

A method for directed evolution and functional cloning of enzymes

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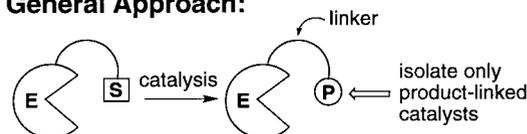
ABSTRACT A general scheme is described for the *in vitro* evolution of protein catalysts in a biologically amplifiable system. Substrate is covalently and site specifically attached by a flexible tether to the pIII coat protein of a filamentous phage that also displays the catalyst. Intramolecular conversion of substrate to product provides a basis for selecting active catalysts from a library of mutants, either by release from or attachment to a solid support. This methodology has been developed with the enzyme staphylococcal nuclease as a model. An analysis of factors influencing the selection efficiency is presented, and it is shown that phage displaying staphylococcal nuclease can be enriched 100-fold in a single step from a library-like ensemble of phage displaying non-catalytic proteins. Additionally, this approach should allow one to functionally clone natural enzymes, based on their ability to catalyze specific reactions (e.g., glycosyl transfer, sequence-specific proteolysis or phosphorylation, polymerization, etc.) rather than their sequence- or structural homology to known enzymes.

The increasing use of enzymes for medical, industrial, and environmental applications has generated considerable interest in the engineering of enzymes with novel properties. One approach that has been used to produce catalysts for a wide variety of chemical reactions involves the generation of catalytic antibodies by “chemical instruction” using, for example, haptens resembling the transition state for the reaction in question (1). More recently, efforts have focused on generating the combinatorial diversity of the immune system *in vitro* (2–4) and developing screens and selections based on catalytic function rather than binding affinity. For example, phage displaying antibodies with an active site cysteine capable of nucleophilic catalysis were selected based on a disulfide exchange reaction that crosslinked phage to a solid support (5). Mechanism-based inhibitors coupled to solid support also have been used to enrich antibodies and enzymes from phage-displayed libraries (6–8). Although protocols based on mechanism-based inhibitors are likely to be quite useful, they have the potential problem that selection depends both on catalytic efficiency and the efficiency with which the reactive product is trapped. Selection therefore may, in some cases, be based on efficient trapping rather than efficient catalysis of the reaction of interest. In addition, one requires the availability of mechanism-based inhibitors that produce highly reactive species that are efficiently quenched in the enzyme active site to avoid nonspecific intermolecular reactions by released trapping agent.

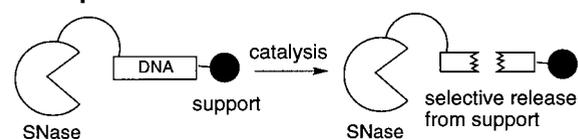
In nature, improved function of an enzyme can confer a selective growth advantage to its host organism. The ability to

directly link substrate turnover with a selective advantage *in vitro* would allow one to evolve enzymes with a broader range of substrates, reactions, and reaction conditions. Here we report the development of a simple method that makes possible the *in vitro* isolation of enzymes for almost any reaction, even in the absence of mechanistic information; all that is required is to specify substrate and product. The method involves covalent attachment of substrate to each potential catalyst in a library, in a configuration that allows intramolecular reaction between catalyst and substrate to occur. Conversion of substrate (S) to product (P) by active catalysts (E) provides the basis for the selection (Scheme 1). This latter step can involve attachment or cleavage of the enzyme from solid support, or alternatively, capture of the reaction product by a product-specific reagent or antibody. Here we describe the development of this methodology and show that it can be used to select an enzyme that carries out a DNA cleavage reaction from a library-like ensemble of phage-displayed proteins.

General Approach:



Example:



Scheme 1

MATERIALS AND METHODS

Synthesis of Compounds. Fluorenylmethoxycarbonyl-*S*-(2-nitro-4,5-dimethoxybenzyl)-*L*-cysteine was synthesized by a variation of the method of Erickson and Merrifield (9). Briefly, 605 mg of *L*-cysteine (5 mmol) was suspended in 100 ml of degassed ethanol/water (2:1), and 1.39 ml of triethylamine (10 mmol) and 1.39 g of 1-(bromomethyl)-2-nitro-4,5-dimethoxybenzene (5 mmol) were added. The mixture was stirred for 10 h at 23°C in the dark under nitrogen and filtered. The filter cake was washed with ethanol and recrystallized from ethanol/water to provide 0.95 g of *S*-(2-nitro-4,5-dimethoxybenzyl)-*L*-cysteine (3 mmol). The recrystallized product (0.8 g) was suspended in 20 ml of water; 0.53 ml of triethylamine (3.8 mmol) was added followed by a solution of 0.9 g of 9-fluorenylmethoxycarbonyl succinate (2.7 mmol) in 12 ml of

Abbreviations: SNase, staphylococcal nuclease; pTp, deoxythymidine-3',5'-diphosphate.

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acetonitrile, and the mixture was stirred for 10 h at 25°C under nitrogen. The product precipitated upon acidification to pH 2–3 with 1 M HCl and evaporation of the acetonitrile. The precipitate was collected on a frit and washed with water and ethylacetate to remove excess HCl and reagent. The resulting crude product (1.13 g) was extensively dried under vacuum and used directly in the synthesis of the base-linker peptide C(GGS)₄AQLKKKLOALKKKNAQLKWKLOALKKKLAQGGC (base sequence underlined, photoprotected cysteine in bold). Solid-phase peptide synthesis was accomplished on an Applied Biosystems 431A synthesizer using Fmoc/HOBt-DCC chemistry and user-devised extended cycles. Crude peptides (≥85% purity) were purified by reversed-phase liquid chromatography to >95%; peptide identity and purity were assessed by electrospray-ionization mass spectrometry (Hewlett-Packard 5989A; all masses were within 0.5 amu of calculated values). Compounds 1–3 were synthesized on an Applied Biosystems DNA synthesizer on a 1 μmol scale with a 3'-biotin group (BiotinTEG CPG, Glen Research, Sterling, VA) and a 5'-thiol (5'-Thiol-Modifier C6, Glen Research) and purified by reverse-phase HPLC after removal from the resin [Rainin Microsorb C18 column, flow 1 ml/min.; solvent A: 50 mM triethylammonium acetate (TEAA), pH 7; solvent B: acetonitrile, linear gradient from 5 to 50% solvent B over 40 min]; the trityl protecting group on the thiol was removed according to the protocol of Glen Research. The products were lyophilized and dissolved in water (1.0 mM final concentration). The conjugate of 1 with the base-linker peptide was prepared as follows: 2 mg (415 nmol) of base-linker peptide was reacted with a 20-fold molar excess of *N,N*-bis(3-maleimidopropionyl)-2-hydroxy-1,3-propanediamine (3.2 mg) in 1 ml of 50 mM sodium phosphate buffer, pH 5.5, for 10 h under nitrogen at 4°C. Compound 4 was purified from the reaction mixture by reverse-phase HPLC [Vydac RP-18 column, flow 2 ml/min; solvent A: 0.1% trifluoroacetic acid (TFA) in water; solvent B: 0.1% TFA in acetonitrile; linear gradient from 10 to 55% solvent B over 35 min], and the product fractions concentrated to approximately 0.3 ml (OD₂₈₀ = 6, compound 4 should not be concentrated to dryness). To 100 μl (138 nmol) of this solution was added 75 μl water, 75 μl of aqueous 1 M aqueous sodium phosphate, pH 7, 30 μl of aqueous 5 M NaCl, and 22 μl (22 nmol) of compound 1, and the reaction was incubated for 10 h under nitrogen at 23°C (to avoid precipitation the reagents should be added in this order). The product was purified by anion exchange chromatography (Mono Q HR 5/5 column (Pharmacia), flow 0.75 ml/min solvent A: 20 mM Tris·HCl, pH 7, solvent B: 20 mM Tris·HCl, pH 7, 2 M NaCl; linear gradient from 20 to 60% B in 7.5 min); on a 10% denaturing polyacrylamide gel the product ran as a single band. Fractions of OD₂₆₀ = 0.3–1 were used directly for the photo-deprotection step (see below). The conjugates of 2 and 3 with the base-linker-peptide were prepared as follows: approximately 200 nmol of either 2 or 3 and a 20-fold excess of bismaleimide were incubated in 1 ml of aqueous 50 mM phosphate buffer, pH 5.5, at 4°C for 15 h. After purification by reverse-phase HPLC and lyophilization, the identity of compounds 5 and 6 was verified by matrix-assisted laser desorption ionization (MALDI)-time of flight (ToF) mass spectrometry. Either 5 or 6 (150 nmol) then was incubated with 100 nmol of base-linker-peptide in 100 μl of 10 mM TEAA, pH 6.5, 100 mM NaCl for 15 h at 4°C. The products were purified by reverse-phase HPLC (Vydac RP-18 column, conditions as described above), lyophilized, and analyzed by MALDI-ToF mass spectrometry (10). The 2-nitro-4,5-dimethoxybenzyl protecting group on the C-terminal cysteine of the three conjugates was removed by photolysis to afford compounds 7–9 as follows: for compound 7, 100 μl of the purified fraction containing the protected conjugate (see above) was degassed thoroughly with argon for 15 min, and then exposed to a mercury lamp (450 W HPLC mercury lamp, Ace-Hanovia;

Pyrex filter, cutoff ≤300 nm) in a septum capped glass vial for 30 min (11). For compounds 8 and 9, 10 nmol of the conjugate was dissolved in 100 μl of 10 mM DTT, degassed, and photolyzed as described above. After 30 min of irradiation no remaining starting material could be detected by MALDI-ToF mass spectrometry. The reaction mixture was separated by HPLC (Vydac RP-18 column, conditions as described above) and the product fractions were lyophilized. The conjugates were stored frozen and used within a week after photo-deprotection to ensure efficient attachment to phage.

Construction of Acid Helper Phage. A *NarI* restriction site was introduced between the third and fourth codon of mature pIII protein of M13K07 helper phage (Promega) by Kunkel mutagenesis (12) with the primer K07-*NarI*-prim (5'-ACAACTTTCAACGGCGCCAGTTTCAGCGG-3') to give *NarI*-helper phage. DNA encoding the amino acids GAAOLEKELOALEKENAOLEWEOALEKELAOGGCPAGA (acid peptide sequence underlined, GGC motif in bold) with a *NarI* restriction site at both ends, was produced by PCR with the plasmid pCRII acid (Ellis L. Reinherz, Dana Farber Cancer Institute, Boston) with the primers *NarI*fw (5'-ACTACAAATTGGCGCCGCTCAGCTCGAAAAAGAGC-3') and *NarI*lbc (5'-AATTATAGGCGCCAGCCGGGCAACCGCCCTGAGCCAGTTCCTTTTCC-3'). The PCR product was digested with *NarI* and inserted into *NarI*-digested *NarI*-helper phage to afford acid helper phage.

Construction of Phagemids Encoding the Staphylococcal Nuclease (SNase)-pIII Fusion and 39-A11 Fab-pIII Fusions. To make the SNase-pIII fusion, PCR was performed on the plasmid pONF1 (13), carrying the gene encoding SNase, with primers 5'-CGCGAATTGGCCAGCCGGCCATGGCCGCAACTTCACTAAA-3' (*SfiI* restriction site underlined) and 5'-TGC AATTGGTGGCGCCGCTTGACCTGAATCAGCGTTG-3' (*NotI* restriction site underlined). The product was digested with *SfiI* and *NotI* and inserted into *SfiI*-*NotI*-digested pFAB-5c.His, a derivative of plasmid pFAB-5c (14), to give phagemid pII78–6. As a negative control the phagemid pComb³H.DA was used. This phagemid (P.G.S. and F. E. Romesberg, unpublished results) carries the 39-A11 Fab antibody (15) fused to the pIII protein. The expression of both the SNase and control protein is driven by the *lac* promoter.

Production of Phage Particles. Phage particles were produced with minor modifications according to Ørum *et al.* (14). Briefly, *Escherichia coli* XL1-blue was transformed with pII78–6 or pComb³H.DA, and shaken at 37°C in 2× yeast/tryptone broth and 100 μg/ml of ampicillin. At an OD₆₀₀ of 0.5, acid helper phage was added to a final concentration of 1.5 × 10⁸ cfu/ml, and incubated at 37°C for 20 min. The cells were pelleted and resuspended in 2× YT, 100 μM isopropyl β-D-thiogalactoside, 100 μg/ml of ampicillin, and 50 μg/ml of kanamycin, and shaken for 14 h at room temperature. Cells were pelleted and phage particles in the supernatant were polyethylene glycol-precipitated, followed by resuspension in TBS (25 mM Tris·HCl, pH 7.4/140 mM NaCl/2.5 mM KCl). Phage titrations were performed with *E. coli* XL1-blue by using standard procedures (16).

Covalent Attachment of Base-Linker-Substrate Conjugates to Phage. Approximately 10⁸ phage particles were incubated in 40 μl buffer A (25 mM Tris·HCl, pH 7.4/140 mM NaCl/2.5 mM KCl, 10 mM EDTA, 0.1% BSA), supplemented with 1 mM 2-mercaptoethylamine and 1 nmol of either base-linker-oligodeoxynucleotide (8), base-linker-deoxythymidine-3',5-diphosphate (pTp) (9), or base-linker-pTpTp (10), at 37°C for 60 min, then polyethylene glycol-precipitated twice and resuspended in buffer A.

Phage Immobilization and Release from Solid Support. Approximately 10⁸ phage particles, covalently attached to the base-linker-substrate conjugates, were incubated with 50 μl of magnetic streptavidin beads (Boehringer Mannheim, biotin binding capacity: 1.5 nmol/ml) in 1 ml of buffer A for 15 min

at 23°C; eight 1-min washes were performed in buffer A with 0.1% Tween 20, followed by two 1-min washes in buffer A. The number of phage immobilized on the beads was determined by suspending the beads in buffer A, and then either directly infecting *E. coli* XL1-blue with the bead suspension and titering or alternatively, infecting after treatment of the beads with DNase 1 (1 unit/μl DNase 1, 10 mM MgCl₂, 20 mM Tris·HCl, pH 8, 23°C for 15 min). Calcium-dependent release (cleavage) from solid support was assessed by suspending beads in buffer B (25 mM Tris·HCl, pH 7.4/140 mM NaCl/2.5 mM KCl, 10 mM CaCl₂, 0.1% BSA), incubating at 23°C for 5 min, and titering the supernatant. Calcium-independent release from the beads (leakage) was determined by resuspending the beads in buffer A, incubating for 5 min at 23°C, and titering the supernatant.

Enrichment of Active Enzymes from a Library-Like Ensemble. Phage particles displaying SNase or 39-A11 Fab were mixed in a 1:100 ratio, and the base-linker-oligodeoxynucleotide conjugate (**8**) was covalently attached. Phage then were immobilized on magnetic streptavidin beads, washed in buffer A, and incubated in buffer B as described above. *E. coli* XL1-blue were infected with the supernatant, and the cells were plated on a Luria-Bertani plate containing 100 μg/ml of ampicillin. Randomly picked colonies were identified as SNase- or control clones by PCR or restriction enzyme digestion.

RESULTS AND DISCUSSION

Selection Scheme. To test the above strategy for directed-enzyme evolution in a phage-display format, it was first necessary to develop a general method for selectively attaching a given substrate to or near a phage-displayed enzyme. Importantly, the substrate must be attached so that it can bind productively in the active site of the conjugated enzyme. Moreover, the substrate should be covalently linked to the phage to ensure that there is no crossover of reaction product between members of the library. One possible strategy involves selective chemical modification of the enzyme or a nearby phage coat protein (e.g., pIII protein) with substrate by a disulfide exchange reaction. For example, a cysteine residue introduced near the active site of SNase through site-directed mutagenesis has been used to selectively introduce unique chemical functionality by a disulfide exchange reaction (17). To apply this method to proteins expressed on filamentous phage, the three single cysteines of the pVI, pVII, and pIX coat proteins first were mutagenized to alanine. The eight buried cysteine residues in the pIII protein were left unchanged, as they likely form structurally important disulfide bridges (18, 19). Unfortunately, repeated attempts to selectively modify unique cysteine residues introduced near the active site of several enzymes displayed on phage, by either disulfide exchange, maleimide addition, or alkylation reactions, resulted in

significant nonspecific labeling of phage coat proteins. No conditions or reagents were found that made possible selective labeling of the pIII fusion protein containing the unique surface cysteine residue. It is likely that the thousands of proteins constituting the phage coat make the specificity requirement for a chemical reaction too great; also, the probability of cysteine misincorporation caused by the intrinsic error rate in protein biosynthesis becomes significant for such a large ensemble of proteins. Alternatively, the cysteine residues in the pIII protein may be accessible to crosslinking reagents.

To circumvent these problems, a two-step process was developed in which chemical crosslinking is preceded by the selective formation of a noncovalent complex at the site of modification (Figs. 1 and 2). The complex is a heterodimeric coiled-coil consisting of a synthetic basic peptide **B** C(GG-S)₄AQLKKKLQALKKKNAQLKWKLQALKKKLQAGGC, to which substrate is covalently coupled before heterodimerization, and an acidic peptide **A**, GAAQLEKELQALEKEN-AQLEWELQALEKELAQGGCPAGA that is expressed as an N-terminal fusion to the pIII coat protein of filamentous phage. The acid and base peptides (underlined) were chosen as dimerization domains because of their small size (30 amino acids) and high tendency to form stable, parallel heterodimeric coiled-coil structures; the acid-acid and base-base homodimers form 10⁵-fold less efficiently than the heterodimer (20–23). Heterodimerization of the synthetic (**B**) and phage-encoded (**A**) peptides should bring the substrate into close proximity of the displayed enzyme and lead to spontaneous disulfide bond formation between cysteines on each of the peptides (Fig. 2). The tripeptide Gly-Gly-Cys was added to the C termini of the acid and base peptides to facilitate formation of a disulfide bridge between the two helices (20–23). The substrate is covalently linked to the basic peptide **B** through a flexible linker to facilitate productive binding of substrate to enzyme (Fig. 1). The acidic peptide **A** is fused to the pIII protein of the phage rather than to the displayed enzyme itself for the following reasons: (i) insertion of the acid peptide sequence into an enzyme might interfere with enzyme function; (ii) the flexible linker of the base-linker-peptide, as well as hinges in the pIII protein and a peptide linker inserted between pIII and the displayed enzyme, should allow many possible orientations of the substrate relative to the enzyme active site; and (iii) it should be possible to use a single helper phage bearing the acid peptide extension to display many enzyme-substrate pairs, rather than having to engineer into each enzyme a functional conjugation site.

Generation of the Acid Helper Phage and Base-Linker-Substrate Conjugate. To attach the base-linker-substrate conjugate to phage we introduced the acidic peptide **A** at the N terminus of pIII protein in the M13K07 helper phage. The enzyme library is fused to the N terminus of the pIII coat protein; this construct is carried in the phagemid. Upon

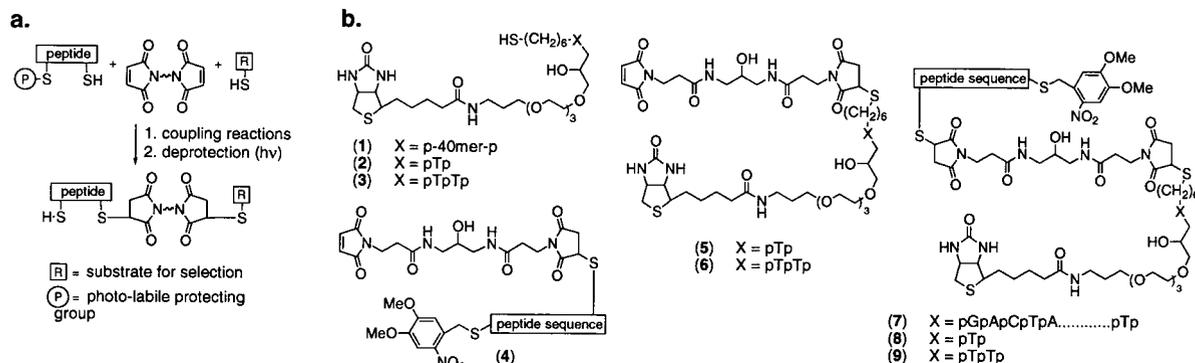


FIG. 1. (a) Structures and (b) synthesis of base-linker-substrate conjugates.

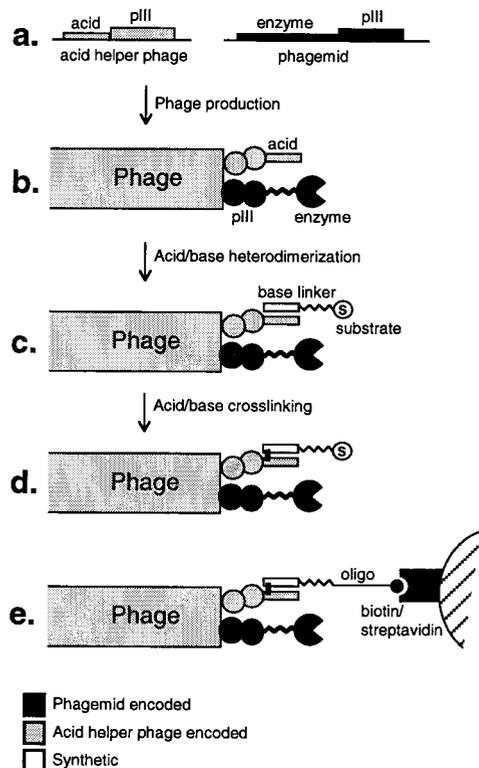


FIG. 2. Covalent attachment of substrate to the pIII protein on phage. (a) DNA encoding the acid peptide sequence and a C-terminal cysteine was fused to the N-terminal end of gene *gIII*, to form the acid helper phage. A phagemid encodes the protein library in fusion with the pIII protein. (b) Phage production leads to phage particles displaying the phagemid encoded protein; the pIII proteins have acid peptide extensions. (c) Coiled-coil formation of the acid and base peptides noncovalently attaches the substrate to the phage pIII protein. (d) Removal of the reducing agent leads to crosslinking of acid and base peptides through their C-terminal cysteines. (e) In the present study phages displaying SNase are attached to streptavidin beads through a 5'-biotinylated, single-stranded oligodeoxynucleotide. Phages displaying active enzyme are released by cleavage of the oligodeoxynucleotide in an intramolecular reaction.

superinfection by helper phage, phage particles are produced containing the phagemid DNA but whose coat consists (with one exception) of proteins encoded by the helper phage genome. The one exception is the pIII protein, present in 4–5 copies at one tip of the phage. During packaging of the phage, both enzyme-pIII fusions and acid peptide A-pIII fusions are produced; the phage particles obtained from a typical preparation carry either one or zero enzyme-pIII fusions plus 3–5 copies of acid peptide A-pIII fusion.

To generate phages bearing an acid peptide-pIII fusion, DNA encoding the acidic peptide A with a C-terminal extension containing a cysteine residue, was introduced into the 5'-end of gene III of the M13K07 helper phage. The resulting acid helper phage particles were immobilized more than 100-fold more efficiently than M13K07 on an ELISA-plate coated with basic peptide B, indicating that the mutant helper phage carry accessible acid peptide extensions on their pIII proteins. Likewise, when *E. coli* containing a phagemid encoding a pIII fusion protein were superinfected with the acid helper phage, the resulting phage particles displayed modified pIII extensions in addition to the pIII fusion protein (Fig. 2). The insertion of the acid peptide did not appear to change the titer or rescue efficiency of the helper phage significantly.

The synthetic base-linker-peptide (B) to which substrate is attached consists of the 12-residue (GlyGlySer)₄ linker followed by the 30 amino acids constituting the base sequence

(Fig. 1). The base-linker peptide also contains cysteine residues at the N and C termini that allow efficient, selective coupling of the peptide to substrates and disulfide bond formation to phage, respectively (Figs. 1 and 2). The C-terminal cysteine of the synthetic peptide initially is protected with the photochemically removable 2-nitro-4,5-dimethoxybenzyl protecting group. This allows substrate to be selectively conjugated by a thiol-specific reaction (e.g., by disulfide exchange, alkylation, or Michael addition reactions) to the free thiol group of the N-terminal cysteine. After substrate conjugation, the C-terminal cysteine is photochemically deprotected in high yield to generate a free thiol available for crosslinking to the acid peptide extension on phage. Because the chemical conjugation of substrate and base-linker peptide, and the crosslinking of this conjugate to phage are carried out separately, many different chemistries and reaction conditions can be used to couple the base-linker peptide and substrate. Moreover, the composition of the conjugate can be purified and characterized (e.g., by mass spectrometry) before it is crosslinked to phage.

SNase as a Model System. The enzyme SNase is a well-characterized enzyme consisting of a single polypeptide chain 149 aa in length (24–30). The enzyme preferentially hydrolyzes the phosphodiester bonds of single-stranded RNA, single-stranded DNA, and duplex DNA at A, U- or A, T-rich regions to generate 3'-phosphate and 5'-hydroxyl termini (24–30). Ca²⁺ is required for enzymatic activity, providing a mechanism for modulating enzyme action. In addition, SNase has successfully been displayed as a pIII fusion protein on phage (31).

Because no reagent, antibody, or receptor is available that can easily distinguish between a single-stranded oligodeoxynucleotide substrate and its cleavage product (a complementary oligonucleotide would be degraded), a selection scheme was developed in which enzymatic cleavage of single-stranded DNA substrate results in release of phage from solid support. In this scheme, one round of selection involves the following steps: (i) attachment of phage displaying SNase to solid support through a single-stranded oligodeoxynucleotide (in the absence of Ca²⁺, inactivating SNase); (ii) removal of unbound phage by washing; (iii) initiation of the cleavage reaction by addition of Ca²⁺, and (iv) isolation of eluted phage. In later rounds of selection, elution can be done under increasingly stringent conditions, e.g., shorter reaction time, lower temperature, and altered pH. Attachment of phage to solid support is carried out by coiled-coil formation between 5'-biotinylated oligodeoxynucleotide-peptide B conjugates and acid peptide A extensions on phage, followed by disulfide crosslinking of the two peptides and immobilization on streptavidin beads (Fig. 1). This scheme, in which the phage is attached to solid support through the substrate, requires that the enzyme or substrate be maintained in an inactive state during attachment to phage, and then be activated by a change in reaction conditions. Such changes can include modulation of pH, addition of cofactors or cosubstrates, and photochemical or chemical activation of the substrate. In the case of biomolecular condensation reactions in which bond formation results in phage immobilization on solid support, it is not necessary to initiate the reaction; the same is true whether capture of active enzymes is by a product-specific reagent, antibody, or receptor.

Covalent Attachment of the Substrate to Phage. Phage displaying either SNase or a control protein (antibody 39-A11 Fab fragment) were prepared by superinfection with the acid helper phage. To evaluate the efficiency of the attachment of base-linker-substrate conjugates to phage, an excess of a control conjugate, "pTp"-peptide B (compound 9), was incubated with the phage. The base-linker-pTp conjugate consists of a biotin moiety, followed by pTp, the flexible peptide linker and base peptide sequence, and a C-terminal cysteine. The base-linker-pTp conjugate is not a substrate for wild-type SNase in solution (pTp is a potent inhibitor of SNase) (32).

Phage and the substrate-peptide **B** conjugate first were incubated with the reducing agent 2-mercaptoethylamine (MEA) to reduce disulfide bonds between cysteines on the phage acid peptide or the synthetic peptide. Then, MEA and free base-linker-pTp were removed by polyethylene glycol precipitation, and magnetic streptavidin beads were added. After 10 washes, the number of phage that were immobilized was determined by infection of *E. coli* XL1-blue with the beads and titering phage. When measured this way, the efficiency of phage immobilization was approximately 10%, for both phage displaying SNase and 39-A11 Fab (Fig. 3).

Next it was determined whether an oligodeoxynucleotide substrate attached to phage displaying SNase would be stable in the absence of Ca²⁺. The base-linker-oligodeoxynucleotide conjugate was attached to phage displaying SNase (in the presence of EDTA), and the immobilization efficiency was determined as above. The efficiency of immobilization was again approximately 10% (Fig. 3), indicating that the tethered oligodeoxynucleotide substrate is not cleaved by SNase in the absence of Ca²⁺. It is possible that the true immobilization efficiency is higher than observed if some of the phage are rendered noninfective when attached to the beads. This notion was tested by addition of DNase I, which should cleave the tethered oligodeoxynucleotide substrate and release the immobilized phage. As can be seen in Fig. 3, most of the

immobilized phage are noninfective, but become infective upon addition of DNase I, indicating that the true immobilization efficiency is about 80% (Fig. 3). If the oligodeoxynucleotide-peptide **B** conjugate is not included, less than 0.01% of the phage become immobilized; if the wild-type M13K07 helper phage is used to superinfect, about 0.3% of phage are immobilized. It thus appears that the two-step protocol for attachment of substrate to phage pIII protein is efficient and highly site specific.

Enzyme-Dependent Cleavage of Phage from Solid Support and Enrichment. To determine whether phage-displayed SNase is capable of specifically cleaving the tethered oligodeoxynucleotide substrate in an intramolecular reaction, Ca²⁺ was added to the immobilized phage to activate the enzyme. Approximately 15% of the phage were released (Fig. 3), in contrast to release of only 0.2% of the control phage displaying Fab 39-A11 (Fig. 3). This experiment demonstrates that SNase cleaves and releases phage from the solid support much more efficiently than the control protein, as expected. However, it appears that a small, but significant, fraction of the phage leak off the support during the assay (this background leakage is observed without Ca²⁺, for both the base-linker-oligodeoxynucleotide and base-linker-pTp conjugates, and for both displayed proteins, Fig. 3). Addition of Ca²⁺ leads to an initial burst of phage release from support; however, the release of phage quickly declines to a level corresponding to the leakage observed without Ca²⁺. This result demonstrates that phage released into solution by intramolecular cleavage events do not release other phage from support as a result of intermolecular cleavage reaction. Crossreactivity therefore does not appear to be significant, even with a very active enzyme like SNase.

The above analysis suggests that it should be possible to enrich phage displaying SNase from a library-like ensemble of phage displaying catalytically inactive proteins. To test this, phage displaying SNase and the Fab 39A-11 control protein were mixed in a ratio of 1:100, crosslinked to the oligodeoxynucleotide-peptide **B** conjugate, and immobilized. After incubation with Ca²⁺, the ratio of recovered phage was 22:18, which corresponds to an enrichment factor slightly higher than 100. This degree of enrichment should be sufficient to isolate an active catalyst from a library of 10¹⁰ members after five rounds of selection and amplification.

The enrichment factor likely can be increased by minimizing background leakage of phage from support. This leakage may result from release of streptavidin from support, or alternatively, reduction or incorrect formation of the disulfide bridge between the synthetic and phage encoded peptides. We currently are exploring these possibilities. Alternatively, the enrichment factor can be increased by increasing the extent of the enzyme-catalyzed cleavage reaction. Under the conditions of phage production, the ratio of pIII expressed from the helper phage relative to the pIII fusion protein expressed from the phagemid is such that most of the phage carry only wild-type pIII proteins; only a minor fraction of the phage carry the protein-pIII fusion. The number of phage that can cleave themselves off can be increased simply by increasing the number of phage that display the enzyme. For the phagemid/helper phage combination described here, we estimate that only about 15% of the phage are monovalent. By appropriate vector design and phage preparation, it should be possible to increase the average display to about one protein per phage. This should increase the cleavage-to-leakage ratio 7-fold, and hence, increase the enrichment factor of active versus inactive enzymes from the present ≈100 to about 700.

To examine whether the selection scheme described here can be used for reactions that involve small molecule substrates, a pTpTp-peptide **B** conjugate (compound **10**) was attached to phage displaying SNase or the control protein. Phage were carried through the enrichment routine described above, and again SNase displaying phages were enriched.

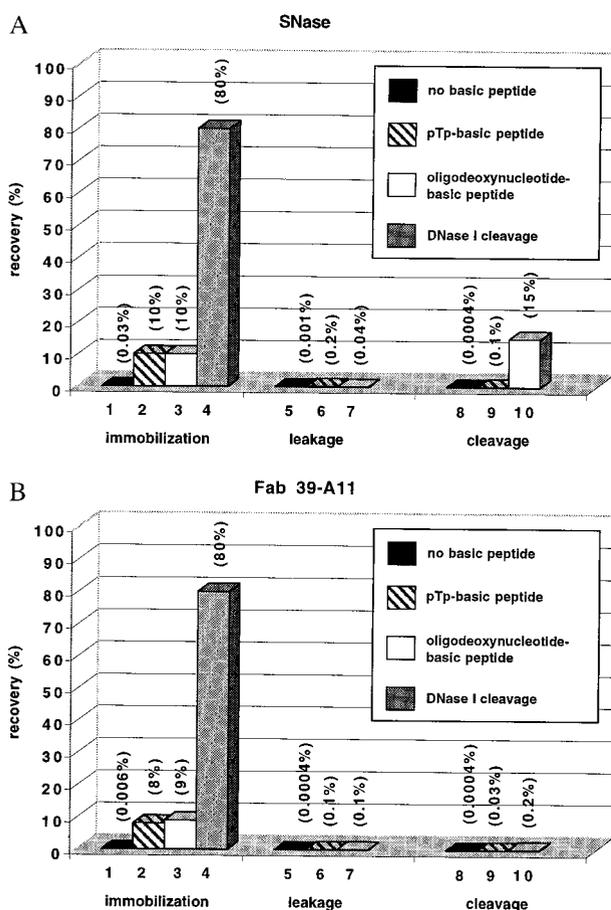


Fig. 3. Immobilization and cleavage of phage from solid support. Either no base-linker, the base-linker-pTp, or the base linker-oligodeoxynucleotide conjugate was crosslinked to (a) phage displaying SNase or (b) the control protein Fab 39-A11. Columns 1-4 show immobilization on streptavidin beads. Immobilization was examined either by phage titering of the beads directly (columns 1-3) or after DNase I treatment of the beads (column 4); columns 5-7 show leakage (release in absence of Ca²⁺); columns 8-10 shows Ca²⁺ induced release (cleavage). The percent recovery is shown in parentheses above the columns.

Matrix-assisted laser desorption ionization-time of flight mass spectrometry was used to show that the pTpTp substrate was cleaved at the phosphodiester bond between the two thymidines; no side products were detected. It thus appears that the methodology is applicable to both macromolecular and small molecule substrates. We currently are exploring the possibilities for isolating novel catalysts from libraries of enzyme or antibody origin.

Most enzyme libraries displayed on phage require superinfection by a helper phage like M13K07. The selection protocol described here therefore can be applied directly to these libraries—one simply needs to prepare phage after superinfection of the phagemid-encoded library with the acid peptide helper phage and conjugate the substrate of choice to the basic peptide **B**. Likewise, this methodology can be applied to populations of structurally diverse proteins. The collection of proteins encoded by a genome is one such population. For example, it should be possible to isolate natural kinases with predefined substrate specificity from a genomic protein library using this selection scheme. This type of functional cloning in which a natural enzyme (and the gene that encodes it) is isolated on the basis of its catalytic activity should be applicable to many reactions catalyzed by natural enzymes.

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