A Method for the Selection of Catalytic Activity Using Phage Display and Proximity Coupling**

Jean-Luc Jestin,* Peter Kristensen, and Greg Winter*

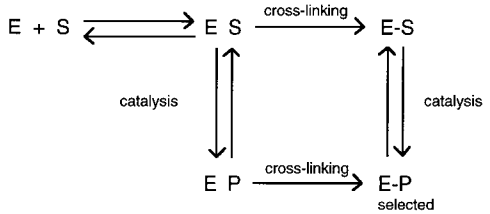
Attempts have been made to endow proteins with catalytic activities by use of either design or selection strategies.[1] Among the selection strategies employed, the use of phage display[2–4] has facilitated the selection of proteins with binding activities.[5] Repertoires of variant polypeptides are displayed on the surface of filamentous bacteriophages by fusion of the gene that encodes the polypeptide to that of a phage coat protein; binding of the phage to ligands coated on a solid phase thereby allows the isolation of those genes that encode polypeptides with ligand binding activities. However it has proved more difficult to apply this technology to the isolation of catalysts.

A first strategy, analogous to the methods used for the isolation of catalytic antibodies by immunization of animals with transition state analogues,[6] involves the selection of the phage by binding of the displayed enzyme to transition state analogues and then the screening of the selected phage for catalytic activities.[7] A second strategy involves the use of the catalytic mechanism to generate a covalent cross-link from the enzyme to a tagged mechanism-based enzyme-inhibitor,[8] to a tagged and highly reactive moiety generated by the catalytic reaction,[9] or to a tagged reaction intermediate.[10] The displayed enzymes are then selected by capture of the phage by using the tag, for example with a biotin tag on a streptavidin-coated surface.

Both strategies have limitations. For example, they require a detailed knowledge of the reaction mechanism and may impose considerable challenges in the design and chemical synthesis of the substrate or transition state analogue. Furthermore such strategies may direct the selection process to a single feature of the catalytic cycle, for example to a single transition state (whereas enzyme catalysis is likely to involve multiple transition states) or to a reaction intermediate (whereas enzyme catalysis also involves product formation and release).[11]

A third strategy has been developed recently, and was reported during the preparation of this paper, which may overcome some of these limitations.[12] It is based on the selection of the product and the use of proximity effects. Thus a tagged substrate was cross-linked to the phage in the proximity of the displayed enzyme; the phage was thereby attached to a solid phase and released by an intramolecular cleavage reaction catalyzed by the displayed enzyme.[12]

We had independently devised a similar approach, as applied here to a synthetic reaction (Scheme 1). It involves two chemically independent reactions, the catalytic reaction



Scheme 1. Our strategy for the selection of catalytically active enzymes (see text for further information). E = enzyme, S = substrate, P = product.

that leads to a product (in this case distinguished by incorporation of a biotin tag) and a chemical cross-linking reaction in which the substrate (and product) is linked to the phage. Selection of the phage by streptavidin beads therefore selects for phages that are chemically attached to the tagged product; these reactions are more likely to be coupled on the same phage since reactions in a *cis* position should be favored by proximity effects over those in *trans* positions.

The sites for cross-linking could be engineered within the enzyme[13] or phage,[12] or more randomly as shown here. Indeed the use of a range of different sites for cross-linking may pose fewer steric constraints for the catalytic reaction, and provide multiple opportunities for a cross-linked substrate to be tagged. We used maleimides in a chemical cross-linking reaction, which are known to react with thiols and, in alkaline solutions, with amino groups,[14] and are therefore capable of reacting with a wide range of sites on the phage and on the displayed enzyme. A covalently bound product between the major coat protein (p8) and *N*-biotinoyl-*N*′-(6-maleimidohexanoyl)hydrazide, was detected by SELDI-MS[*]. We believe that either of the two amino groups (the N-terminal Ala-1 and the residue Lys-8) are involved (data not shown in the table).[15]

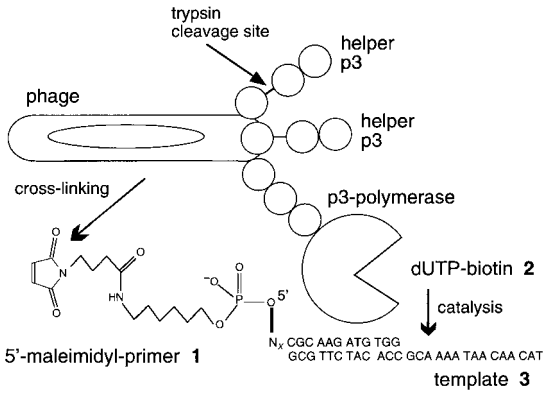
We tested the strategy using DNA polymerases in view of their central role in molecular evolution. We therefore introduced a maleimidyl group at the 5′ end of a DNA primer (Scheme 2); the product was tagged by addition of biotinylated dUTP to the 3′ end of the primer by the catalytic action of the polymerase. We cloned the Klenow and Stoffel fragments of DNA polymerase I from *Escherichia coli* and *Thermus aquaticus* for display by fusion to the pIII coat protein of filamentous bacteriophage (see experimental section). Both fragments lack the 5′ → 3′ exonuclease domains; the Stoffel fragment also lacks a 3′ → 5′ exonuclease activity.[16]

[*] Dr. J.-L. Jestin, Dr. G. Winter, Dr. P. Kristensen
Centre for Protein Engineering and Laboratory of Molecular Biology
Medical Research Council
Hills Road, CB2 2QH Cambridge (UK)
Fax: (+44) 1223-402-140
E-mail: jjestin@pasteur.fr
gw@mrc-lmb.cam.ac.uk

[**] We are grateful to I. Lavenir for SELDI-MS data; H. Griffin for the anti-Klenow scFv; P. Holliger for pTTQ18-Taq; G. Williams for pSV-$V_{NP}\gamma$PolIk; and D. Neri, F. Sait, M. Farrow, and M. Gait for advice. We also thank P. Nielsen for a training period for J.L.J., EMBO for a postdoctoral fellowship (for J.L.J., ALTF 624 – 1995), and the Danish Natural Science Research Council for a postdoctoral fellowship (for P.K.).

[*] Abbreviations: SELDI = surface-enhanced laser desorption and ionization; MALDI = matrix-assisted laser-desorption and ionization; PBS = aqueous solution of 0.1 M NaCl and 25 mM Na_2HPO_4 at pH 7.5, unless otherwise stated; TEN = aqueous solution of 0.3 M Tris-HCl, 33 mM EDTA, and 1.3 M NaCl at pH 9.0; dNTP = deoxynucleotide triphosphate; BSA = bovine serum albumin; EDTA = ethylenediaminetetraacetic acid; scFv = single-chain variable antibody fragment; ELISA = heterogeneous enzyme assay.

  1433-7851/99/3808-1124 $ 17.50+.50/0



Scheme 2. Selection of catalytically active DNA polymerases obtained by phage display and proximity coupling. If the polymerase is active, then 5′-maleimidyl-derivatized primer **1** is extended with biotinylated dUTP **2** and dNTPs in a template **3** dependent manner. The maleimidyl group can react with the phage coat, and thereby allow the capture of catalytically active phage polymerases onto streptavidin-coated beads. N_{13} = TAA CAC GAC AAA G; $N_{50} = T_{50}$

We decided to use "monovalent" display,[4] in which the fusion protein is cloned on a phagemid (pHEN1)[17] and then rescued by a helper phage. The polymerase fragments were shown to be displayed on the phage (after rescue with the helper phage) by binding of the phage to wells coated with anti-polymerase antibodies (as detected by ELISA). The phages were also analyzed by Western blot with anti-p3 or anti-polymerase antibodies. This confirmed the presence of the fusion protein, but also indicated contamination by free polymerase. Presumably this arises by secretion from the bacterial host through incomplete suppression of the amber stop codon or by cleavage from the phage surface. This was removed by a further step of ultracentrifugation or by size-exclusion chromatography. The purified phages were assayed for DNA polymerase activity in a primer/template extension assay with radioactively labelled α^{32}P-dCTP (see experimental section) and found to be active (data not shown in the table).

However, the polymerase-p3 fusion protein was poorly incorporated into the phage compared to the p3 protein as indicated by Western blots (data not shown in the table). This appeared to be a consequence of extensive incorporation of p3 from the helper phage, as shown by our use of a helper phage (KM13) in which the p3 protein of the helper phage (but not that of fusion protein) can be cleaved with trypsin so as to render it incapable of mediating infection.[18] Thus after proteolysis only those phages that had incorporated the fusion protein are infective; from the loss in titre after proteolysis we estimated that only one phage particle in a thousand had incorporated the fusion protein. We anticipated that the selection process, which relied on tagging by polymerase *cis*, would be compromised by such a great excess of phages that lacked the polymerase but were available for tagging *trans*. We therefore treated selected phages with trypsin to destroy the infectivity of those lacking the displayed polymerase.

We incubated the phage that displayed the Stoffel fragment with primer **13** (Table 1, which comprised a 5′ maleimidyl group and a 3′ biotinylated nucleotide. After incubation the phage could be captured on streptavidin-coated beads, with a yield of about 1 – 5 % of infectious phage. This shows that the primer can be chemically cross-linked to the phage, presumably through the p8 protein as shown for the *N*-biotinoyl-*N*′-(6-maleimidohexanoyl)hydrazide. We then incubated phage with primer **1b**, which comprised a 5′ maleimidyl group in the presence of biotin – dUTP **2** and template **3**. Capture of the phage was dependent on the presence of **1b, 2**, and **3** (Table 2).

Table 1. Deoxyoligonucleotide sequences.[a]

3: AAA TAC AAC AAT AAA ACG CCA CAT CTT GCG
4: TGG TCC CGG CGC AAG ATG TGG CGT
5: CGC AAG ATG TGG CGT
6: GTG GAC GCC ACA TCT TGC G
7: TTC AGA TAC CCA TGG CCT CTG GTG GCG GTG GC**T GT**G GTG GCG GTG GCA GCC CCA AGG CCC TGG AGG AGG CC
8: GAG TCA TTC TGC GGC CGC CTC CTT GGC GGA GAG CCA GTC C
9: TTC AGA TAC CCA TGG CCC AGG TGA TTT CTT ATG ACA ACT ACG TC
10: GAG TCA TTC TGC GGC CGC GTG CGC CTG ATC CCA GTT TTC GCC
11: CTC TCC CAG GAG CTA GCC ATC C
12: ACA CCC CCT CCA TGA CCT CCT TGG CCA GCC GGG CCA CGG CCT CCG CCC TCT CTT TTG GGG CCT CGA GGA CCA GTG CGG CGT GGA CCT GAA GGA GCA TCC
13: TTT CGC AAG ATG TGG CGT
14: GTG CCA GAC CTA GAG GCC
15: CTA TGC GGC CCC ATT CA
16: ACA GCT ATG ACC ATG ATT ACG CC
17: AAT AGG TGT ATC ACC GTA CTC AGG.

[a] Restriction sites are underlined. The TGT codon in primer **7** noted in bold encodes a cysteine at the N-terminus of the Stoffel-p3 fusion protein. Deoxyoligonucleotide **4** contains a 5′-biotinyl group and deoxyoligonucleotide **13** a 5′-maleimidyl group and a 3′-biotinyl group.

Table 2. Selection of catalytically active Stoffel-phage particles.

φ_i[a] [tu]	φ_f[b] [tu]	Yield [%]	Conditions[c]
8.4×10^5	2.0×10^4	2.4	
3.6×10^5	1.0×10^2	0.028	– primer **1b**
4.4×10^5	3.0×10^2	0.068	– biotinylated dUTP **2**
4.8×10^5	3.0×10^2	0.062	– template **3**
4.4×10^9	4.0×10^6	0.091	– trypsin
1.5×10^9	5.5×10^5	0.037	– trypsin, – primer **1b**

[a] φ_i and φ_f denote the number of transformation units (tu) prior and after the selection. [b] Yield = φ_f/φ_i. [c] with primer **1b**, biotinylated dUTP **2**, template **3**, and trypsin.

This observation shows that the primer tagged by the enzyme can be cross-linked to the phage. The addition of trypsin greatly reduced the infectivity of phages before and after capture. This suggests that many of the phages that are captured nevertheless lack the phage polymerase. The effect of trypsin, in eliminating these phages, is to greatly improve the capture yield as defined in Table 2.

We then used the selection process to select active from denatured polymerase (Table 3). We mixed Klenow-phage and Stoffel-phage particles, heated the mixture at 60 °C to favor the denaturation of the Klenow fragment, and then undertook a round of selection. Without heating we enriched the Klenow phage twofold; with heating we enriched the Stoffel phage 14-fold. The enrichment of the Stoffel phage

Table 3. Selection of Stoffel phage from Klenow phage.

Phage particles[a] (Stoffel/Klenow)	Enrichment in Stoffel	Conditions
$(27/5)_i$ $(21/10)_f$	0.39	pH 7.5, 25 °C
$(3/56)_i$ $(23/30)_f$	14	pH 7.5, 60 °C/25 °C[b]
$(18/17)_i$ $(110/4)_f$	26	pH 8.5, 60 °C/25 °C[b]
$(18/17)_i$ $(22/6)_f$	3	pH 8.5, 60 °C/25 °C[b]; Taq 1U

[a] Ratios (Stoffel/Klenow) indicate the relative proportion of Stoffel phage and Klenow phage for the mixture before (i) and after selection (f). [b] The phage particles were treated at 60 °C for 3 min, then under catalytic and cross-linking conditions at 60 °C for 3 min followed by 1 h at 25 °C.

was improved (26-fold) at higher pH values. As expected, the addition of free polymerase resulted in a loss of enrichment (to threefold).

We also used the selection process to select between phages that displayed Stoffel fragments that bind to DNA but have different catalytic activities (Table 4). Two highly conserved

Table 4. Selection of Stoffel phage with different catalytic activities.

Phage particles[a] (WT/M)	Enrichment	Catalytic conditions
$(4/44)_i$ $(35/10)_f$	35	**1b** 5 μM, 60 °C/25 °C[b]
$(4/44)_i$ $(30/15)_f$	20	**1b** 0.5 μM, 60 °C/25 °C[b]
$(4/44)_i$ $(16/32)_f$	5	**1b** 50 nM, 60 °C/25 °C[b]
$(5/43)_i$ $(27/21)_f$	11	**1b** 0.5 μM, 25 °C
$(5/43)_i$ $(40/8)_f$	43	**1b** 0.5 μM, 35 °C
$(5/43)_i$ $(34/14)_f$	21	**1b** 0.5 μM, 45 °C
$(5/41)_i$ $(45/3)_f$	123	**1a** 5 μM, 35 °C
$(24/23)_i$ $(20/22)_f$	1	**13** 1.3 μM, 60 °C/25 °C[b]
$(38/10)_i$ $(9/33)_f$	0.071	no catalysis, DNA binding only[c]

[a] The ratios of wild-type Stoffel (WT) or mutant Stoffel (M) as in Table 3. [b] Polymerization and cross-linking at 60 °C for 3 min followed by 1 h at 25 °C. [c] See experimental section.

carboxylate residues are located at the active site of family I DNA polymerases such as the Klenow and Stoffel fragments.[19] One of them (D882 in the Klenow and D785 in the Stoffel fragment) has been shown to bind a metal ion and to be directly involved in catalysis in homologous polymerases.[20] Mutation of either residue in the Klenow polymerase leads to a major loss in catalytic activity (k_{cat}).[21] We engineered the homologous mutations in the Stoffel fragment (D785A, D786A). As expected, mutant Stoffel phage proved much less active than the wild-type in a primer-extension assay (not shown). When the mutant Stoffel phage was mixed with the wild-type Stoffel phage, we obtained a 35-fold enrichment for the latter after the process of catalytic selection (Table 4). Using slightly different conditions (T = 35 °C instead of 25 °C and primer **1a** instead of **1b**), the enrichment of the Stoffel fragment could be improved to 123-fold over the less active mutant one.

The selection in favor of the wild-type Stoffel polymerase appears to reflect the catalytic rather than the binding step since the concentration of the DNA primer/template (9 μM) is greatly in excess of its dissociation constant from Taq DNA polymerase (1.5 nM).[22] The mutant Stoffel fragment appears to have an even better binding affinity than the wild-type Stoffel fragment. Thus when we mixed the mutant and wild-type Stoffel phage and selected for binding to duplex DNA we obtained a 14-fold enrichment for the mutant in contrast to the selection in favor of wild-type under catalytic conditions.

Our results show that it is possible to base a selection process for enzymes that catalyze a synthetic reaction on the proximity of chemical cross-linking and catalytic tagging reactions on the same phage particle. The sites need not be adjacent; indeed the sites for catalysis and cross-linking may be separated by as much as 5.1 nm in primer **1a**. Here we let both reactions run together; the order of reactions (tagging then cross-linking, or cross-linking then tagging; Scheme 1) will depend on the relative rates of the cross-linking and tagging reactions and in turn on such factors as the concentrations of tagged substrate, the binding affinity of the enzyme for tagged substrate, and the chemical reactivity of the cross-linking moiety. In principle it should be possible to force the order of reactions, for example by first adding the reactive substrate, and later adding the tag. It should also be possible to adjust the selection procedure according to the kinetic parameters.

The strategy of proximity coupling seems likely to be general, as demonstrated now for both a cleavage[12] and here for a synthetic reaction. It is illuminating to compare key technical features of the approach used here with that recently described.[12] Each made use of helper phages engineered within the p3 protein but for very different purposes. We introduced a protease cleavage site to focus the selection process on those phages that displayed polymerase, whereas Pedersen and colleagues introduced a site of specific attachment for the substrate of the nuclease. We used random cross-linking of the maleimidyl group (probably to lysine residues in the major coat protein p8), whereas Pedersen and colleagues used disulfide bond formation with an engineered cysteine of the helper p3. It may be possible to combine these different features to advantage, or indeed to use the principle with other display[23] or compartmentation strategies.[24] This may facilitate their use for the evolution of enzymes in vitro.

Experimental Section

Deoxyoligonucleotides (Table 1) were prepared by solid-phase synthesis on a DNA synthesizer (394, Applied Biosystems) except **4**, which was provided by Eurogentec. The 5′-biotinyl group was introduced from 1-(dimethoxytriphenylmethyl)oxy-3-O-(*N*-biotinyl-3-aminopropyl)-triethyleneglycolylglyceryl-2-O-(2-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramidite and the 3′-biotinyl group from 1-(dimethoxytriphenylmethyl)oxy-3-O-(*N*-biotinyl-3-aminopropyl)-triethyleneglycolylglyceryl-2-O-succinoyl modified pore glass. The 5′-aminohexyl modification was introduced with a 6-((4-monomethoxytriphenylmethyl)amino)hexyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramidite (Glen Research).

The deoxyoligonucleotide containing a 6-((4-monomethoxytriphenylmethyl)amino)hexyl group was purified on a C_{18} reversed-phase HPLC column (Waters) prior to deprotection. The lyophilized product was dissolved in 0.1 mL of $NaHCO_3$ at pH 8.5, and added to a suspension of succinimidyl 4-(*N*-maleimidyl)butyrate (Prochem, Rockford, IL) in DMF (35 mL). After 2 h at 25 °C, the deoxyoligonucleotide containing a maleimidyl group was purified on a C_{18} reversed-phase HPLC column, lyophilized, and characterized by MALDI-TOF-MS (Voyager DE, Perseptive Biosystems); m/z: calcd (found): **1a**: 9000.9 (9000.4), **3**: 9146.0 (9138.2), **4**: 7994.4 (7985.7), **13**: 6460.5 (6458.9).

The product bound covalently between the major coat protein (p8) of the phage (m/z 5235.7) and *N*-biotinoyl-*N*′-(6-maleimidohexanoyl)hydrazide was detected by SELDI-MS; m/z: calcd (found): 5687.2 (5689.5).

  1433-7851/99/3808-1126 $ 17.50+.50/0 *Angew. Chem. Int. Ed.* **1999**, *38*, No. 8

The DNA encoding the Stoffel fragment was amplified by PCR by using the template pTTQ18-Taq and primers **7** and **8**; it was cloned as a Nco1 – Not1 fragment into the phagemid pHEN1[17] to give the pHEN1 – Stoffel fragment. Similarly, pHEN1 – Klenow was made from the PCR amplification product of the Klenow fragment by using the template pSV – $V_{NP}\gamma$PolIk and primers **9** and **10**. The phagemid pHEN1 – Stoffel mutant is a derivative of pHEN1 – Stoffel whose Nhe1 – Xcm1 fragment was replaced by the PCR amplification product by using primers **11** and **12**. Note that a free cysteine residue near the C-terminus of the Klenow fragment is lacking in the Stoffel fragment. A free cysteine was introduced at the N-terminus of the Stoffel fragment. These residues are on the far side of the polymerase away from the active site.

Phage particles were produced as described previously,[3] except that the helper phage KM13 was used instead of VCSM13.[18] The phage particles were purified by ultracentrifugation in a CsCl gradient followed by dialysis.[3, 25] Size-exclusion chromatography on a column (Econo-Pac, Biorad) loaded with Sephacryl (S300, Pharmacia-Biotech) was used to purify phage polymerases (5×10^{12} tu, 10^{13} tu mL^{-1}) from free contaminating polymerase.

For ELISA, an anti-Klenow scFv isolated from a phage antibody repertoire or an anti-Taq antibody (Taqstart, Clontech) was used for coating and an anti-M13-horseradish peroxidase (Pharmacia Biotech) used for detection in a standard protocol.[3]

To test the catalytic activity of phage polymerases, we used a primer extension assay; 7×10^8 phage polymerases (about 7×10^{11} infective phage particles) were incubated for 10 min at 25 °C (Klenow phage) or 51 °C (Stoffel phage) in an aqueous solution (10 μL) of 15 or 150 μM of primer **5** annealed to template **6**: 250 μM dNTP, 1.5 mM $MgCl_2$, 33 nM α^{32}P-dCTP, 3.10^6 Ci mol^{-1} in a buffer containing 50 mM Tris-HCl, 50 mM KCl, 1 mM DTT at pH 7.5 (Klenow phage) or 10 mM Tris-HCl, 50 mM KCl at pH 9 (Stoffel phage). The reaction was stopped by addition of EDTA (0.1M, 5 μL). Labelled oligonucleotides were detected on an polyacrylamide gel (21 %) in denaturing conditions.

In a typical selection for catalysis, phage polymerases (10^{10} infective particles, corresponding to about 10^7 infective particles after pIII cleavage with trypsin in 17 μL) in PBS were mixed with 5′-maleimidyl containing 9 μM primer **1**, 50 μM template **3**, 90 μM dATP, 0.9 μM each of dGTP and dCTP, and 0.9 μM biotinylated deoxynucleotide triphosphate **2** (Boehringer, Mannheim), 9 mM $MgCl_2$, and let react for one hour at 35 °C and pH 8.5. An aliquot of the phage mixture (8 μL) was then added to streptavidin-coated superparamagnetic beads (Dynabeads M-180, Dynal, 200 μL) in TEN buffer (190 μL). The mixture of phages and beads was mixed for 30 min at room temperature. The beads were then washed seven times with TEN buffer (200 μL) during 35 min and the selected phage mixture linked to the beads was finally resuspended in PBS (100 μL). The selected phage mixture (100 μL) or an aliquot of the initial phage mixture (5 μL) was incubated for 5 min at 37 °C after addition of one tenth in volume of bovine pancreas trypsin (10 g L^{-1}, Sigma). To each mixture, 1 mL of *E.coli* TG1 in 2xTY at a density of about 8×10^8 cells mL^{-1} was added and incubated at 37 °C for 15 min. The cells were plated on ampicillin-containing plates and grown overnight at 37 °C.

The conditions used in the selection of phage polymerases by binding to DNA, were as follows: Streptavidin-coated beads (200 μL) were mixed for 30 min at room temperature with the 5′-biotinyldeoxyoligonucleotide **4** (0.3 nmol) and template **3** (0.3 nmol) in TEN buffer (100 μL). The beads were then washed three times with a PBS solution adjusted at pH 8.5 with triethylamine prior to addition of a mixture of Stoffel phage and Stoffel phage/mutant (10^{10} infective phage particles) in a PBS solution at pH 8.5. The resulting suspension was mixed for 25 min at room temperature. The beads were then washed four times with a PBS solution at pH 8.5 and trypsin was added as described previously.

The ratios between Stoffel phage and Stoffel phage mutant particles before and after selection were determined by PCR-fingerprinting as follows: Cells from single colonies were added to 19 μL of a polymerization mixture (pH 8.8) containing 0.25 mM dNTP, 0.5 μM primers **14** and **15**, 1 U Taq polymerase, 0.05 U Pfu polymerase, 20 mM Tris-HCl, 2 mM $MgSO_4$, 0.1 % Triton X-100, 0.1 g L^{-1} bovine serum albumin, 10 mM KCl, and 10 mM $(NH_4)_2SO_4$. The amplification was made using the temperature/time sequence (°C/min): (94/10) (94/1; 61/1; 72/2)$_{30}$ (65/10). The restriction enzyme *AluI* (4U, New England Biolabs) was then added to the PCR products (9 μL) mixed with a buffer (9 μL, pH 7.9) containing 10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, and the solution was incubated at 37 °C for 3 h. The resulting DNA mixtures were loaded on a agarose gel (2 %) for electrophoresis. The DNA band patterns obtained were characteristic of the polymerases: the Stoffel fragment contains a GAC GAG CTG sequence encoding amino acids 785 – 787 whereas the mutant Stoffel fragment contains a GCC GCA CTG sequence and lacks the AGCT *AluI* restriction site. The ratios between the number of Stoffel-phage and Klenow-phage particles were determined using the same protocol except that the amplification was done with primers **16** and **17** and the digestion using the restriction enzyme *PvuII* (9U, New England Biolabs) prior to analysis on a agarose gel (1 %).

Received: September 15, 1998 [Z12418IE]
German version: *Angew. Chem.* **1999**, *111*, 1196 – 1200

Keywords: enzyme catalysis · in vitro selection · molecular evolution · phage display · polymerases

[1] a) A. J. Kirby, *Angew. Chem.* **1996**, 108, 770 – 790; *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 707 – 724; b) E. Quemeneur, M. Moutiez, J. B. Charbonnier, A. Menez, *Nature* **1998**, *391*, 301 – 304.
[2] G. P. Smith, *Science* **1985**, *228*, 1315 – 1317.
[3] J. McCafferty, A. Griffiths, G. Winter, D. J. Chiswell, *Nature* **1990**, *348*, 552 – 554.
[4] S. Bass, R. Greene, J. A. Wells, *Proteins Struct. Funct. Genet.* **1990**, *8*, 309 – 314.
[5] a) G. Winter, *FEBS Lett.* **1998**, *430*, 92 – 94; b) P. A. Nygren, M. Uhlen, *Curr. Opin. Struct. Biol.* **1997**, *7*, 463 – 469.
[6] R. A. Lerner, P. G. Schulz, *Science* **1995**, *269*, 1835 – 1842.
[7] a) L. O. Hansson, M. Widersten, B. Mannervik, *Biochemistry* **1997**, *36*, 11252 – 11260; b) I. Fujii, S. Fukuyama, Y. Iwabuchi, R. Tanimura, *Nat. Biotechnol.* **1998**, *16*, 463 – 467.
[8] P. Soumillion, L. Jespers, M. Bouchet, J. Marchand-Brynaert, G. Winter, J. Fastrez, *J. Mol. Biol.* **1994**, *237*, 415 – 422.
[9] K. D. Janda, L. C. Lo, C. H. L. Lo, M. M. Sim, R. Wang, C. H. Wong, R. A. Lerner, *Science* **1997**, *275*, 945 – 948.
[10] C. Gao, C. H. Lin, C. H. L.Lo, S. Mao, P. Wirsching, R. A. Lerner, K. D. Janda, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 11777 – 11782.
[11] a) F. Hollfelder, A. J. Kirby, D. S. Tawfik, *Nature* **1996**, *383*, 60 – 63; b) M. Baca, T. S. Scanlan, R. C. Stephenson, J. A. Wells, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 10063 – 10068.
[12] H. Pedersen, S. Hölder, D. P. Sutherlin, U. Schwitter, D. S. King, P. G. Schultz, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 10523 – 10528.
[13] D. Neri, D. Tawfik, S. Demartis, F. Viti, A. Huber, G. P. Winter (Medical Research Council), *Isolation of enzymes*, WO-A PCT/GB97/01153 [Chem. Abstr. **1997**, *128*, 1458].
[14] S. S. Wong, *Chemistry of protein conjugation and cross-linking*, CRC Press, Boston, London, **1991**, pp. 34 – 35.
[15] D. A. Marvin, R. D. Hale, C. Nave, M. Helmer-Citterich, *J. Mol. Biol.* **1994**, *235*, 260 – 286.
[16] a) A. Kornberg, T. A. Baker, *DNA replication.*, 2nd ed., Freeman, New York, **1992**; b) K. R. Tindall, T. A. Kunkel, *Biochemistry* **1988**, *27*, 6008 – 6013; c) F. C. Lawyer, S. Stoffel, R. K. Saiki, K. Myambo, R. Drummond, D. H. Gelfand, *J. Biol. Chem.* **1989**, *264*, 6427 – 6437.
[17] H. R. Hoogenboom, A. D. Griffiths, K. S. Johnson, D. J. Chiswell, P. Hudson, G. Winter, *Nucleic Acids Res.* **1991**, *19*, 4133 – 4137.
[18] P. Kristensen, G. Winter, *Folding Design* **1998**, *3*, 321 – 328.
[19] M. Delarue, O. Poch, N. Tordo, D. Moras, P. Argos, *Protein Eng.* **1990**, *5*, 461 – 467.
[20] a) S. Doublié, S. Tabor, A. M. Long, C. C. Richardson, T. Ellenberger, *Nature* **1998**, *391*, 251 – 258; b) J. R. Kiefer, C. Mao, J. C. Braman, L. S. Beese, *Nature* **1998**, *391*, 304 – 307.
[21] A. H. Polesky, M. E. Dahlberg, S. J. Benkovic, N. D. F. Grindley, C. M. Joyce, *J. Biol. Chem.* **1992**, *267*, 8417 – 8428.
[22] F. B. Perler, S. Kumar, H. M. Kong, *Adv. Protein Chem.* **1996**, *48*, 377 – 435.
[23] J. Hanes, L. Jermutus, S. Weber-Bornhauser, H. R. Bosshard, A. Plückthun, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 14130 – 14135.
[24] D. S.Tawfik, A. D. Griffiths, *Nat. Biotechnol.* **1998**, *16*, 652 – 656.
[25] W. O. Salivar, H. Tzagoloff, D. Pratt, *Virology* **1964**, *24*, 359 – 371.

  1433-7851/99/3808-1127 $ 17.50+.50/0