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IN VITRO SELECTION FOR CATALYTIC TURNOVER FROM A LIBRARY OF β-LACTAMASE MUTANTS AND PENICILLIN-BINDING PROTEINS.

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Abstract: A library of mutants of the RTEM ß-lactamase displayed on phage was created; it contained penicillin binding proteins (PBPs) as well as a small fraction of active ß-lactamases. The library was submitted to a selection process to extract the ß-lactamases i.e. the enzymes that turnover efficiently. This was achieved by a two steps procedure. In the first step, the ß-lactamases were labelled by reaction with a biotinylated suicide inhibitor while the PBPs were blocked by incubation in the presence of benzylpenicillin. In the second step, the labelled active phage-enzymes were separated by affinity chromatography on streptavidin coated beads.

Over the last few years, protein engineering has witnessed extensive technological developments with great impacts on elucidation of structure / function relationships and generation of catalysts endowed with new properties. Random mutagenesis together with the phage display technology offers the potential to discover mutants with new anticipated skills. Indeed, through the cloning of a foreign gene between the sequences encoding the signal peptide and mature g3p of a filamentous phage, bioactive peptides [1] or folded proteins [2,3] can be displayed on phage, then libraries of mutants of the displayed protein can be created. The crucial step for finding interesting mutants in these libraries remains an efficient and suitable selection protocol.

Several enzymes have been displayed in their active conformation on phage and tools have been developed to select them (for a review see ref. 4.). Phage particles expressing alkaline phosphatase were shown to bind to an immobilized competitive inhibitor and to be eluted with product [5]. Panning on a substrate analog has been successfully used to select, after several rounds of error prone mutagenesis and selection, a nuclease with modified properties (lower k_{cat} and higher k_{cat}/K_m) [6]. Attempts have also been made to modify the specificity of a phage displayed glutathione transferase by introduction of random mutations in the region of the binding site of electrophilic substrate; after selection by binding to transition state analogs, functional mutants with binding properties were isolated but they had limited catalytic potential [7].

All these examples use selection procedures based on complexation abilities. However, they all present limitations for obtaining efficient catalysts. As engineering of new enzymes has obviously to deal with catalysis criteria, selection should be performed on the basis of catalytic activity. *In vivo* selection can be used to find enzymes with improved catalytic efficiencies; glutathione transferase mutants more efficient in detoxication *versus* a specific drug were indeed isolated in this way [8]. Nevertheless, *in vivo* selection may be unappropriate for many activities. We have previously developed a reliable *in vitro* two steps process aiming at selecting phage displayed enzymes on the basis of their catalytic activity [9]. In this approach, phage-enzymes are first labelled using a bifunctional organic compound, featuring a mechanism-based inhibitor linked to a biotin moiety. In a second step biotin-labelled phages are recovered by affinity chromatography on immobilized streptavidin. This method has recently been shown to be efficient in the selection of the most active ß-lactamase from a mixture of wild type

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enzyme and known mutants [10]. Covalent labelling has also been used to select from a library of phage displayed antibodies a catalyst featuring a cystein properly located for thioester cleavage [11].

Many enzymes operate by covalent catalysis through an intermediate. Efficient catalysis then requires fast rates for both its formation and decomposition. A proper selection technique should be able to select from a library of mutants those that turnover and exclude those that accumulate a stable intermediate. Recently, we became interested in the relationships between DD-peptidases and ß-lactamases. These enzymes both interact with ß-lactam antibiotics but differ in the efficiency of turnover of these substrates. The first ones form stable acyl-enzymes with them; accordingly, they are also known as penicillin binding proteins (PBPs). The second ones hydrolyse these antibiotics efficiently in two steps going through an acyl-enzyme. To analyse the origin of this difference, we created a library of mutants of the RTEM ß-lactamase on phage and hoped to find in that library a large proportion of fast acylating and slowly deacylating enzymes i.e. PBPs. Preliminary experiments indicated that relatively active ß-lactamases were also present in this library. Although an *in vivo* selection protocol could be used to extract these ß-lactamases, we decided to develop an *in vitro* technique to select them. This would test the potential of this approach in achieving a selection for turnover, a problem of more general interest.

Material and Methods.

Library construction : The phage library was constructed from fd-Bla$^+$ vector described previously [9] : the ß-lactamase RTEM gene was cloned within the minor coat protein gene III (g3p) region, downstream of the signal peptide and upstream of the mature protein. The enzyme is expressed on phage surface, fused to g3p protein, the proteins being connected by a linker containing the factor Xa cleavage site. BbsI non-palindromic sites were introduced on both sides of the 163-171 region. Mutagenesis was performed by cassette replacement of the wild type sequence using a 81-mer oligonucleotide.A library of $> 9*10^6$ transformants was obtained. The detailed construction and characterization of the library will be described elsewere.

Selection of phages displaying active ß-lactamases : The concentration of phages was measured from the absorbance at 265 nm using for the phages an extinction coefficient equal to $8.4.10^7$ $M^{-1}.cm^{-1}$. A mixture of 5.10^{12} phages / ml was incubated in the presence of 10^{-4} M benzylpenicillin during 2 h at room temperature, in 50 mM phosphate buffer (pH 6,86) containing 1% Bovine Serum Albumin (BSA). 10^{-5} M of biotinylated suicide inhibitor (1) was then added and the phages labelled for 3 minutes at room temperature. Phages were precipitated twice using 1/5 (vol/vol) polyethylene glycol (20% polyethylene glycol 6000 / 2,5 M NaCl). Phages were finally resuspended in Tris NaCl buffer containing 2% skimmed milk. 450 μl of labelled phages were incubated 16 h at room temperature under gentle stirring with 50 μl coated magnetic beads ($6,7*10^8$ beads / ml). After 10 washes with TBS containing 0,1% Tween 20, elution of phages was performed by proteolytic cleavage of the peptide linker between the enzyme and g3p with 1 ml TNCB (50 mM Tris.HCl, 100 mM NaCl, 1 mM $CaCl_2$ buffer) containing 5 μg of factor Xa.

Titration of the phages : The overall concentration of tetracyclin transducing units was determined through infection of TG1 cells : infected cells were able to grow overnight at 37°C on tetracyclin as the phage genome contains the resistance gene. Titration of the phages displaying active ß-lactamases was carried out on plates containing ampicillin (50 mg/l); the cells were grown for 3 days at 23°C.

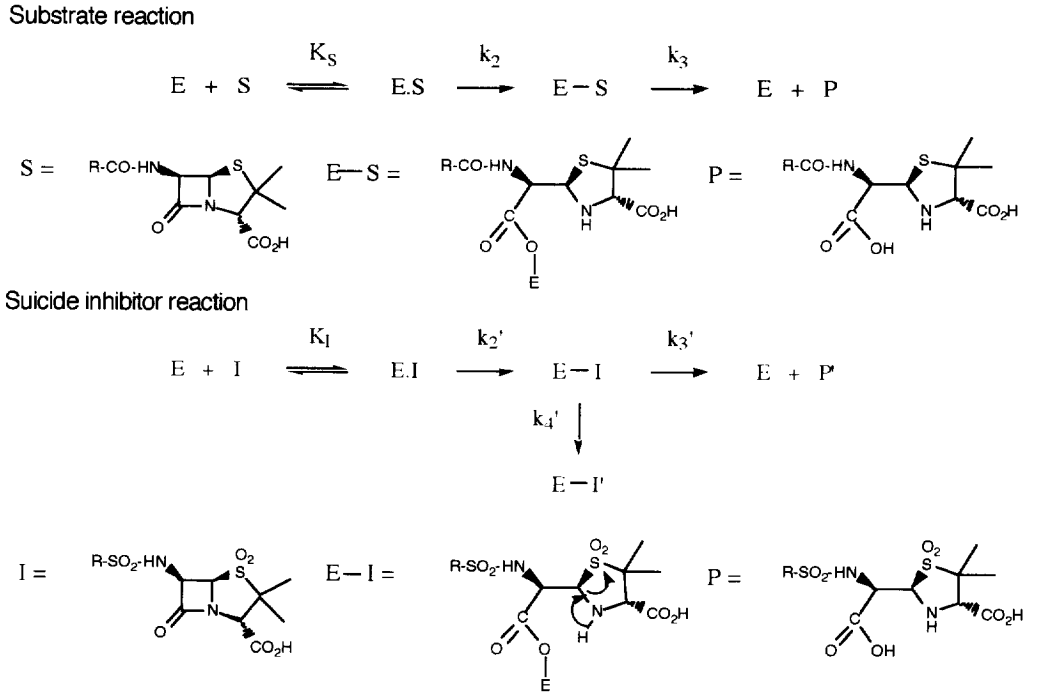
Activity measurements : Hydrolysis of benzylpenicillin (Sigma) was followed spectrophotometrically at 232 nm in 50 mM phosphate buffer pH 6,86 in the presence of phages purified by precipitation with polyethylene glycol as above.

Results and discussion.

Starting from a phage displaying the RTEM ß-lactamase [9], we have constructed a library of mutants in which the wild type sequence was replaced by a cassette encoding mutations from residues 163-171. This region of the enzyme, in the so called ω-loop, contains glutamic acid 166, a residue assumed to act as an acid-base catalyst in the class A ß-lactamases but whose contribution is thought to be more important for deacylation than acylation; it is absent in the corresponding PBPs [12-14]. In the library, the replacements were designed according to sequence alignments between class A ß-lactamases and the *E. coli* PBP4 [15,16]. Glu was not allowed at position 166, it was replaced by Asn, Asp, Tyr and His; these substitutions were designed to force evolution from ß-lactamases towards PBPs. The wild type residues in the other positions were also replaced by 2 to 12 residues. Theoretically, this library contains $5*10^5$ different variants [17]. Sequencing of randomly picked clones indicated that the expected residues were present.

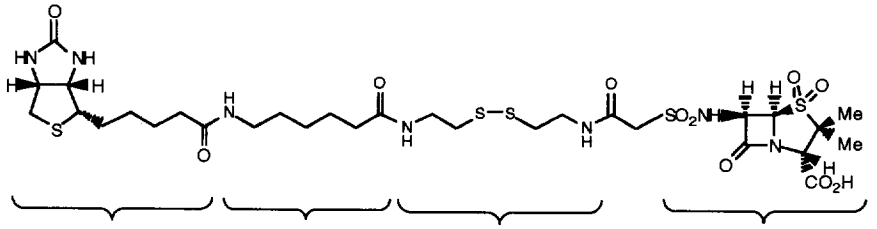
Initial characterization of the library indicated that a significant proportion of the clones were PBPs as they were labelled with radioactive penicillin but also that a very small proportion of them were active ß-lactamases as the bacteria harbouring these phages became penicillin resistant. The library of phages was used to infect a culture of *E. coli.* TG1 and the cells were plated on Petri dishes containing either 5 mg/l of tetracyclin (the phage genome also contains the tetracyclin resistance gene) or 50 mg/l of ampicillin. The cells were grown overnight at 37°C [18]. From the ratio of the numbers of colonies growing on ampicillin and tetracyclin containing media, the percentage of phages displaying a ß-lactamase activity was obtained : it amounted to $6*10^{-4}$ % (6 ppm).

To select *in vitro* the phages displaying active ß-lactamases from a mixture containing a large proportion of



Scheme 1.

penicillin binding proteins, we have developed a modification of the two steps protocol described earlier in which the active phages are labelled in a first step by incubation with a biotinylated suicide inhibitor under competition conditions and the labelled phages recovered in the second step by affinity chromatography. The modification takes into account the difference between the PBPs and the ß-lactamases in their interaction with normal substrates and suicide inhibitors (scheme 1). With normal substrates, the only pathway from the acyl-enzymes is hydrolysis; the rate constant k_3 similar to k_2 for ß-lactamases; it is much smaller than k_2 for PBPs. The same relationships between k_2 and k_3 are also valid with suicide substrates but then the acyl-enzyme (E-I) can undergo a ß-elimination across the liberated amine to generate a very electrophilic species; this species then blocks the active site (pathway k_4 to E-I') [19]. To extract the ß-lactamases from the mixture, we will run the labelling with the biotinylated suicide inhibitor (1) in the presence of a large excess of benzyl-penicillin; the later will block all the PBPs in their acyl-enzyme form; as the ß-lactamases turnover, they will remain free to react with the inhibitor. The selection is thus run under conditions of counter-selection against the PBPS.



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Three rounds of selection for ß-lactamase activity were applied to the 163-171 library. A mixture of $5*10^{12}$ phages / ml was preliminarily incubated with 10^{-4} M of benzylpenicillin during 2 h, then labelled with 10^{-5} M of 1 for 3 minutes. The eluted phages were amplified and the percentage of phages displaying active ß-lactamases was evaluated by plating on ampicillin and tetracyclin containing media as above. The results are summarized in table 1.

Round	Percentage of ß-lactamase	ppm	Elution yield	Enrichment factor
0	$6*10^{-4}$	6	-	-
1	$2,56*10^{-2}$	256	$5,5*10^{-2}$	43
2	0,21	2 160	$7,5*10^{-2}$	8,5
3	1,95	19 500	$2,9*10^{-1}$	9

Table 1.

The selection procedure allowed to enrich the library in phages endowed with ß-lactamase activity. After 3 rounds, the fraction of active phages grew from 0,0006 % to approximately 2%. One further round was applied to the mixture, but a maximum of 2% ß-lactamases-phages was recovered; this is because the active enzymes hydrolyse all the benzyl-penicillin used for counter-selection before labelling. Consequently, we decided to

perform a fourth round on a mixture containing 50 times less phages (10^{11}) that were first incubated for 1 h with $5*10^{-4}$ M benzylpenicillin. The percentage of phages displaying a ß-lactamase activity was then increased from 2% to 24,5% (table 2) :

Round	Percentage of ß-lactamase	ppm	Elution yield	Enrichment factor
4	24,5	245 000	$5,9*10^{-2}$	12,5

Table 2

Sequencing a panel of randomly picked active phages led to the systematic observation of a Glu at position 166; the 6 ppm of active phages in the starting library corresponded to a misincorporation of mutation not intended at position 166.

The catalytic properties of selected mutants were further investigated. TG1 cells were infected by fd-Bla$^+$ (wild type) and by phages issued from the third and the fourth rounds of selection. *In vivo* selection was performed by growing the cells on ampicillin containing plates (50 mg/l) either for 3 days at 23°C or overnight at 37°C. 30 to 100 times more clones were able to grow at 23°C than at 37°C [18]. Active phages were grown in liquid cultures at 23°C during 2 days and purified as described. Their catalytic activity was assayed by following the hydrolysis of benzylpenicillin at 232 nm [20] at phages concentrations ranging from $2*10^{-10}$ to 10^{-9} M. The activity of clones selected at 23°C was significantly lower than that of clones grown at 37°C as the *in vivo* selection pressure was higher at 37°C than at 23°C. The catalytic efficiencies of 10 mutants of round 3 and round 4 selected at 37°C were evaluated. Average results are given in Table 3. It is interesting to note that K_m is not significantly changed in the course of selection whereas catalytic efficiency seems to be concurrently increased.

	K_m (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m (μM^{-1} sec^{-1})
Wild type	120	2000	16,7
Mutants Round 3	95 ± 7	240 ± 12	2 ± 0.6
Mutants Round 4	60 ± 9	290 ± 13	5 ± 0.8

Table 3

The efficiency of the selection depends, on one hand, on the structure of relevant suicide inhibitor, and, on the other hand, on the selection procedure itself. Control of phage-enzymes labelling is done by adjustment of experimental conditions, such as labelling time, concentration of phages and label, and by introduction of a competition reaction with a non biotinylated inhibitor to counter-select the mutants that do not turnover. This counter selection is introduced because the activation of the mechanism based inhibitor requires only the opening of the ß-lactam ring in the acylation step; labelling without counter-selection would extract from the library all the enzymes that are acylated efficiently but deacylate slowly. The activation of a suicide inhibitor by the first step of an enzymatic multistep mechanism may be a rather general characteristic. The strategy presented here may be generalized as sensitive selection procedure for turnover selection.

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17. The following residues were allowed at the indicated positions : 163 : A,V,D,E; 164 : N,Q,S,T,K,R,W; 165: C,S,L,F,Y; 166: D,N,H,Y; 167: A,P; 168: A,G,D,E; 169: A,V,L,S; 170: G,S,N,D,E,K,R; 171: A,G,V,E.
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