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In Vitro Selection for Catalytic Activity with Ribosome Display

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Abstract: We report what is, to our knowledge, the first in vitro selection for catalytic activity based on catalytic turnover by using ribosome display, a method which does not involve living cells at any step. RTEM-β-lactamase was functionally displayed on ribosomes as a complex with its encoding mRNA. We designed and synthesized a mechanism-based inhibitor of β-lactamase, biotinylated ampicillin sulfone, appropriate for selection of catalytic activity of the ribosome-displayed β-lactamase. This derivative of ampicillin inactivated β-lactamase in a specific and irreversible manner. Under appropriate selection conditions, active RTEM-β-lactamase was enriched relative to an inactive point mutant over 100-fold per ribosome display selection cycle. Selection for binding, carried out with β-lactamase inhibitory protein (BLIP), gave results similar to selection with the suicide inhibitor, indicating that ribosome display is similarly efficient in catalytic activity and affinity selections. In the future, the capacity to select directly for enzymatic activity using an entirely in vitro process may allow for a significant increase in the explorable sequence space relative to existing strategies.

Naturally occurring enzymes catalyze a wide variety of chemical reactions and are increasingly used in pharmaceutical, industrial, and environmental applications as a result of their high reactivities and specificities. However, the direct improvement of biocatalysts remains challenging, and the yet more ambitious goal of developing enzymes with new catalytic functions still seems almost elusive. Although our knowledge of structure−function relationships of enzymes has significantly increased, rational protein design is still a difficult task, especially for improved catalysis. The recently developed strategy of directed evolution can be used as a complement to rational design. In directed evolution, a protein function of interest is evolved in the laboratory by mimicking Darwinian evolution in multiple successive rounds of diversification (library generation) with subsequent selection or screening (reviewed in refs 1−3).

Screening, which involves the analysis of single protein variants, can be automated for high-throughput protocols but remains laborious and time-consuming, therefore limiting the size of libraries which can be handled.[4] As opposed to screening, selection methods sample the entire library in a single experimental step. Such experiments require a direct coupling of the phenotype, which is to be selected for, and its encoding genetic information, the genotype.

For the selection of enzymatic activities, three general approaches can be distinguished: methods performed entirely in vivo, those working "partially" in vitro, and those performed completely in vitro.[1] In the in vivo approach, a genetic library encoding enzyme variants is transformed into cells where the variants are expressed and selection takes place. Because selection protocols are generally based on a growth advantage, e.g. complementation of an auxotrophy or resistance to a cytotoxic compound,[5−8] in vivo selection of catalysis is limited to those activities giving rise to a growth advantage. Moreover, microbial genomes have evolved to deal with environmental selection pressure. The expression host can therefore be surprisingly "creative" in escaping selection pressure, such as by higher expression of a poor catalyst or the use of alternative metabolic pathways, completely by-passing the enzymatic activity that is the actual target of selection.

Partially in vitro methods can offer an alternative to in vivo methods. In these methods, the library is also introduced into cells, resulting in display of the protein of interest, usually on the surface of phage,[9] bacteria, or yeast.[10] Selection then occurs

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(1) Griffiths, A. D.; Tawfik, D. S. *Curr. Opin. Biotechnol.* **2000**, *11*, 338−353.
(2) Olsen, M.; Iverson, B.; Georgiou, G. *Curr. Opin. Biotechnol.* **2000**, *11*, 331−337.
(3) Soumillion, P.; Fastrez, F. *Curr. Opin. Biotechnol.* **2001**, *12*, 387−394.
(4) Joo, H.; Lin, Z.; Arnold, F. H. *Nature* **1999**, *399*, 670−673.
(5) Orencia, M. C.; Yoon, J. S.; Ness, J. E.; Stemmer, W. P.; Stevens, R. C. *Nat. Struct. Biol.* **2001**, *8*, 238−242.
(6) Altamirano, M. M.; Blackburn, J. M.; Aguayo, C.; Fersht, A. R. *Nature* **2000**, *403*, 617−622. Retraction, *Nature* **2002**, *417*, 468.
(7) Crameri, A.; Raillard, S. A.; Bermudez, E.; Stemmer, W. P. *Nature* **1998**, *391*, 288−291.
(8) MacBeath, G.; Kast, P.; Hilvert, D. *Science* **1998**, *279*, 1958−1961.
(9) Dunn, I. S. *Curr. Opin. Biotechnol.* **1996**, *7*, 547−553.
(10) Mendelsohn, A. R.; Brent, R. *Science* **1999**, *284*, 1948−1950.

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in vitro, that is, outside the cell, allowing fine-tuning of the selection pressure and selection conditions.

Both entirely in vivo or partially in vitro techniques require a transformation step, limiting the applicable library size to cellular transformation efficiencies. Typically, transformation efficiencies of 10^7-10^8 cells/μg DNA for yeast and 10^9-10^{10} cells/μg DNA for *Escherichia coli* (*E. coli*)[11,12] are achievable. A considerable amount of work arises from the need of ligating and transforming such a library after each round of randomization. Furthermore, cytotoxic proteins cannot be displayed at all. Technologies working completely in vitro can overcome these limitations, because no living cells are involved at any step.

Two approaches that are carried out completely in vitro can be distinguished. In one, a compartmentalization of individual variants is achieved in water-in-oil emulsions.[13] Since the in situ detection of fluorescent products and direct optical sorting of such droplets, harboring the catalytic proteins, have not yet been reported, the physical link between genotype and phenotype is still required, and the emulsions have to be broken up before an affinity selection or sorting can be performed. The other approach makes use of the concomitant presence of mRNA and nascent protein at the ribosome during an in vitro translation for coupling of genotype and phenotype.[14] Here, the most prominent techniques are ribosome display;[15,16] mRNA display, also termed "in vitro virus"[17] or "mRNA−protein fusion";[18] and "ribosome-inactivation display system".[19] Below, we will describe the first application of ribosome display to the selection of catalytic activity in an effort to increase the library sizes accessible beyond those in previously published accounts with in vivo or partially in vitro methods.

To select catalysts in vitro, catalysis must be correlated or coupled to binding. Although reversible binding of enzymes to transition state analogues has been used frequently in the selection of novel catalysts,[20,21] a more direct selection for enzymatic turnover can be achieved by the use of mechanism-based (or "suicide") inhibitors.[22] These compounds are substrate analogues that, upon turnover, are converted into a reactive species that binds with the enzyme in a covalent manner, thus causing irreversible inhibition. Mechanism-based inhibitors can thus be used for labeling active enzyme molecules. The selection of β-lactamase displayed on phage with a biotinylated mechanism-based inhibitor has been reported.[23−26] In these reports,

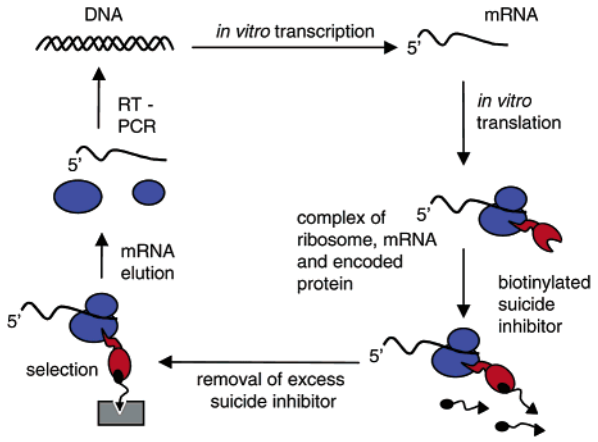


Figure 1. Principle of ribosome display selection for catalytic activity. DNA encoding the protein of interest is transcribed and translated in vitro. Because the mRNA carries no stop codon and translation is stopped with high Mg^{2+} concentrations, stable ternary complexes of mRNA (black), ribosome (blue) and tethered nascent protein (red) are formed. These can be used directly for selection with a biotinylated suicide inhibitor. Excess inhibitor is removed by gel filtration, and the labeled complexes are captured with avidin coated magnetic beads. After washing to remove any untrapped ribosomal complexes, the selected complexes are destroyed to elute the mRNA. Finally, reverse transcription (RT) and PCR are used to amplify the genetic information of the selected clones.

active enzyme was enriched relative to less active mutants and relative to penicillin binding protein. The elution of covalently trapped clones from the matrix to which they had been linked is generally achieved by cleaving within a linker region, either chemically between the suicide inhibitor and the affinity tag or enzymatically between the displayed protein and the phage.

Even though entirely in vitro display techniques have great potential for selection of catalytic activity, this has not previously been reported. We believe that ribosome display is particularly well suited to applications based on mechanism-based inhibition, because ribosome display offers an elegant solution for elution of the selected genetic information. Because genotype and phenotype are coupled in a noncovalent complex at the ribosome (Figure 1), dissociation of these complexes and therefore elution of the genetic information is easily achieved.

In the present study, we use ribosome display for selection of catalytic activity with a suicide inhibitor. We have chosen β-lactamase as a model enzyme, and we displayed it on the ribosome in complex with its encoding mRNA. We have devised a synthetic scheme for the preparation of a biotinylated mechanism-based inhibitor of β-lactamase. We then applied the inhibitor to the selective enrichment of active, displayed enzyme. We show that selection of enzymes is possible entirely in vitro, combining the advantages of both the in vitro technology of ribosome display and direct selection for catalytic turnover with a suicide substrate to select for protein catalysts.

Results and Discussion

In this study we assessed the potential of ribosome display for enzyme selection based on catalytic activity and on binding specificity. In such an in vitro selection technology, the theoretical library size would be limited only by the number of active ribosomes in the reaction. As a model system, we displayed RTEM β-lactamase in a ribosomal complex with its encoding mRNA. We describe a rapid synthesis of a biotinylated mechanism-based β-lactamase inhibitor for activity selection.

(11) Inoue, H.; Nojima, H.; Okayama, H. *Gene* **1990**, *96*, 23−28.
(12) Sidhu, S. S.; Lowman, H. B.; Cunningham, B. C.; Wells, J. A. *Methods Enzymol.* **2000**, *328*, 333−363.
(13) Tawfik, D. S.; Griffiths, A. D. *Nat. Biotechnol.* **1998**, *16*, 652−656.
(14) Amstutz, P.; Forrer, P.; Zahnd, C.; Plückthun, A. *Curr. Opin. Biotechnol.* **2001**, *12*, 400−405.
(15) Hanes, J.; Plückthun, A. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 4937−4942.
(16) He, M.; Taussig, M. J. *Nucleic Acids Res.* **1997**, *25*, 5132−5134.
(17) Nemoto, N.; Miyamoto-Sato, E.; Husimi, Y.; Yanagawa, H. *FEBS Lett.* **1997**, *414*, 405−408.
(18) Roberts, R. W.; Szostak, J. W. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 12297−12302.
(19) Zhou, J. M.; Fujita, S.; Warashina, M.; Baba, T.; Taira, K. *J. Am. Chem. Soc.* **2002**, *124*, 538−543.
(20) Schultz, P. G.; Lerner, R. A. *Science* **1995**, *269*, 1835−1842.
(21) Xu, J.; Deng, Q.; Chen, J.; Houk, K. N.; Bartek, J.; Hilvert, D.; Wilson, I. A. *Science* **1999**, *286*, 2345−2348.
(22) Hilvert, D. *Annu. Rev. Biochem.* **2000**, *69*, 751−793.
(23) Soumillion, P.; Jespers, L.; Bouchet, M.; Marchand-Brynaert, J.; Winter, G.; Fastrez, J. *J. Mol. Biol.* **1994**, *237*, 415−422.
(24) Vanwetswinkel, S.; Marchand-Brynaert, J.; Fastrez, J. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 239.
(25) Vanwetswinkel, S.; Avalle, B.; Fastrez, J. *J. Mol. Biol.* **2000**, *295*, 527−540.
(26) Avalle, B.; Vanwetswinkel, S.; Fastrez, J. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 479−484.

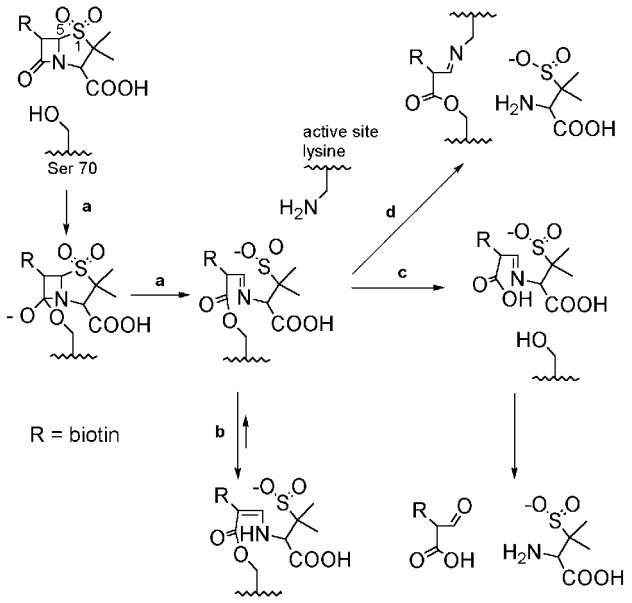


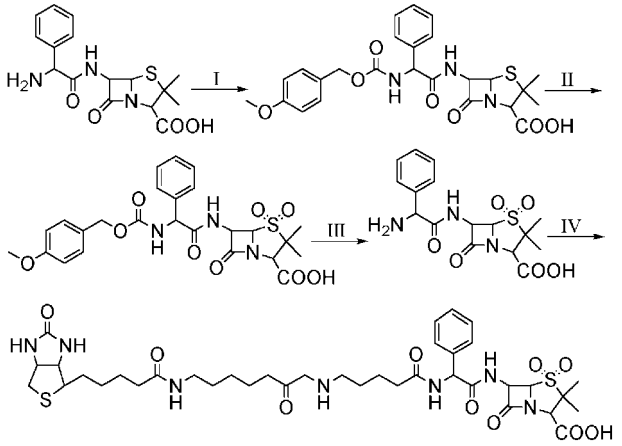
Figure 2. Proposed interaction of ampicillin sulfone with the active site serine β-lactamase.[27,28] The active site serine attacks the carbonyl group of the lactam ring, and before a water molecule can attack, the sulfone in position 1 acts as a leaving group from carbon 5 and evokes the formation of an acyl-enzyme intermediate (a). This acyl-enzyme intermediate is in an equilibrium with a more stable tautomer (b), resulting in transient inhibition. A normal deacylation, triggered by water, can also occur, and no inhibition is seen (c). Finally, transamination with a lysine in the active site can occur (d), which results in an irreversibly inactivated enzyme, bound covalently to the inhibitor and the biotin moiety.

We demonstrate that the enzyme folds on the ribosome to its correct three-dimensional structure and is active there. Furthermore, we demonstrate selection based on activity as well as selection for affinity with this system.

Rapid Synthesis of a Bifunctional Mechanism-Based Inhibitor: Biotinylated Ampicillin Sulfone. Penicillin analogues with an electron withdrawing group in position 1 (Figure 2) are known to lead to opening of the thiazolidine ring after nucleophilic attack of the β-lactam ring by the active-site serine of type-A β-lactamase, forming an acyl-enzyme intermediate.[27,28] The attack of a second active-site nucleophilic side chain on this reactive intermediate results in a covalently inhibited enzyme (Figure 2). Clavulanic acid and penicillanic acid sulfone are well-characterized mechanism-based inhibitors of type-A β-lactamase that follow this mechanism.[29,30] By tethering biotin to such an inhibitor, an affinity handle is generated that links biotin exclusively to active enzyme molecules as a consequence of enzymatic turnover. The resulting irreversible trapping allows for the possibility to select directly for catalytic activity.[23]

We developed a four-step synthesis for biotinylated ampicillin sulfone, a β-lactamase suicide inhibitor, with 64% overall yield. The synthesis of ampicillin sulfone involved only protection of the amino group of ampicillin, oxidation of the sulfur in the thiazolidine ring to the sulfone, followed by deprotection (Scheme 1). Since the compound is highly sensitive to hydrolysis at the lactam ring, the choice of the protecting group proved to be of utmost importance. Only protection with 4-methoxybenzyloxycarbonyloxyimino-2-phenyl-acetonitrile (MOZ-ON), but not with benzylchloroformate or paranitrobenzylchloroformate, yielded intact ampicillin sulfone upon deprotecting. In a fourth step, the biotin moiety was added to yield the biotinylated suicide inhibitor (Scheme 1). Our synthesis is simpler than that of a similar penicillanic acid sulfone, because we do not need a disulfide bond in the linker region between the penicillin and the biotin moiety.[31] This disulfide bridge was required in the context of phage display selection of β-lactamase to release the covalently trapped phages by cleaving with DTT. In contrast, ribosome display does not require elution of the covalently trapped and displayed protein, because the genetic information coding for the displayed protein can easily be released by chelating the Mg^{2+} ions so as to disrupt the ribosomal complex.

Scheme 1. Synthesis of Biotinylated Ampicillin Sulfone, a Mechanism-Based Inhibitor for Selection of Active Site Serine β-Lactamases.[a]



[a] (I) H_2O, CH_2Cl_2 (4:1); $NaHCO_3$; MOZ-ON. (II) H_2O, $KMnO_4$. (III) CH_2Cl_2, TFA (10:1). (IV) H_2O, EZ-Link Sulfo-NHS-LC-LC-Biotin (Pierce)

Mechanism-Based Enzyme Inhibition. The ability of ampicillin sulfone to irreversibly inhibit β-lactamase was tested by steady-state kinetic assays. The inhibition pattern of the RTEM β-lactamase from *E. coli*, obtained directly from in vitro translation, was compared to that of the β-lactamase from *Enterobacter cloacae*. Both enzymes behaved identically. The enzyme inhibition profile as a function of time showed a dramatic fall of activity within the first 20 min, followed by a slower activity decay (Figure 3). These biphasic inhibition kinetics are typical for mechanism-based inhibitors of β-lactamases, such as clavulanic acid, 6-(methoxymethylene)penicillanic acid and 6-β-((carboxy)methylsulfonamido)penicillanic acid sulfone.[27,32] The kinetics have been interpreted as being due to a first inhibition phase where the enzyme is transiently inhibited, while in a slower second phase, the compound inhibits the enzyme irreversibly (Figure 2). Since not every turnover results in enzyme deactivation (Figure 2), the inhibitor-to-enzyme ratio needed for complete inhibition is a measure for inhibitor potency. From various inhibition experiments, we determined that this ratio is 8×10^4 for ampicillin sulfone, which is about an order of magnitude higher than that for the previously characterized β-lactamase suicide inhibitor sulbactam.[28,33] This reduced inhibition efficiency is most likely due to the fact that

(27) Fisher, J.; Charnas, R. L.; Knowles, J. R. *Biochemistry* **1978**, *17*, 2180−2184.
(28) Fisher, J.; Charnas, R. L.; Bradley, S. M.; Knowles, J. R. *Biochemistry* **1981**, *20*, 2726−2731.
(29) Brenner, D. G.; Knowles, J. R. *Biochemistry* **1984**, *23*, 5833−5839.
(30) Charnas, R. L.; Knowles, J. R. *Biochemistry* **1981**, *20*, 3214−3219.
(31) Vanwetswinkel, S.; Fastrez, J.; Marchand-Brynaert, J. *J. Antibiot.* **1994**, *47*, 1041−1051.
(32) Brenner, D. G.; Knowles, J. R. *Biochemistry* **1981**, *20*, 3680−3687.
(33) Imtiaz, U.; Billings, E. M.; Knox, J. R.; Mobashery, S. *Biochemistry* **1994**, *33*, 5728−5738.

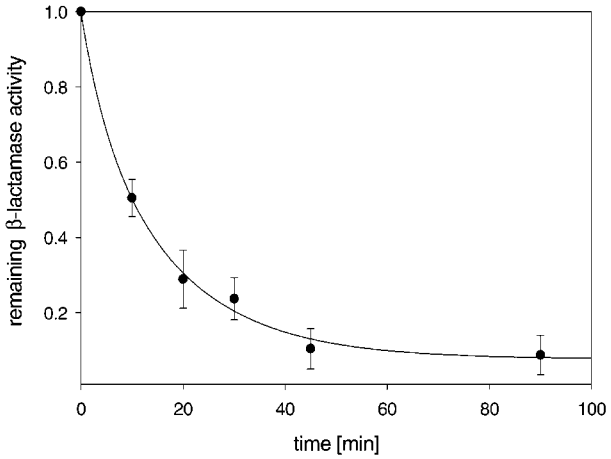


Figure 3. Time course of RTEM-β-lactamase inhibition by ampicillin sulfone. β-lactamase (10^{-9}−10^{-10} M, from in vitro translation) was incubated with ampicillin sulfone (1 mM) for different time periods at room temperature, and the remaining activity was measured in nitrocefin assays. The data, given as activity relative to that at time = 0, were fitted as double exponential decay. Similar inhibition curves have been reported for other β-lactamase suicide inhibitors.[27,31,34] The data for this plot were obtained from three independent experiments.

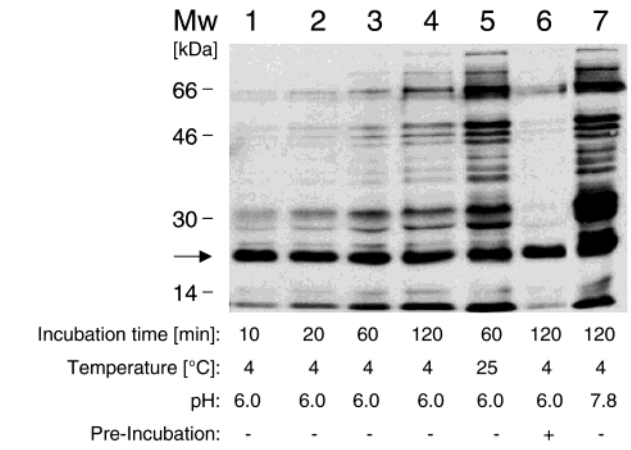


Figure 4. Analysis of nonspecific labeling of proteins by ampicillin sulfone. A protein mix, here *E. coli* S30 extract, was incubated with biotinylated ampicillin sulfone (0.1 mM). The proteins were separated from excess inhibitor by gel filtration, followed by SDS−PAGE and Western blotting for detection of biotin with avidin−alkaline phosphatase conjugate. The arrow indicates the signal obtained from pyruvate carboxylase, a biotinylated *E. coli* protein in the extract, used as internal standard. Lanes 1−5 and 7 show different labeling conditions and incubation times. In lane 6, the protein mix was preincubated with 1 mM ampicillin sulfone at room temperature for 1 h. The nonspecific labeling is most likely caused by the attack of nucleophilic surface residues on the lactam ring and has been reported also for ampicillin.[35]

ampicillin sulfone is derived from ampicillin, a very good substrate of β-lactamase that is rapidly hydrolyzed.[28] The covalent nature of the inhibition was confirmed by dialysis of the inhibited enzyme. Even upon extensive dialysis, no recovery of activity could be detected, whereas the noninhibited enzyme remained fully active (data not shown). The biotinylated form of the inhibitor gave results comparable to ampicillin sulfone itself in all assays (data not shown). These results confirm that the biotinylated form of ampicillin sulfone acts as a bona fide mechanism-based inhibitor of β-lactamase and could be used for selection of β-lactamase activity.

Prevention of Nonspecific Protein Labeling by Ampicillin Sulfone. Besides its reactivity, we also investigated the specificity of the suicide inhibitor. Although hydrolysis of the lactam ring, leading to formation of the reactive species, will be greatly accelerated at the active site of the enzyme, the labile nature of the lactam ring may allow for labeling outside of the active site. In addition, ampicillin is known to react nonspecifically with ϵ-amino groups of proteins in a covalent manner, a cause for ampicillin allergies.[35] Nonspecific labeling could lead to significant background in selection rounds by the suicide inhibitor reacting with inactive enzyme displayed on the ribosome or with ribosomal proteins. We thus investigated possible nonspecific binding in order to minimize it. Aliquots of *E. coli* S30 extract were incubated with biotinylated ampicillin sulfone under various conditions and for different incubation times. The proteins were then separated from excess suicide inhibitor by gel filtration. The resulting samples were analyzed by Western blotting. The results showed significant labeling of proteins other than β-lactamase (Figure 4). Nonspecific labeling also occurred when incubating the biotinylated suicide inhibitor with purified control proteins, such as citrate synthase, GFP (green fluorescent protein), anti-GCN4-single-chain Fv, and M13 helper phage (data not shown). The signal intensity of nonspecific labeling increased with time (lanes 1−4), temperature (lane 5), and higher pH (lane 7) and could be competitively inhibited by preincubation with ampicillin sulfone (lane 6) (Figure 4). These observations are in agreement with the published results on β-lactamase selections with phage display using similar mechanism-based inhibitors.[23−26] In all the phage display experiments, the labeling time was short (<30 min), the pH was kept below 6, and there was at least 1% BSA present, presumably to reduce nonspecific labeling. Under those conditions, the authors reported the selection of highly active β-lactamase over less active mutants or penicillin binding protein, which binds but does not hydrolyze the β-lactam ring.

The broadly based nonspecific labeling of proteins that are not linked to their genotype, as illustrated above, is not likely to be detrimental to the selection of displayed enzyme. However, it suggests that nonspecific labeling of inactive displayed enzyme and of ribosomal proteins may also occur under certain conditions. To favor specific labeling in our in vitro system, the selection for enzymatic activity of β-lactamase was carried out at pH 6.0 and at 4 °C for short times (10 min to 1 h) (10 min in Figure 4, lane 1). These conditions are also compatible with those required for ribosome display.

Ribosome-Displayed β-Lactamase is Active. In ribosome display, selection is performed with a ternary complex of mRNA, ribosome and displayed protein (Figure 1). For this strategy to be successful, the protein must fold to its native three-dimensional structure before the polypeptide is released from the ribosome. Furthermore, the ribosome-bound protein must be active. To allow the displayed protein of interest to exit the ribosomal peptide channel and fold into its active conformation, the encoding gene was fused to a 171-amino acid-long C-terminal fusion partner TolA of *E. coli* serving as a tether. To demonstrate functionality of β-lactamase in the ribosomal complex, the complexes were separated by gel filtration from enzyme released during translation, under conditions securing complex stability (see Experimental Procedures). Usually such protein-ribosome complexes are purified by sucrose gradient

(34) Brenner, D. G.; Knowles, J. R. *Biochemistry* **1984**, *23*, 5839−5846.
(35) Schneider, C. H.; De Weck, A. L. *Nature* **1965**, *208*, 57−59.

Table 1. Activity of Ribosome-Displayed β-Lactamase

	sum of free and ribosome-displayed β-lactamase[a]	ribosome-bound β-lactamase[b]	ribosome-displayed β-lactamase after release[c]	background activity in complex fraction[d]
rel enzyme activity[e]	100 ± 4.8	50 ± 5.1	54 ± 7.0	17 ± 5.8

[a] Total activity measured after in vitro translation. [b] After gel filtration (activity of ribosomal complex fraction). [c] Complexes destroyed by RNAse A treatment after gel filtration (activity of ribosomal complex fraction). [d] Complexes destroyed by RNAse A treatment prior to gel filtration (activity of ribosomal complex fraction). [e] Data from three independent experiment measured in triplicates.

centrifugation.[36−38] The use of small gel filtration columns is a rapid and gentle alternative and allows the parallel processing of a large number of samples. Thus, we were able to quantify the amount of active β-lactamase in ribosomal complexes (Table 1). An aliquot of this fraction was treated with RNase A, destroying the ribosomal complex, thus releasing the enzyme. No significant increase in activity was measured, indicating that ribosome-bound β-lactamase is fully active, and no additional active enzyme molecules are obtained by releasing them from the ribosome (Table 1). When the RNase A treatment was carried out prior to the gel filtration procedure, however, some background activity was detected in the complex fraction (Table 1), either from insufficient complex destruction or incomplete separation by gel filtration. These results confirm that the tethered β-lactamase can fold to its correct three-dimensional structure and can catalyze substrate turnover while still bound to the ribosome. Comparing the total activity after in vitro translation of the ribosome display construct prior to gel filtration to the amount of ribosome-displayed activity, we estimated the percentage of β-lactamase in ribosomal complexes to be ~50% of total β-lactamase produced. We presume that the other half is released from the ribosome by hydrolysis of RNA or proteolysis of the tether. The possibility that a fraction of the produced protein is bound to the ribosome in an inactive state and does not refold upon complex destruction remains, but that seems unlikely, because β-lactamase is known to fold efficiently.[39] Although co-translational folding and activity on the ribosome have been demonstrated for firefly luciferase in different experiments,[36−38] we show here that β-lactamase also folds and acquires activity on the ribosome, thereby fulfilling the major requirements for ribosome display selections.

Affinity Selection and Selection for Activity Are Equally Efficient. We extended our analysis of β-lactamase functionality in the ribosome display format by performing selection rounds based on affinity and catalytic activity. The β-lactamase inhibitory protein (BLIP) binds near the active site of β-lactamase with high affinity ($K_d = 0.6$ nM)[40] and had been used in affinity selection with phage display.[41,42] In contrast to His-tagged BLIP, gpHDBLIP, a C-terminal fusion of BLIP to the His-tagged protein D (gpHD),[43] could be expressed at high levels in inclusion bodies and could be refolded efficiently. The fusion partner, gpHD, not only improved the expression of BLIP very significantly but also provides a His tag, which we used for purification of the fusion protein and its immobilization for affinity selection. We performed selection rounds with active ribosome-displayed β-lactamase on either immobilized gpHD-BLIP or the immobilized biotinylated suicide inhibitor. The experiments were carried out as described[44] with some modifications to adapt the system for activity selection, notably lower pH (pH 6.0) and the presence of 1% BSA, for reasons discussed above. After in vitro translation and incubation on ice, aliquots of ribosomal complexes displaying β-lactamase were incubated for 1 h with gpHDBLIP or biotinylated ampicillin sulfone. Excess ampicillin sulfone or unbound gpHDBLIP was removed by gel filtration, and the complexes selected with the biotinylated suicide inhibitor were captured with avidin-agarose, whereas the ones binding to gpHDBLIP were immobilized via a tetra-His antibody which was captured with protein G-PLUS agarose. Extensive washing removed unbound complexes. The mRNA was eluted, and after RT and PCR, the yields were quantified according to band intensities on an agarose gel (Figure 5). We observed identical results with affinity- and activity-based selections, indicating that the ribosome-displayed β-lactamase is active in terms of both binding and enzymatic activity and that selection for activity and affinity in this system are equally efficient.

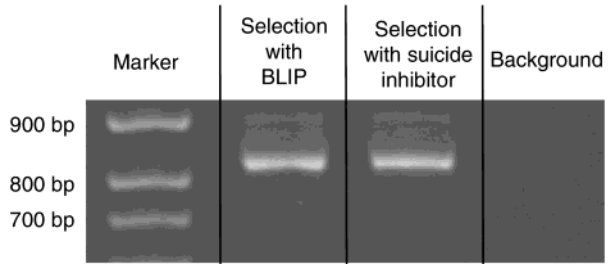


Figure 5. β-Lactamase selection for activity and affinity. The DNA yield after one round of ribosome display by affinity with gpHDBLIP (BLIP), by biotinylated ampicillin sulfone (suicide inhibitor) or with beads alone (background) were compared. Only active β-lactamase was used, resulting in PCR amplification of a band at 834 bp. Lane M indicates DNA molecular weight marker. Background binding to the beads is very weak, and the efficiency of selection for affinity and activity are comparable.

Selection of Active β-Lactamase over an Inactive Mutant Completely In Vitro. To assess the efficiency and specificity of selection for activity with ribosome display, the enrichment of active β-lactamase over an inactive point mutant was tested. The serine residue at position 70 of the active enzyme, whose hydroxyl group carries out the nucleophilic attack of the lactam ring (Figure 2), was replaced by alanine, yielding an inactive mutant enzyme with otherwise unimpaired structure.[45−48] Ri-

(36) Kolb, V. A.; Makeyev, E. V.; Spirin, A. S. *J. Biol. Chem.* **2000**, *275*, 16597−16601.
(37) Makeyev, E. V.; Kolb, V. A.; Spirin, A. S. *FEBS Lett.* **1996**, *378*, 166−170.
(38) Frydman, J.; Erdjument-Bromage, H.; Tempst, P.; Hartl, F. U. *Nat. Struct. Biol.* **1999**, *6*, 697−705.
(39) Vanhove, M.; Guillaume, G.; Ledent, P.; Richards, J. H.; Pain, R. H.; Frere, J. M. *Biochem. J.* **1997**, *321*, 413−417.
(40) Albeck, S.; Schreiber, G. *Biochemistry* **1999**, *38*, 11−21.
(41) Huang, W.; Petrosino, J.; Palzkill, T. *Antimicrob. Agents Chemother.* **1998**, *42*, 2893−2897.
(42) Huang, W.; Zhang, Z.; Palzkill, T. *J. Biol. Chem.* **2000**, *275*, 14964−14968.
(43) Forrer, P.; Jaussi, R. *Gene* **1998**, *224*, 45−52.
(44) Hanes, J.; Jermutus, L.; Plückthun, A. *Methods Enzymol.* **2000**, *328*, 404−430.
(45) Sigal, I. S.; DeGrado, W. F.; Thomas, B. J.; Petteway, S. R., Jr. *J. Biol. Chem.* **1984**, *259*, 5327−5332.
(46) Dalbadie-McFarland, G.; Neitzel, J. J.; Richards, J. H. *Biochemistry* **1986**, *25*, 332−338.

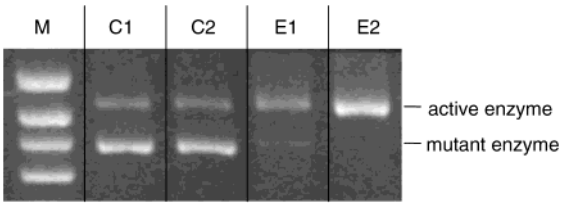


Figure 6. Restriction analysis for detection of enrichment of active β-lactamase with ribosome display. The results of two independent experiments are shown, where E1 represents a usual result and E2, the most successful result obtained. In each experiment, an *Msc*I digest of the DNA pool was carried out after one round of enrichment for enzymatic activity of β-lactamase with ribosome display**.** The round was performed with a ratio of inactive mutant to active β-lactamase of 10:1. After RT, the β-lactamase gene was amplified (834 bp) by PCR and digested with *Msc*I, resulting in cleavage of only the DNA encoding the inactive mutant (662 bp + 172 bp). Lane M indicates DNA molecular weight marker. Lane C1 shows the input mRNA directly reverse-transcribed and amplified by PCR without undergoing a display cycle. C2 shows the background of a standard enrichment round without biotinylated ampicillin sulfone present. E1 and E2 represent two independent enrichment rounds performed with biotinylated ampicillin sulfone. The enrichment factors were estimated to be 110 for E1 and 1400 for E2.

bosome display was performed with RNA encoding active and mutant β-lactamase mixed in a ratio of 1:10. After translation, biotinylated ampicillin sulfone was added, and after 30 min, excess suicide inhibitor was removed by gel filtration. The biotin-labeled complexes were captured with streptavidin magnetic beads, and the mRNA was eluted and amplified by RT and PCR. Five completely independent selection experiments were performed. Enrichment of the active enzyme was quantified by two independent methods: first, an *Msc*I restriction site had been introduced along with the S70A mutation, allowing restriction fragment analysis to distinguish the PCR products encoding active and inactive enzyme. Figure 6 illustrates a representative enrichment (lane E1, factor of 110) as well as the best enrichment obtained (lane E2, factor of 1400). To independently determine the magnitude of the enrichment factor, gene pools after one round of ribosome display were sequenced for the 2 experiments illustrated in Figure 6, and the signal intensities of the respective bases were analyzed, giving enrichment factors of 110 and 250 (data not shown). The fact that the enrichment factor varied within 1 order of magnitude in different selection experiments is most likely due to the small amount of inhibitor used, giving a low yield difficult to quantify, and the inhibitor concentration was not increased to prevent unspecific labeling.

In summary, all experiments clearly showed enrichment of active enzyme over inactive mutant. We concluded that after only one round of mechanism-based selection with ribosome display, an enrichment >100-fold was achieved. To demonstrate that the enrichment was, indeed, due to specific labeling of active β-lactamases, several control experiments were performed. To ascertain that enrichment was not an artifact of RT and PCR, input mRNA was directly applied to this procedure, showing no enrichment of the active species (Figure 6, lane C1). To rule out mRNA stability during translation as enrichment source, a second control was performed. A normal ribosome display round was performed using the same ratio of input mRNA but in the absence of biotinylated ampicillin sulfone. Here, no enrichment of active enzyme was detected (Figure 6, lane C2). The possibility of different translation efficiencies of mutant and active construct was ruled out by performing radioactive translations and comparing protein yields after SDS−PAGE and autoradiography. The band intensities were identical for both constructs (data not shown). It follows that active β-lactamase is efficiently enriched with ribosome display in vitro due to its catalytic activity.

Conclusions

In conclusion, we have shown for the first time that ribosome display may be used for selection of enzymes based on their catalytic activity. It follows that this strategy is ideally suited to performing directed evolution of enzymes. This novel potential of ribosome display was demonstrated by selecting β-lactamase based on its enzymatic activity. We show that the enzyme β-lactamase folds correctly on the ribosome and is active in the ternary complex, thus fulfilling the major requirements for ribosome display. To select for enzymatic activity of β-lactamase with ribosome display, we synthesized ampicillin sulfone, a mechanism-based inhibitor of β-lactamase, which can be derivatized with biotin, yielding a bifunctional activity label. This label was applied to ribosome display experiments, in which active β-lactamase could be enriched over an inactive mutant >100-fold/selection round. The efficiency of selection for activity was comparable to that of affinity selection, which was carried out with immobilized BLIP.

Taken together, we demonstrate that ribosome display, a technique that combines the advantages of very rapid selection rounds with the use of large libraries, known to be capable of evolving high affinity binders, may also be applied to the selection of catalytic proteins. Therefore, we believe this method will have great potential for directed evolution of enzymes and the selection of new catalytic proteins.

Experimental Section

Synthesis of Biotinylated Ampicillin Sulfone. Ampicillin was obtained from Roche Diagnostics, and 4-methoxybenzyloxycarbonyloxyimino-2-phenyl-acetonitrile (MOZ-ON) and other chemicals were from Fluka. IR spectra were measured on a Perkin-Elmer 297 spectrometer; NMR spectra, on a Bruker ARX-300 (^{1}H) and a Bruker ARX-75 (^{13}C) spectrometer; and mass spectra, on a Perkin-Elmer Sciex API III$^+$ ESI-MS.

Ampicillin (1.5 g, 4.02 mmol) was dissolved in 4 mL of H_2O. One equiv of MOZ-ON (1.25 g, 4.02 mmol) in 4 mL dioxane was added.[49] The pH of the reaction mixture was adjusted to 7.8. After stirring for 6 h, the mixture was treated with 8 mL of H_2O (pH 7.8) and extracted with EtOAc (3×, 100 mL). The pH was adjusted to 3.0 with 10% H_3PO_4, and the aqueous phase was extracted with EtOAc (5×, 100 mL). The combined EtOAc extractions were dried with $NaSO_4$ and evaporated in vacuo. MOZ-ampicillin (1.58 g, 3.08 mmol, 76.5% yield) was collected as an egg white powder: IR ($CHCl_3$ [cm^{-1}]) 3405, 2960, 1785, 1725, 1695, 1612, 1515, 1495; ^{1}H NMR (300 MHz, DMSO): δ 1.42, 1.55, 3.74 (3× 3H, 3s), 4.23 (1H, s), 4.98−4.99 (2H, m), 5.38−5.41 (1H, m), 5.50−5.54 (2H, m), 6.91 (2H, d like m, J = 6.9), 7.24−7.35 (5H, m), 7.44−7.53 (2H, m), 7.88 (1H, br d, J = 7.9), 8.99 (1H, br d, J = 7.7); ^{13}C NMR (DMSO): δ 26.4, 30.2, 54.9 (3q), 57.4, 58.1 (2d), 63.5 (s), 65.4 (t), 67.1, 70.2 (2d), 113.6 (2C, d), 125.4 (d), 127.1, 128.0 (2× 2C, 2d), 128.6 (1C, s), 129.5 (d), 137.7, 155.6, 158.9, 168.9, 170.4, 173.2 (s); MS: 514.2 *m*/*z*.

(47) Jacob, F.; Joris, B.; Frere, J. M. *Biochem. J.* **1991**, *277*, 647−652.
(48) Mazzella, L. J.; Pazhanisamy, S.; Pratt, R. F. *Biochem. J.* **1991**, *274*, 855−859.
(49) Chen, S. T.; Wang, K. T. *Synthesis* **1989**, 36−37.

MOZ-ampicillin (513 mg, 1.00 mmol) was dissolved in 3.6 mL of cold H_2O, and the pH was adjusted to 7.0. $KMnO_4$ (190 mg, 1.2 mmol) was dissolved in 5 mL H_2O and treated with 65 μL 10% H_3PO_4. The $KMnO_4$ solution was added dropwise to the first solution on ice, while maintaining the pH between 6.8 and 7.3 with 10% H_3PO_4 and 10% NaOH. After 40 min, the reaction mixture was brought to pH 8.0 and filtered over Celite, and the filtrate was acidified to pH 3.0 with 10% H_3PO_4.[50] The product was extracted with EtOAc (5×, 100 mL, dried with $NaSO_4$, and concentrated in vacuo to afford 495 mg (91% yield) of MOZ-ampicillin sulfone as an egg white powder: IR ($CHCl_3$ [cm^{-1}]) 3410, 2960, 1810, 1730, 1700, 1612, 1515, 1495, 1328, 1118; ^{1}H NMR (DMSO): δ 1.33, 1.46, 3.74 (3 × 3H, 3s), 4.36 (1H, s), 4.97 (2H, s), 5.31 (1H, d, J = 4.5), 5.51 (1H, d, J = 8.3), 5.90 (1H, dd, J = 4.4, 8.8), 6.90 (2H, d, J = 8.6), 7.28−7.31 (5H, m), 7.35−7.50 (2H, m), 7.97 (1H, br d, J = 8.5), 8.65 (1H, br d, J = 8.8); ^{13}C NMR (DMSO): δ 17.4, 19.8, 55.1 (3q), 56.8, 59.1, 63.7 (3d), 64.6 (s), 65.4 (d), 67.3 (t), 113.8, 127.0 (2× 2C, 2d), 128.4 (s), 128.7 (d), 129.1, 129.9 (2× 2C, 2d), 136.0, 156.0, 159.5, 168.9, 170.6, 173.6 (6s); MS: 546.2 *m*/*z*.

A solution of MOZ-ampicillin sulfone (220 mg, 0.40 mmol) in 2 mL of CH_2Cl_2 and 0.2 mL of TFA was stirred at room temperature for 30 min.[49] The solvent was removed in vacuo. The product was triturated with ether that was removed as the supernatant after centrifugation. This procedure was repeated twice, and the product was dried in vacuo. Ampicillin sulfone (150 mg, 0.39 mmol, 97% yield) was isolated as a pale yellow powder: IR (KBr [cm^{-1}]): 3390, 2980, 1802, 1674, 1525, 1458, 1433, 1376, 1323, 1116; ^{1}H NMR (DMSO): δ 1.32, 1.42 (2× 3H, 2s); 4.33 (1 H, s); 5.31−5.29 (2H, m); 5.95 (1 H, dd, J = 8.4, 4.5,); 7.30−7.54 (5H, m); 9.12 (1H, d like m, J = 8.5); ^{13}C NMR (DMSO): δ 17.0, 19.1 (2q); 54.7, 55.6, 63.1 (3d); 63.7 (s), 64.6 (d), 127.4, 128.7 (2× 2C, 2d), 129.1 (d), 132.9 167.7, 168.0, 173.1 (4s), MS: 382.2 *m*/*z*.

EZ-LinkSulfo-NHS-LC-LC-Biotin (7.6 mg, 1.14 × 10^{-2} mmol, Pierce) was dissolved in 7 mL borate buffer pH 7.8. Ampicillin sulfone (7.1 mg, 2.3 × 10^{-2} mmol) was added. This solution was sonicated and stirred for 1 h at room temperature. Tris(hydroxymethyl)aminomethane base (2 mg, 1.7 × 10^{-2} mmol) was added, stirred for an additional 10 min, and lyophilized to afford 71.5 mg of a white powder (as a complex with borate) from which 30 mg was dissolved in MeOH and applied to a silica gel column (1 g, in a Pasteur pipet). The product was eluted with 10 mL of MeOH, which was removed in vacuo. The product was obtained quantitatively as a white powder from ampicillin sulfone. MS: 834.4 *m*/*z*.

Inhibition Studies. β-Lactamase (from *E. cloacae* (Fluka) or *E. coli* R-TEM β-lactamase from in vitro translation) in concentrations from 10^{-9} to 10^{-10} M (as determined by activity measurements,[51] and assuming that the specific activity of the in vitro translated β-lactamase is similar to that of the bacterially expressed enzyme) was incubated at room temperature in 50 mM potassium phosphate buffer (pH 6.0, 5% DMSO), with or without 1 mM ampicillin sulfone. DMSO is required to dissolve ampicillin sulfone. Aliquots of 500 μL were taken after 10, 20, 30, 45, and 90 min and were mixed with 500 μL of 0.4 mM nitrocefin (Becton Dickinson) in 50 mM potassium phosphate buffer (pH 6.0, 1% DMSO). Reaction kinetics were followed at OD_{486} during 2 min at room temperature with a Perkin-Elmer Lambda 20 spectrophotometer.[51] To show the covalent nature of the inhibition, a sample (500 μL) of inhibited and, as a control, not inhibited enzyme were dialyzed against 500 mL potassium phosphate buffer (50 mM, pH 7) in "Slide-A-Lycer 10K" devices (molecular weight cut-off, 10 kD, 0.5−3 mL sample volume) (Pierce) for 2 h before activity was measured.

Western Blot Analysis. *E. coli* S30-extract (as used for ribosome display [44]) was diluted 2-fold in 50 mM potassium phosphate buffer (pH 6.0, 1% DMSO) and incubated with 0.1 mM biotinylated ampicillin sulfone on ice. Aliquots were taken after various time points and excess inhibitor was removed by gel filtration (NAP-500 column, Pharmacia, according to the manufacturer's instructions). Alternatively, one aliquot was incubated at 25 °C for 2 h while another was kept on ice at a pH 8 for 2 h. For inhibition studies, one aliquot was preincubated with 1 mM ampicillin sulfone for 1 h at room temperature prior to incubation with biotinylated ampicillin sulfone (as described above). The proteins were separated by SDS−PAGE (12%) and blotted onto a membrane (Immunoblot, Millipore). The membrane was blocked with milk, and the presence of the biotinylated suicide inhibitor was detected with an avidin−alkaline phosphatase conjugate (Pierce).

DNA Constructs for the Model System. The β-lactamase gene encoding the double cysteine to alanine mutant was PCR amplified from the plasmid pAP5__2C__2A [51] with the primers SDA-BLA 5′-(AGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATA TATCCATGGACTACAAAACCAGCCAGAAACGCTGGTGAAAGT), which codes for the Shine Dalgarno box and the beginning of the β-lactamase gene, and BLArev 5′-(ACACAGGCCCCCGAGGCCCAATGCTTAATCAGTGA), annealing at the end of the β-lactamase gene and introducing a unique *Sfi*I restriction site, while removing the stop codon. To create an inactive β-lactamase to use as a negative control for activity, the active site serine was mutated to alanine (S70A) by PCR mutagenesis. A unique *Msc*I restriction site was added at the site of the mutation to allow for discrimination of the genes encoding active and inactive enzyme. In parallel, a 171 amino acid portion of the *tol*A gene (residues 131 to 302) was amplified by PCR from chromosomal *E. coli* DNA in order to serve as a C-terminal tether for the displayed β-lactamase. The primers used were tolAforSFI 5′-(TATATGGCCTCGGGGGCCGAATTCCAGAAGCAAGCTGAAG), introducing an *Sfi*I-site, and tolAmrev 5′-(CCGCACACCAGTAAGGTGTGCGGTTAGCTCACCGAAAATATCATC), which introduced a RNA-stabilizing 3′-loop. The inactive S70A mutant and the active β-lactamase were fused to the *tol*A spacer by *Sfi*I digestion−ligation. The resulting constructs were further amplified by PCR with the primers tolAmrev and T7B 5′-(ATACGAAATTAATACGACTCACTATAGGGAGACCACAACGG), which introduced an RNA stabilizing 5′-loop and the T7 promoter for in vitro transcription. Both constructs were verified by DNA sequencing.

Separation of Ternary Complexes from Free β-Lactamase by Gel Filtration. For ribosome display, the in vitro transcription and in vitro translation of the constructs were carried out essentially as previously described.[44] Following a 10-min translation in 110 μL, the reaction was stopped by 4-fold dilution in ice-cold wash buffer (WB; 50 mM potassium phosphate buffer, pH 6.0; 150 mM NaCl; 50 mM $MgCl_2$). A 250-μL aliquot was treated with 2.5 μL RNase A (Qiagen, 10 mg/mL in WB) for 180 min at 4 °C to release ribosome-bound β-lactamase, while another aliquot was treated with WB only. An aliquot (200 μL) of each solution was applied to a 1-mL gel filtration column (CL-4B, Pharmacia) that had been equilibrated with 10−20 mL of ice-cold WB. Fractions of 100 μL were collected, and the enzyme activity was assayed in a nitrocefin assay (200 μM nitrocefin in WB buffer, pH 6.0, 1% DMSO). In addition, an aliquot of diluted translation mix was treated with RNaseA for 1 h at room temperature without subsequent gel filtration. The β-lactamase activity of this sample was determined in order to quantify the total activity, as free enzyme, resulting from translation.

Enrichment for Catalytic Activity with Ribosome Display. RNA coding for active as well as inactive S70A mutant β-lactamase was mixed in a ratio of 1 to 10 (10 μg total of RNA in 10 μL water) and translated in 110 μL for 10 min.[44] Translation was stopped by 4-fold dilution into ice-cold WB with 1% BSA (bovine serum albumin, Sigma). Biotinylated ampicillin sulfone (stock solution, 0.01 M in DMSO) was added to a final concentration of 0.01 mM. A control sample contained the same final concentration of DMSO but no biotinylated ampicillin sulfone. After 30 min at 4 °C, the reaction was stopped by removal of the excess biotinylated ampicillin sulfone by

(50) Johnson, D. A.; Panetta, C. A.; Cooper, D. E. *J. Antibiot.* **1963**, *28*, 1927−1928.
(51) Laminet, A. A.; Plückthun, A. *EMBO J.* **1989**, *8*, 1469−1477.

gel filtration (250 μL ribosomal complexes, 1 mL CL-4B column, as described above). Avidin-coated agarose beads (100 μL) (Roche Diagnostics), equilibrated in ice-cold WB containing 6% biotin-depleted sterilized milk,[44] were added to the flow-through (500 μL) and gently shaken at 4 °C for 30 min to capture the labeled complexes. The beads were then washed five times with 500 μL of ice-cold WB. During this procedure, the beads were transferred to a new prechilled tube. To elute the mRNA, 250 μL EB20 buffer (50 mM Tris-HCl, pH 7.5, 20 mM EDTA) was added, and the solution was gently shaken for 10 min at 4 °C before centrifugation (2 min, 2000*g*). The supernatant (200 μL) was used for mRNA purification (High Pure RNA Isolation Kit, Roche Diagnostics). Reverse transcription (RT) and PCR was carried out as described,[44] except that the primers used were SD-BLA and BLArev.

Monitoring the Enrichment of Active β-Lactamase Relative to the Inactive S70A Mutant. The product from RT-PCR was purified with a spin column (QIAquick, Qiagen) according to the manufacturer's protocol. The DNA was subsequently analyzed by restriction analysis with *Msc*I or by sequencing. After digestion with *Msc*I, the ratio of active (uncleaved) to mutant (cleaved) β-lactamase was estimated from the relative intensity of bands on a 2.5% Metaphor agarose gel (FMC) using the program NIH image. DNA sequencing was carried out with 10 ng PCR product according to the manufacturer's protocol (SequiTherm EXCEL *II*, Epicenter Technologies). The enrichment factor was estimated by determining the relative signal intensities of the 2 nucleotides that were mutated in the inactive S70A enzyme, with NIH image.

***β*-Lactamase Inhibitory Protein (BLIP) Expression as pD Fusion.** We constructed a fusion protein of BLIP with protein D, carrying an N-terminal His_6 tag (gpHD). The BLIP (Swiss-Prot P35804) gene was amplified by PCR and cloned into the vector pAT39 [43] between the *Bam*HI and *Hind*III sites to yield gpHDBLIP, consisting of gpHD, a Gly-Ser linker (GSGGGSGGGG), and BLIP. The fusion protein was amplified from pAT39 by PCR and ligated into the vector pET40-b (Novagen), carrying a kanamycin resistance gene. Plasmids were verified by sequencing and subsequently used for expression in the *E. coli* strain BL21 (DE3) [pLysS] (Stratagene) in 1 L of LB medium containing 100 μg/mL kanamycin. Expression was induced with IPTG (1 mM) at $OD_{600} = 0.8$. The cells were grown for 3 h at 37 °C and harvested by centrifugation. The cell pellet was resuspended in PBS (10 mM sodium phosphate buffer, pH 7.4, 140 mM NaCl, 15 mM KCl), and the cells were lysed by sonication. The resulting crude extract was centrifuged (5 min, 6000 g, 4 °C), and the pelleted inclusion bodies were washed once with PBS and resuspended with a solution containing 6 M GdnHCl, 0.1 M Tris, pH 8.0, and 10 mM imidazole. The solubilized inclusion bodies were applied to a Ni−NTA column (15 mL) (Qiagen) and washed with GdnHCl buffer. The protein was refolded on the column by replacing the GdnHCl-buffer with refolding buffer (20 mM Tris pH 8, 500 mM NaCl, 20% glycerol, GSH (0.2 mM)/GSSG (1 mM)) in a linear gradient and eluted with 250 mM imidazole in refolding buffer.[52] The protein was dialyzed against PBS and concentrated to 2.5 mg/mL. One liter of *E. coli* culture yielded 60 mg of gpHDBLIP, which was frozen in liquid nitrogen and stored at −80 °C. The protein gave a single band in SDS−PAGE upon staining with Coomassie-Blue. The β-lactamase inhibitory activity of gpHDBLIP was quantified in β-lactamase inhibition experiments as described for ampicillin sulfone (data not shown).

Comparison of Affinity Selection and Selection for Catalytic Activity. Ribosome display was carried out as described above, but only with active β-lactamase. After stopping the translation, the stopped mix was incubated for 4 h on ice. A 200-μL aliquot was treated for 1 h on ice with biotinylated ampicillin sulfone (final concentration, 0.01 mM), while another aliquot was treated with gpHDBLIP (final concentration, 0.3 μM). The selection procedure for the aliquot treated with biotinylated ampicillin sulfone was as described above. A gel filtration step was performed, as described for the fraction with the suicide inhibitor. The ribosomal complexes binding to gpHDBLIP were captured via the His_6 tag by incubating the flow-through with 10 μL of Tetra-His antibody (Qiagen, 0.1 μg/μL, in WB) and 100 μL of protein G PLUS-Agarose (Santa Cruz) equilibrated in WB in the presence of 4% sterilized milk powder. The agarose was washed as described for the magnetic beads. Rescuing of the mRNA, RT, and PCR was carried as out described above.

Abbreviations: RT, reverse transcription; PCR, polymerase chain reaction; GSH and GSSG, the reduced and oxidized forms of glutathione, respectively; GdnHCl, guanidine hydrochloride; bp, base pairs; BLIP, β-lactamase inhibitory protein; *E. coli*, *Escherichia coli*; GFP, green fluorescent protein; gpHD, His-tagged gene product D from phage lambda; IPTG, isopropyl-β-D-thiogalactoside; PAGE polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; MOZ-ON, 4-methoxybenzyloxycarbonyloxyimino-2-phenyl-acetonitrile.

Note Added in Proof: After this paper was submitted, it was reported (Takahashi, F.; Ebihara, T.; Mie, M.; Yanagida, Y.; Endo, Y.; Kobatake, E.; Aizawa, M. *FEBS Lett.* **2002**, *514*, 106−110) that 4 random point mutants of dihydrofolate reductase, which had been obtained in a ribosome display selection for binding to the inhibitor methotrexate, were active. This suggests that enrichment of active enzyme variants may also be possible simply by binding to an inhibitor under some circumstances, not requiring the turnover-based selection reported here, even though inactive mutants that still bind to an inhibitor are certainly conceivable.

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(52) Holzinger, A.; Phillips, K. S.; Weaver, T. E. *BioTechniques* **1996**, 20, 804−806, 808.