylated substrate (entry 7). The increase in fd-βLII versus fd-DOG1 on immobilized product is again in the same range as in the other control experiments. On acidic elution from product-coated beads (glycine buffer at pH 2.5), only 1.1×10^{-3}% of fd-βLII phages are recovered (background level); ten times more phages (1.2×10^{-2}%) are recovered from substrate-coated beads under the same conditions. This confirms that fd-βLII phages bind better to substrate than to product, but also that zinc elution is more efficient (% elution $= 5 \times 10^{-1}$%) for releasing the phages.

In the second set of model experiments (Table 1, entries 8 – 11), selection was attempted between fd-βLII and fd-Bla(−), a mutant of fd-Bla(+) displaying the inactive S70A mutant of the TEM-1 β-lactamase.[9] An enrichment of fd-βLII is again observed whose efficiency depends on the number of copies of metallo-β-lactamase on the phage surface. The enrichment factors are lower than in the selection versus fd-DOG1. This is anticipated as fd-Bla(−) is capable of binding to the immobilized substrate and is retained more efficiently on the beads off which it is slowly washed during the catalytic elution of fd-βLII. In the control experiment where no inactivation of fd-βLII was performed, no enrichment is observed; a weak enrichment of fd-Bla(−) is observed on elution without zinc ions.

In the third set of model experiments, selection was attempted between phages displaying the wild-type β-lactamase and two mutants of medium or very low activity. Their specific activity (k_{cat}/K_m values measured in the presence of 100 μM $ZnCl_2$) is reduced compared to that of the wild-type enzyme ($7.3 \times 10^5\,M^{-1}\,s^{-1}$) either because a ligand of the zinc ion has been replaced (mutant H86S, $k_{cat}/K_m = 8.9 \times 10^4\,M^{-1}\,s^{-1}$) or because the general base has been mutated (mutant D90N, $k_{cat}/K_m < 100\,M^{-1}\,s^{-1}$).[31] The genes encoding the mutants were inserted into fd-DOG1 to give phage-bound enzymes whose apparent specific activities (moles of PenG hydrolyzed per second and mole of phage-bound enzyme at a substrate concentration of 10^{-3} M) were 1.2 s^{-1} and $< 0.1\,s^{-1}$, respectively, for the H86S and the D90N mutants (as compared to 630 s^{-1} for the wild-type enzyme prepared under identical conditions). Two selection experiments were run with mixtures containing the following percentages of the wild-type, H86S and D90N phages, respectively: 3/15/82% or 1/15/84%. After a single round of selection, the mean activity of the mixtures increased by a factor of 18 (from 18 s^{-1} to 320 s^{-1}) or 38 (from 8 s^{-1} to 300 s^{-1}), respectively. Plating the colonies producing the eluted phages on media containing both cefotaxime and tetracycline versus plating on media containing tetracycline alone indicated that 80% of them were wild-type fd-βLII.

A library of 5×10^6 mutants was then created by error-prone PCR.[27] Sequencing of seven clones indicated a mean number of 2.6 mutations per βLII gene. This level of mutation is deleterious to activity as the mean activity of the library produced at 23 °C was 1.6% of the activity of the wild-type. When this library was submitted to two rounds of selection, its activity increased sevenfold after the first round and 58-fold after the second round. Eleven clones were randomly picked from plate and the phage-bound enzymes were prepared. Their sequences were determined. The activities of the phage-bound enzymes were measured as a function of PenG concentration up to 7.5 mM. Fitting of the data afforded k_{cat} and K_m values. The data are listed in Table 2. The specificity constants, k_{cat}/K_m, ranged from 20% to 170% of the wild-type value (recovered once from the selection). All except two phage-bound enzymes have activities similar or higher than that of the wild-type. The stabilities of the displayed enzymes towards irreversible denaturation were determined by following the rate of disappearance of activity as a function of the time of incubation of the phage-bound enzymes at various temperatures between 55 and 85 °C. The data obtained at 65 °C are listed in Table 2. All the mutants were less stable than the wild-type enzyme on phage.

Table 2. *Properties of mutant phage-bound enzymes selected from a library generated by error-prone PCR.*

Mutant	Mutations	k_{cat} [s^{-1}]	K_m [mM]	$t_{1/2}$ [s][a]
1	I83T, K216T	1160 ± 78	1.0 ± 0.2	100
2	L52S, V213A	792 ± 92	0.8 ± 0.3	200
3	E141G, Y144C	796 ± 43	0.6 ± 0.1	75
4	L52S, N204Y	673 ± 63	0.77 ± 0.25	400
5	K10E, N132D, E141A	755 ± 45	1.3 ± 0.23	300
6	V82D, K117R, S192P	240 ± 33	1.5 ± 0.6	180
7	E69V, K74N, V156E, L225I	830 ± 155	1.3 ± 0.7	360
8	L52S, L124P, R199Q, V213A	707 ± 57	0.68 ± 0.2	310
9[b]	V55G, K99R, V155A, K198R	576 ± 33	0.9 ± 0.17	350
wt	–	727 ± 86	0.9 ± 0.35	> 2400

[a] Half-life for denaturation at 65 °C. [b] This mutant was isolated twice.

Discussion

A selection protocol based on catalytic elution has been developed and tested by using a phage-displayed metallo-β-lactamase as a model system. The experiments were based on the assumption that, on extraction of the metal ion cofactor, the enzyme would retain its ability to bind its substrate and that the activity would be restored on zinc salt addition. Under these conditions, inactivated phage-bound enzymes would be adsor-

bed on a solid support on which substrate is immobilized and released on regain of enzyme activity because the enzymes would have a lower affinity for their products than for their substrates. In the first set of model experiments, selection was attempted between fd-βLII, a phage displaying the wild-type enzyme, and fd-DOG1 or fd-Bla(−), phages displaying no foreign protein or an inactive mutant of the serine β-lactamase TEM-1, respectively. Enrichment of fd-βLII is observed not only compared to fd-DOG1 but also compared to fd-Bla(−), a phage showing affinity for the immobilized substrate. The efficiency of selection appears to depend on the number of copies of enzyme per phage. This observation is probably related to the fact that the enzyme under investigation does not have a high affinity for its substrate ($K_m \approx 0.6$ mM; the affinity of the zinc-free enzyme is not known). Consequently, phages displaying only a single copy of the enzyme will be poorly adsorbed on the support and more easily washed away. Phages with more than one copy of bound enzyme will bind more efficiently as a consequence of the avidity phenomenon. This property is likely to be shared by most phage-bound enzymes.

A method of selection should ideally be able to extract the most active enzymes from a library. To test this potentiality of the method, the selection protocol was applied to a mixture of mutants of different activities and to a library of low-activity mutants generated by error-prone PCR.

In the selection from a mixture of wild-type fd-βLII and two mutants, the most active enzyme is clearly preferentially selected. Although we could not measure the relative percentages of H86S and D90N in the eluate, we can put a limit on the maximum enrichment of the H86S mutant by assuming that all the non-wild-type phage-bound enzymes eluted are this medium-activity mutant: $Er \leq 3$. There are two possible reasons for the preferential selection of the wild-type phage-bound enzyme: a higher activity, responsible for a more efficient catalytic elution, or a higher level of display leading to a better adsorption on the substrate-coated beads. Indeed, the ratio of specific activities of the H86S mutant versus the wild-type enzyme is lower with the phage-bound enzymes than with the free enzymes. This indicates that the mutant is less efficiently displayed, probably because it is less stable than the wild-type enzyme. Analysis of the phage proteins by Western blotting confirms that the level of display of the mutant is approximately ten times lower than that of the wild-type enzyme (data not shown).

Mutagenic PCR creates a library of mutants of variable properties. Some will feature active and stable enzymes, others will have a disrupted active site, others will be incorrectly folded or will have a less stable tertiary structure. The last two types of mutants are likely to be proteolytically removed before or during phage morphogenesis and will be disfavored in the selection. Part of the activity increase on selection simply reflects the potential of the protocol to discard them. Determination of the properties of a few selected clones shows that the selection does not tend to extract weakly active clones from the library. Most phage-bound enzymes had activities similar or even higher than the wild-type enzyme, and the lower stability of these enzymes did not prevent them from being selected. Although it might have been anticipated that active phages could bring about the elution of weakly active or inactive ones during the progressive transformation of immobilized substrate into product, this factor does not appear to prevent the selection of the most active phages even if it might limit the efficiency of the selection. The fact that, after the washing step discarding the unbound phages, the phage concentration ($\leq 10^8$ mL^{-1}) reaches the same order of magnitude as the microbead concentration (8×10^8 particles per mL, coated with ca. 10^5 biotin sites per bead), and that both phages and beads diffuse slowly, may contribute to limiting the cross-elution of inactive by active phages.

The mutants that have been characterized feature from two to four mutations. None of them is close to the active site; 70% concern residues located at the surface of the protein. Five mutations involve buried residues: V59G (mutant 9), V82D (mutant 6), I83T (mutant 1), V155A (mutant 9), and V156E (mutant 7). These mutations are likely to destabilize the folded structure as they replace buried hydrophobic residues by residues capable of hydrogen bonding or, worse, acidic residues, or because they create cavities. These mutants do not appear to be much less stable than the other ones. It is possible that other mutations could contribute a compensatory stabilization (e.g. the K117R mutation at the C-terminal end of helix H3). Two mutations in mutant 5 (N132D and E141A) involve residues that are close together in space; the E141A mutation compensates for the destabilization that would result from the electrostatic repulsion between D132 and E141 (the side chain of N132 forms a hydrogen bond with the carboxylate of E141). A detailed interpretation of the effect of these mutations on stability and activity would, however, require extensive mechanistic, thermodynamic, and structural studies on the free enzymes.

In conclusion, the method described allows the selection of active phage-bound metallo-β-lactamase enzymes from a mixture of mutants. It is expected that it can be applied to other metalloenzymes.

Experimental Section

Materials: Deoxyoligonucleotides were purchased from Eurogentec (Belgium). Phages fd-Bla(+) and fd-Bla(−) have been described previously.[9] The synthesis of the biotinylated penicillin derivative has been described.[26] The following culture media were used: medium A: 10 g select peptone 140 (Gibco BRL), 5 g yeast extract (Merck), and 5 g NaCl per liter, medium B: 20 g LB broth base (Gibco BRL) per liter. The selective media LB-tet and LB-tet-cef contained 7.5 mg L^{-1} tetracycline and 7.5 mg L^{-1} tetracycline plus 0.1 mg L^{-1} of cefotaxime, respectively.

Construction of the phages fd-βLII and mutants: The nucleotide sequence encoding the mature *Bacillus cereus* 569/H/9 zinc-β-lactamase was isolated from plasmid pCIP32[28] by PCR amplification with introduction of *Apa*LI and *Not*I restriction sites using primers BLII3 (5′-TCGTCGTCGCGGCCGC**ACGCCCCTCAAT**CCCTTTTAATAAATCCAATG-3′) and BLII5 (5′-CGACACGTGCACAAAAGGTAGAGAAAAC-3′) (10 min at 94 °C; 30 cycles of 1 min at 94 °C, 1 min at 54 °C, 90 s at 72 °C; 10 min at 72 °C). The 3′ primer (BLII3) used for amplification is a 48-mer complementary over 17 bases to the 3′ end of the zinc-β-lactamase gene. It contains an extension that replaces the stop

codon by a glycine (bases in italics) and introduces the restriction site *Not*I for cloning (underlined bases) as well as four codons encoding an Ile-Glu-Gly-Arg sequence recognized and cleaved by factor Xa (bases in bold). The 5′ primer (BLII5) is a 28-mer complementary over 19 bases to the 5′ end of the β-lactamase gene. It contains a GCA codon (Ala) that replaces the first codon TCA (Ser) of the sequence coding for mature βLII and allows for the introduction of the restriction site *Apa*LI for cloning (underlined bases). After amplification and restriction by *Apa*LI and *Not*I, the βLII gene was inserted between the corresponding sites of phage fd-DOG1.[22] Phage fd-DOG1 is a derivative of fd-tet carrying a tetracycline resistance gene in the intergenic region and a polylinker between the sequences encoding the signal peptide and the mature form of coat protein g3p. This construct was used to transform *Escherichia coli* strain TG1. The transformed cells were plated on LB-tet-agar medium. Colonies were collected and grown in an LB-tet medium to prepare the fd-βLII phages. For the H86S and D90N mutants, the genes were recovered by PCR from derivatives of pCIP32[31] and inserted into fd-DOG1 according to the same protocol. The mutants were characterized by complete sequencing of the βLII gene. The library of randomly distributed mutants was constructed similarly except that the βLII gene was amplified by 30 cycles of PCR under conditions of error-prone mutagenesis.[27]

Preparation of the phages: Phages were prepared at three different temperatures, 23, 30, and 37 °C, from infected cells grown in 250 mL of LB-tet medium for 64, 16, and 16 h, respectively. After centrifugation of the cells, the phages were precipitated with 4% (w/v) PEG, 0.5 M NaCl (final concentration) and resuspended in water (10 mL). The solutions were filtered on a 0.45-μm Millex-GV unit (Millipore) to remove contaminating cells and insoluble material. The phages were precipitated and resuspended in water (1 mL). The concentration of phage solutions were determined by measuring the absorbance at 265 nm (A_{265}). The extinction coefficients were: for fd-βLII = $9.7 \times 10^7\,M^{-1}\,cm^{-1}$ (1 A_{265} unit = 6.2×10^{12} phages per mL), for fd-DOG1 = $9.09 \times 10^7\,M^{-1}\,cm^{-1}$. These values were calculated from the extinction coefficient published for fd[29] adapted for difference in phage size and composition.

Analysis of the phage proteins: Solutions of 10^{12} fd-βLII phages in 15 μL were denatured by boiling for 10 min in the presence of 5 μL of sample buffer (4 mL water, 1 mL 0.5 M Tris-HCl (pH 6.8), 1.8 mL glycerol, 1.6 mL 10% (w/v) SDS, 0.4 mL β-mercaptoethanol, 0.2 mL 0.05% (w/v) bromophenol blue). After boiling, the 20-μL samples were loaded on a 10% (w/v) denaturing (SDS) polyacrylamide gel and separated (miniprotean II apparatus, Biorad). The proteins were then transferred onto nitrocellulose membranes (minitrans blot, Biorad) and detected with a mouse anti-g3p antibody (Eurogentec), followed by a goat anti-mouse antibody conjugated to alkaline phosphatase (Biorad). The fusion protein βLII-g3p was detected at an apparent molecular weight of 93 kDa, while free g3p, derived from fd-DOG1, appears at an abnormal molecular weight of 64 kDa.[30]

Infectivity of fd-βLII phages: A solution containing approximately 10^{12} fd-βLII phages per mL (concentration determined from A_{265}) was diluted 10^3 – 10^4-fold, and 100 μL were used to infect 1 mL of *E. coli* TG1 cells in exponential growth phase (OD at 600 nm ≈ 0.6). The cells were incubated without shaking at 37 °C for 30 min. Different dilutions of the infected cells were then plated on LB-tet-agar medium and grown at 37 °C overnight. The ratio between the number of tetracycline-resistant colonies and phages in the starting solution was used to determine the infectivity of the phages.

In vivo selection of colonies expressing fd-βLII phages: The β-lactamase II activity of infected colonies was detected on LB-agar medium containing tetracycline (7.5 mg L^{-1}) and cefotaxime (0.1 mg L^{-1}).

In vitro selection of the phages displaying active βLII: Streptavidin-coated magnetic particles (50 μL diluted to 1 mL per experiment) from Boehringer Mannheim (binding capacity: 350 pmol of free biotin per mg of particles) were washed three times with TBS buffer (0.05 M Tris, 0.15 M NaCl, pH 7.5) and blocked for 1 h with 2% (w/v) of blotting grade blocker (nonfat dried milk, Biorad) in TBS buffer. The magnetic beads were then saturated with the biotinylated penicillin derivative **1** by incubation in a 10^{-5} M solution of **1** for 30 min, and they were washed twice with TTBS (TBS containing 0.1% (w/v) Tween 20 and 10^{-4} M EDTA) before use. Mixtures of approximately 5×10^{12} phages per mL containing various proportions of fd-βLII and fd-DOG1 or fd-Bla(−) in TBS or citrate (50 mM, pH 7) buffers were incubated for 5 min with EDTA (10^{-3} M final concentration). 1 mL of this mixture was then incubated for 30 min with the magnetic beads. These were washed five times with TTBS to remove unbound phages. The elution of the phages was performed by addition of 1 mL of 50 mM citrate buffer (pH 7) containing 11 mM $ZnCl_2$, which restored the βLII activity and led to the hydrolysis of the complexed substrate. The concentrations of the loaded and eluted phage solutions were measured by infection of a culture of TG1 cells as described above.

Activity of the fd-βLII phages: The hydrolysis of benzylpenicillin (Sigma) in the presence of the purified fd-βLII phages was followed spectrophotometrically at 232 nm in 50 mM citrate buffer (pH 7) containing 11 mM $ZnCl_2$ at 25 °C.

Rates of irreversible denaturation of phage-displayed β-lactamase mutants: The phage-bound enzymes were incubated at the denaturation temperature in the buffer used for the activity measurements for 0 – 25 min and were quickly cooled down to 20 °C before measurement of the residual activity.

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[32] Abbreviations: A_{265} = absorbance at 265 nm; βLII = *Bacillus cereus* β-lactamase II derived from strain 569/H/9; fd-βLII = fd phage displaying *Bacillus cereus* β-lactamase II; fd-Bla(+) and fd-Bla(−) = fd phage displaying TEM-1 β-lactamase or the inactive mutant S70A, respectively; fd-DOG1 = fd-tet phage featuring a polylinker multiple cloning site; fd-tet = fd phage containing a gene coding for tetracycline resistance; g3p = product of gene 3 of phage fd; PCR = polymerase chain reaction; PEG = polyethylene glycol; PenG = benzylpenicillin; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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