

Affinity maturation of a *Taq* DNA polymerase specific affibody by helix shuffling

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The possibility of increasing the affinity of a *Taq* DNA polymerase specific binding protein (affibody) was investigated by an α -helix shuffling strategy. The primary affibody was from a naive combinatorial library of the three-helix bundle Z domain derived from staphylococcal protein A. A hierarchical library was constructed through selective re-randomization of six amino acid positions in one of the two α -helices of the domain, making up the *Taq* DNA polymerase binding surface. After selections using monovalent phage display technology, second generation variants were identified having affinities (K_D) for *Taq* DNA polymerase in the range of 30–50 nM as determined by biosensor technology. Analysis of binding data indicated that the increases in affinity were predominantly due to decreased dissociation rate kinetics. Interestingly, the affinities observed for the second generation *Taq* DNA polymerase specific affibodies are of similar strength as the affinity between the original protein A domain and the Fc domain of human immunoglobulin G. Further, the possibilities of increasing the apparent affinity through multimerization of affibodies was demonstrated for a dimeric version of one of the second generation affibodies, constructed by head-to-tail gene fusion. As compared with its monomeric counterpart, the binding to sensor chip immobilized *Taq* DNA polymerase was characterized by a threefold higher apparent affinity, due to slower off-rate kinetics. The results show that the binding specificity of the protein A domain can be re-directed to an entirely different target, without loss of binding strength.

Keywords: affibody/affinity maturation/phage display/staphylococcal protein A/*Taq* DNA polymerase

Introduction

From libraries of peptides and proteins, variants capable of binding desired target molecules can be efficiently selected and identified using, for example, *in vitro* selection technologies such as phage display (Dunn, 1996; Smith and Petrenko, 1997; Hoogenboom *et al.*, 1998), ribosomal display (Hanes and Pluckthün, 1997; He and Taussig, 1997) peptides on plasmids (Schatz, 1993) or bacterial display (Georgiou *et al.*, 1997). For such selections, a correlation between library size (complexity) and the likelihood of isolating binders of higher affinities ($K_D = 10^{-8}$ M or lower) has been theoretically considered (Perelson and Oster, 1979) and experimentally demonstrated (Griffiths *et al.*, 1994; Vaughan *et al.*, 1996; Aujame *et al.*, 1997). From 'standard' sized (10^6 – 10^8 members) naive, non-biased libraries of antibody fragments (Griffiths *et al.*, 1993) or protein domain libraries (Tramontano *et al.*,

1994; McConnell and Hoess, 1995; Nord *et al.*, 1997), dissociation constants in the range of $K_D = 10^{-5}$ – 10^{-7} M are typical affinities of isolated binders. Although the construction of phage libraries of higher complexities has been described using, for example, 'brute force' principles of scale-up (Vaughan *et al.*, 1996) or *in vivo* recombination (Cre-lox) (Griffiths *et al.*, 1994), in most cases, the library diversity is still a limiting factor for the isolation of high affinity variants.

A previously described strategy to circumvent the need for large naive libraries to isolate high-affinity antibody fragments is the harvesting of immunoglobulin-encoding sequences from donors immunized with the target of interest (Clackson *et al.*, 1991; Hoogenboom *et al.*, 1998). However, such biased libraries are of limited use for the isolation of antibodies to a wide range of targets. Alternatively, binders of higher affinities can be obtained through affinity maturation of lead binders ('first generation' binders) isolated from naive libraries. This approach includes selection of variants from hierarchical libraries constructed on the basis of sequences of already identified binders. For antibodies, different methods for construction of such libraries for the isolation of high-affinity variants have been described involving, for example, heavy and/or light chain shuffling (Marks *et al.*, 1992; Schier *et al.*, 1996a), CDR re-randomization (Yang *et al.*, 1995; Schier *et al.*, 1996b), step-wise sexual PCR (Cramer *et al.*, 1996) or by using a bacterial mutator strain (Low *et al.*, 1996). Similar principles, although directed to other relevant portions of the structure, have also been applied to the increase of affinities of non-immunoglobulin proteins, including scaffold proteins (Martin *et al.*, 1996) or peptides (Wrighton *et al.*, 1996) obtained from primary selections from combinatorial libraries, or naturally occurring binding proteins (Lowman and Wells, 1993; Ballinger *et al.*, 1998).

We have previously described the phage display-facilitated selection of novel binding proteins (affibodies) from combinatorial libraries of the three-helix bundle, 58 residue Z domain derived from staphylococcal protein A (SPA), in which 13 amino acid positions distributed over the first two helices were targeted for randomization (Nord *et al.*, 1995, 1997) (Figure 1). Using standard technology, the library sizes obtained were approximately 4.5×10^7 members, which only corresponds to a minute fraction of the number of clones (approximately 10^{19}) required for a full sampling of the variability. The affinities observed for binders to the different targets investigated so far have all been in the micromolar (K_D) range, albeit with different binding kinetics (Nord *et al.*, 1997). The moderate affinities obtained for selected affibodies could possibly be a consequence of the sparse library sizes or reflect a conceptual limitation of the approach involving the randomization of a discontinuous 'paratope' distributed over two separate α -helices.

In this study, we have investigated if affibody variants with higher affinities for the target *Taq* DNA polymerase could be isolated from a hierarchic library, constructed in analogy with

principles applied on the affinity maturation of antibody fragments, involving shuffling of individual VH or VL domain chains. Here, the re-randomization was instead selectively directed to one of the two α -helices making up the *Taq* DNA polymerase binding surface of the affibody (helix shuffling).

Materials and methods

Strains and plasmids

Escherichia coli strain RRIAM15 (Rüther *et al.*, 1981) was used as host during library construction and phagemid work. Strain RV308 (Maurer *et al.*, 1980) was used as host for production of soluble affibodies and strain BL21(DE3) (Novagen, Inc., Madison, WI) for the production of the Aff2–*Taq* DNA polymerase fusion protein. Phagemid vector pKN1 was used for library constructions as described earlier (Nord *et al.*, 1995) and the vector pAff2c (Nilsson *et al.*, 1996), encoding a multiaffinity fusion partner containing an *in vivo* biotinylated domain, a hexahistidyl sequence and a serum albumin binding protein (ABP), was employed for intracellular production (T7 system) of *Taq* DNA polymerase.

Construction of the hierarchical library

The library was constructed based on the sequence of a previously described *Taq* DNA polymerase binding protein ($Z_{Taq4:8}$), selected from a library of the Z domain derived from staphylococcal protein A (Nord *et al.*, 1997). For library constructions, a solid phase gene assembly strategy was employed as described earlier (Nord *et al.*, 1995) where all the oligonucleotides used were as described for Z-lib 1 except for the oligo GUEL8 (5'-CAAAGAAGCTGGGTTGGGC-GACCTGGGAGATCTTCAACTTACCTA-3'), encoding the sequence of helix one, which was kept unchanged in the hierarchical library. Briefly, 350 ng *NheI/Esp31*-digested PCR product, encoding helices one and two [NN(G/T) randomization at six positions] was ligated into 6 μ g *MluI/NheI*-restricted pKN1 phagemid. The ligation mixture was digested with *SacI* and thereafter purified by extraction with phenol/chloroform/isoamylalcohol (25:24:1), washed twice with chloroform, ethanol precipitated and finally dissolved in 30 μ l sterile water. Samples of 2.5 μ l ligation mix were electroporated into 100 μ l electrocompetent RRIAM15 cells. Cells were grown in 1 ml SOC medium Tryptone Soy Broth (TSB) medium supplemented with 2% glucose, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 10 mM NaCl and 2.5 mM KCl for 1 h and plated on TYE (15 g/l agar, 8 g/l NaCl, 10 g/l tryptone and 5 g/l yeast extract) medium supplemented with 100 μ g/ml ampicillin and 2% glucose (TYE–Amp–Glu) and grown overnight at 37°C. Colonies were pooled in TSB medium supplemented with 100 μ g/ml ampicillin and 2% glucose (TSB–Amp–Glu) and frozen at –80°C in aliquots with addition of glycerol to a final concentration of 30%. The frequency of religated phagemid vector was determined by PCR screening using phagemid-specific primers RIT27 and NOKA-2. The diversity of the library was analysed by solid-phase DNA sequencing (Hultman *et al.*, 1991) using the same set of oligonucleotides but with a biotinylated version of NOKA-2.

Preparation of phage stocks

TSB–Amp–Glu (100 ml) was inoculated with approximately $0.5\text{--}2 \times 10^9$ cells from the constructed library or from between selection rounds and incubated with shaking at 37°C to give an A_{600} of 0.5. About 5×10^{10} M13K07 helper phage particles (New England Biolabs, Beverly, MA) were added to 10 ml

and incubated 30 min without shaking at 37°C. The superinfected cells were spun down and used to inoculate 100 ml TSB medium supplemented with 100 μ g/ml ampicillin, 25 μ g/ml kanamycin and 100 μ M isopropyl- β -D-thiogalactoside (IPTG). The culture was grown at 30°C for approximately 15 h before it was pelleted by centrifugation and subjected to rounds of PEG/NaCl precipitation. The phages were re-dissolved in 1 ml phosphate buffered saline (PBS; 50 mM phosphate, 100 mM NaCl, pH 7.2) and filtered through a 0.45 μ m filter. This procedure routinely resulted in a phage titre of $10^{11}\text{--}10^{12}$ phages/ml.

Selections

Selections were performed against an *in vivo* biotinylated Aff2–*Taq* DNA polymerase (Aff2–*Taq*) fusion protein, produced and purified as described earlier (Nilsson *et al.*, 1997), either in solution or immobilized onto streptavidin coated paramagnetic beads (Dynabeads® M280-SA, Dynal AS, Oslo, Norway).

Solid phase selections

For each panning round, a 5 mg portion of beads was washed twice in washing/binding buffer (W/B; 1 M NaCl, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA), followed by incubation with 40 μ g Aff2–*Taq* protein in 310 μ l storage solution (100 mM KCl, 100 μ M EDTA, 20 mM Tris–HCl, pH 8.0, 0.5% Tween 20 and 50% glycerol) overnight at 4°C, resulting in a target protein concentration of approximately 4 μ g Aff2–*Taq*/mg beads. Approximately 100 μ l phage stock was added to 5 mg beads with immobilized Aff2–*Taq*, previously washed four times in PBS with 0.1% Tween 20 (PBST), in a pretreated (PBST with 0.1% gelatin) Eppendorf tube together with 5 μ l 2% gelatin (final concentration 0.1%). The panning mixture was incubated with rotation overnight at 4°C and subsequently washed 10 times with 1 ml PBST. The beads were transferred to a new pretreated tube followed by 5 washes in 1 ml PBST. Bound phages were eluted with 500 μ l 0.1 M glycine–HCl, pH 2.2 for 20 min and the eluate was neutralized with 50 μ l 1 M Tris–HCl and 450 μ l PBS. Eluted phages were used to infect 10 ml log phase RRIAM15 cells for 20 min which were subsequently plated on TYE–Amp–Glu agar plates. This panning procedure was repeated during four rounds of selection.

Soluble selections

Eppendorf tubes, phage stocks and beads were pretreated with PBST containing 0.1% gelatin for 1 h before use in selections. Aff2–*Taq* protein was added to 100 μ l phage stock (PBS and 0.1% gelatin) to a final concentration of 10 nM or 1 nM and incubated on a rotator for 2.5 h at room temperature. After washing, 0.5 mg beads in PBS (washed twice in PBST) were added to the phage mix to a total volume of 200 μ l and incubated for 15 min as before. The beads were immediately washed 10 times with 1 ml portions of PBST and transferred to a new tube before bound phages were eluted during 10 min and neutralized and used for reinfection as described above.

Protein expression and purification

After four rounds of selections, 10 randomly picked clones from each protocol were subjected to solid-phase DNA sequencing (Hultman *et al.*, 1991; Nord *et al.*, 1997). Plasmid DNA from selected clones were prepared using a standard alkali lysis protocol (Sambrook *et al.*, 1989). Purified plasmid (phagemid) constructs were transformed into *Escherichia coli* strain RV308 for production of soluble affibody–ABD fusion proteins as follows. Overnight cultures in TSB–Amp supplemented with 5 g/l yeast extract at 37°C were used to inoculate (diluted

1:200) 100 ml TSB–Amp in baffled shake flasks and allowed to grow until $A_{600\text{nm}}$ reached 1 before protein production was induced with IPTG to a final concentration of 1 mM. Cultures were further grown at 25°C for 24 h before subjected to an osmotic shock treatment to release periplasmic proteins (Nygren *et al.*, 1988). Periplasmic fractions were loaded onto human serum albumin (HSA)–Sepharose columns for affinity chromatography (Nygren *et al.*, 1988). Proteins were analysed by SDS–PAGE on 20% polyacrylamide gels stained with Coomassie brilliant blue R-250 using the Pharmacia Phast™ system (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and the protein concentrations were determined spectrophotometrically at $A_{280\text{nm}}$.

Binding studies using BIAcore

Selected affibodies were affinity ranked using surface plasmon resonance (SPR) employing a BIAcore 2000 instrument (Biacore AB, Uppsala, Sweden). AmpliTaq DNA polymerase (Perkin Elmer, Norwalk, CT) or polyclonal hIgG (used as control) (Pharmacia & Upjohn AB, Stockholm, Sweden) was immobilized in different flow cells by amine coupling according to the manufacturer's recommendations onto the carboxylated dextran layer on surfaces of a CM-5 chip (research grade) resulting in approximately 2100 and 5900 resonance units (RU), respectively. A third flow cell surface was activated and deactivated for use as a blank during injections. Twelve newly selected *Taq* DNA polymerase binding affibody variants and the original $Z_{Taq4:8}$ affibody were injected over the surfaces at a concentration of 50 nM, diluted in HEPES buffered saline (HBS; 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.5% surfactant P-20) in duplicates in random order at a constant flow rate of 30 $\mu\text{l}/\text{min}$ for 7 min. After each injection, the surfaces were regenerated with 0.05% SDS. The five affibodies showing the highest equilibrium responses were selected for further binding analyses.

K_D values of selected affibodies were determined using a CM-5 chip surface containing 1500 RU of immobilized AmpliTaq DNA polymerase. Samples of *Taq* DNA polymerase binding affibodies were injected (duplicates in random order) at different concentrations ($Z_{Taq4:8}$: 0.75 nM to 25 μM ; second generation variants: 0.75 nM to 7.5 μM) at a flow rate of 20 $\mu\text{l}/\text{min}$. Injections were made during 5 min and the surfaces were regenerated using 0.05% SDS. Binding curves were based on the equilibrium responses obtained and K_D values were calculated using the BIAevaluation 2.1 software (Biacore AB). Kinetic rate constants were calculated using BIAevaluation 2.1 software assuming a one-to-one binding model.

Dimerization of the $Z_{TaqS1-1}$ affibody

The gene encoding the $Z_{TaqS1-1}$ variant was amplified in eight 50 μl PCR reactions using oligonucleotides NOKA-6 (5'-CCCCGTCGACCGTAGACAACAAATTCAACAAAG-3') and NOKA-7 (5'-CCCCCTCGAGCTTTTCGGCGCCTGAG-CATC-3') introducing recognition sites for the restriction enzymes *SalI* and *XhoI*, respectively. Fragments were pooled, ethanol precipitated, restricted and agarose gel purified before ligation into the vector pKN1- $Z_{TaqS1-1}$, restricted with *XhoI* and dephosphorylated. Transformants were analysed by solid phase DNA sequencing and plasmid DNA of the resulting construct pKN1-di- $Z_{TaqS1-1}$ was prepared using a Jetstar mini-prep column (Genomed, Inc., NC). Di- $Z_{TaqS1-1}$ –ABD fusion protein was produced and purified as described above. A comparative binding analysis between $Z_{TaqS1-1}$ –ABD and di- $Z_{TaqS1-1}$ –ABD fusion proteins was performed using a CM-5

	1	Helix 1	20	Helix 2	40	Helix 3	
Z wt	VDNKFNK	SQQNAPYELH	LPNLNE	EQRNFIQSLKD	DPSQ	SANLLAEAKLND	QAPK
$Z_{Taq4:1}$	-----	-KGE-VV--FR	-----	R-VK--A--Y-	-----	-----	-----
$Z_{Taq4:8}$	-----	-LGN-TW--FH	-----	F-AA--R--R-	-----	-----	-----
Beads 2 μM							
1/8 Z_{Taq} B2000-1	-----	-----	-----	G-S-VR--A--R-	-----	-----	-----
1/8 Z_{Taq} B2000-3	-----	-----	-----	A-VS--G--P-	-----	-----	-----
1/8 Z_{Taq} B2000-4	-----	-----	-----	G-G-VQ--H--R-	-----	-----	-----
1/8 Z_{Taq} B2000-5	-----	-----	-----	V-VE--D--R-	-----	-----	-----
1/8 Z_{Taq} B2000-6	-----	-----	-----	S-AA--Y--R-	-----	-----	-----
1/8 Z_{Taq} B2000-7	-----	-----	-----	S-VQ--R--Y-	-----	-----	-----
1/8 Z_{Taq} B2000-8	-----	-----	-----	A-VS--G--R-	-----	-----	-----
1/8 Z_{Taq} B2000-11	-----	-----	-----	A-VL--S--R-	-----	-----	-----
Solution 10 nM							
3/10 Z_{Taq} S10-2	-----	-----	-----	G-S-AA--Y--R-	-----	-----	-----
2/10 Z_{Taq} S10-3	-----	-----	-----	A-VS--G--R-	-----	-----	-----
1/10 Z_{Taq} S10-4	-----	-----	-----	S-VA--H--R-	-----	-----	-----
1/10 Z_{Taq} S10-5	-----	-----	-----	G-VK--A--H-	-----	-----	-----
1/10 Z_{Taq} S10-6	-----	-----	-----	G-S-AA--Y--L-	-----	-----	-----
1/10 Z_{Taq} S10-7	-----	-----	-----	Q-VR--H--R-	-----	-----	-----
1/10 Z_{Taq} S10-8	-----	-----	-----	G-VQ--H--R-	-----	-----	-----
Solution 1 nM							
8/10 Z_{Taq} S1-1	-----	-----	-----	G-V-VK--D--R-	-----	-----	-----
2/10 Z_{Taq} S1-2	-----	-----	-----	G-VQ--H--R-	-----	-----	-----

Fig. 1. Sequences of first and second generation *Taq* DNA polymerase specific affibody variants. Alignment of deduced amino acid sequences of the wild-type Z domain, two primarily identified *Taq* DNA polymerase binding affibodies ($Z_{Taq4:1}$ and $Z_{Taq4:8}$) and variants selected during the affinity maturation using different selection protocols.

sensor chip containing 2200 RU of immobilized AmpliTaq DNA polymerase and 4600 RU hIgG in different flow cells. A blank surface (200 RU) was prepared through activation/deactivation for use as an additional control. For calculations of binding kinetic parameters, samples of $Z_{TaqS1-1}$ and di- $Z_{TaqS1-1}$ –ABD fusion proteins were injected at random order during 5 min at a flow rate of 50 $\mu\text{l}/\text{min}$ at concentrations ranging between 0.32 nM and 25 μM ($Z_{TaqS1-1}$) or 64 pM and 5 μM (di- $Z_{TaqS1-1}$).

Results

Library design and selections

From a phagemid-displayed combinatorial library of the 58 residue, three-helix bundle staphylococcal protein A domain analogue Z, the selection of two affibody variants ($Z_{Taq4:1}$ and $Z_{Taq4:8}$) showing micromolar affinities (K_D) to *Taq* DNA polymerase have been previously described (Nord *et al.*, 1997). An alignment of their amino acid sequences showed that out of the 13 positions subjected to randomization using NN(G/T) degenerate codons, two positions in helix one [positions 10 (G) and 17 (F)] and a single position in connection to helix 2 [position 24 (G)] were identical in the two selected affibodies (Figure 1). Despite the relatively extensive amino acid substitutions compared with the wild-type Z domain, studies using circular dichroism spectroscopy had shown that the secondary structure contents in the two affibodies closely resembled that of the parental wild-type Z domain, suggesting similar overall α -helical content (Nord *et al.*, 1997; Tashiro *et al.*, 1997).

To investigate if related affibody variants could be isolated with higher binding affinities to *Taq* DNA polymerase, an affinity maturation strategy involving the construction of a secondary library followed by re-selection against the *Taq* DNA polymerase target was performed. A hierarchical phagemid library based on the affibody $Z_{Taq4:8}$ was constructed such that helix one (containing two identities compared with the $Z_{Taq4:1}$ affibody) was kept as initially selected from the naive library whereas the six positions previously variegated in helix two were re-randomized using NN(G/T) degenerate codons

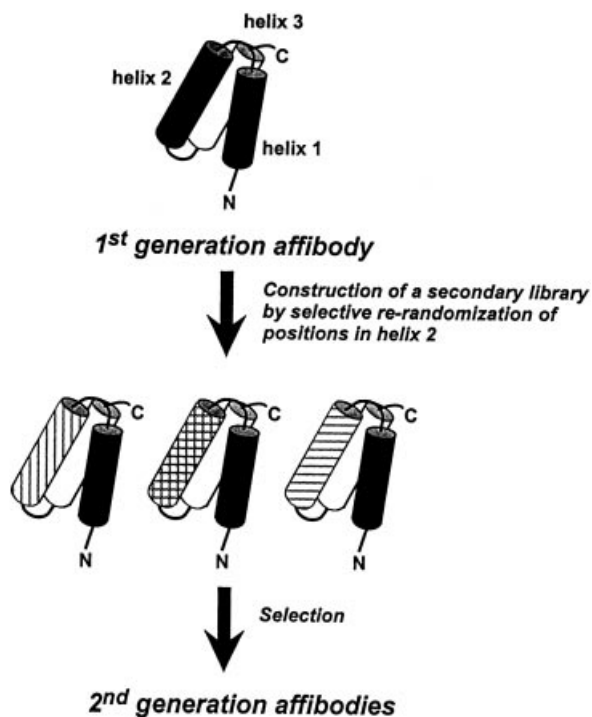


Fig. 2. The helix shuffling strategy. A primary affibody, selected from a naive library of the three-helix bundle Z domain constructed through combinatorial mutagenesis of 13 surface located positions in helices one and two, is subjected to affinity maturation using a helix shuffling strategy. From a secondary library, constructed through selective re-randomization of one of the helices (here helix two) involved in the binding interaction, second generation affibodies are selected.

including all 20 amino acids (Figure 2). This strategy should potentially allow for the selection of novel helix two variants contributing to *Taq* DNA polymerase binding, from a more extensively sampled diversity than present in the naive library.

The resulting library of approximately 1.6×10^7 members was subsequently subjected to affinity selections at different stringencies against a biotinylated *Taq* DNA polymerase fusion protein, either in solution (10 or 1 nM concentration) or with the target protein immobilized onto paramagnetic streptavidin beads (2 μ M concentration). After four rounds of selection, 10 clones were randomly picked from each selection strategy and analysed by DNA sequencing (Figure 1). The results showed that with increasing selection stringency, an increased convergence towards consensus sequences could be observed. The use of a high concentration (2 μ M) of bead-immobilized target protein for four rounds of selections did not result in any enrichment of frequent variants, whereas the use of low concentrations of the target protein in solution during selections resulted in the enrichment of a fewer number of variants, represented several times, e.g. $Z_{TaqS10-1}$ (3/10) and $Z_{TaqS1-1}$ (8/10). Interestingly, for 27/28 sequenced variants, a glycine residue present in both the original clones was re-selected at position 24. Also for other positions, amino acids initially found in the two parental affibodies were frequently re-selected, for example, valine or alanine at position 27, lysine or alanine at position 28 and arginine at position 35. A few variants were isolated regardless of the selection strategy, e.g. $Z_{TaqS1-1}$ from selections with 1 nM of the target in solution which was also found using bead-immobilized target protein (variant $Z_{TaqB2000-5}$), although at a lower frequency.

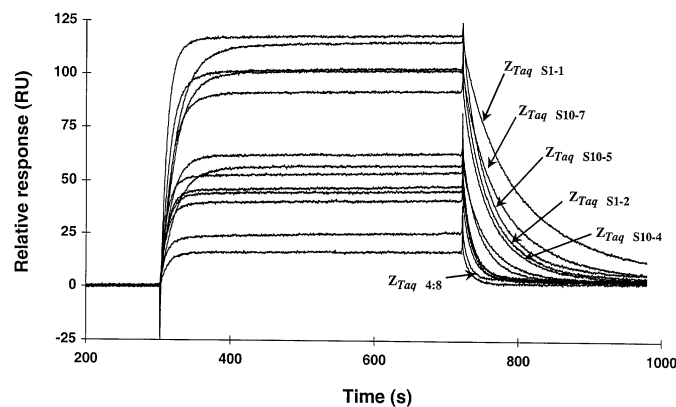


Fig. 3. Affinity ranking of twelve selected affibody variants. Overlay plot of sensorgrams obtained for twelve affinity matured *Taq* DNA polymerase specific affibodies when injected at an approximate concentration of 50 nM over a sensor chip surface with immobilized *Taq* DNA polymerase.

Biosensor binding analyses

To determine binding affinities using biosensor measurements, 12 variants were chosen for further analyses and produced as soluble affibody-ABD fusion proteins from their respective phagemid constructs and HSA-affinity purified employing the 5 kDa serum albumin binding fusion partner. Expression levels were in the range of 6–30 mg/l shake-flask culture and an SDS-PAGE analysis showed that the purified proteins were of expected size (approximately 13 kDa, data not shown). To obtain an initial ranking of binding affinities, the 12 affibodies were separately injected at an approximate concentration of 50 nM over a *Taq* DNA polymerase coated sensor chip surface, using the first generation $Z_{Taq4:8}$ affibody as reference. An overlay plot of recorded sensorgrams shows that all except one of the injected second generation affibodies bind to the target with higher affinities than the reference variant, as indicated by their higher equilibrium (plateau) responses (Figure 3). From a visual inspection of the post-injection parts of the sensorgrams, it could be seen that the interactions between the target and the newly selected variants were characterized by slower off-rate kinetics (Figure 3).

An analysis of complete binding curves, constructed from equilibrium responses obtained from injections of affibodies at different concentrations (0.75 nM to 7.5 μ M), showed that for five of the variants the affinities (K_D) were in the range of 30–50 nM (Figure 4; Table I). This corresponds to up to a 15-fold increase in affinity for the *Taq* DNA polymerase target as compared with the first generation $Z_{Taq4:8}$ binder. Size exclusion chromatography analysis of the affibody variant with the highest affinity for *Taq* DNA polymerase (clone $Z_{TaqS1-1}$), showed that the protein did not form higher aggregates, which otherwise could influence on the affinity determinations (data not shown). From calculated kinetic rate constants, determined from analyses of injection (on-rate) and post-injection phases (off-rate), it could be concluded that the increase in affinity primarily was a result from significantly slower off-rates (Table I).

Increasing the apparent affinity by affibody multimerization

To investigate if the apparent affinity for the *Taq* DNA polymerase target could be further increased by linking two affibody moieties in a dimeric construct, the affibody variant with the highest affinity for *Taq* DNA polymerase (clone $Z_{TaqS1-1}$) was produced as a genetically fused (head to tail)

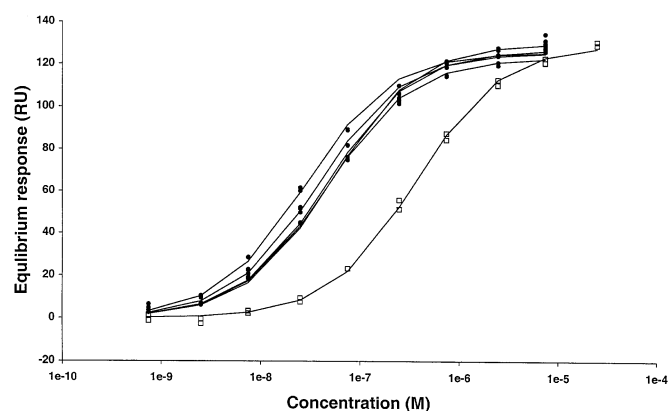


Fig. 4. Binding curves for the five affinity matured affibodies. Overlay plot of binding curves obtained after injection of different concentrations of the five second generation affibodies (●) showing the highest affinities for *Taq* DNA polymerase. As reference, also the ancestral; $Z_{Taq4:8}$ affibody (□) was included in the study.

Table I. Binding data for affinity matured affibodies

Clone	K_D^a (nM)	k_{diss} ($s^{-1} M^{-1}$)
$Z_{Taq4:8}$ (reference)	370	0.10
$Z_{TaqS1-1}$	25	0.014
$Z_{TaqS1-2}$	52	0.022
$Z_{TaqS10-4}$	48	0.024
$Z_{TaqS10-5}$	46	0.025
$Z_{TaqS10-7}$	37	0.017
di- $Z_{TaqS1-1}$	8 ^b	0.0025

^aData obtained from equilibrium response analyses.

^bData obtained from kinetic analyses (k_{on} and k_{diss}).

Note: affinity data for the dimeric construct is dependent on the density of immobilized target.

dimeric fusion protein, di- $Z_{TaqS1-1}$ -ABD and analysed for binding to sensor chip immobilized target protein. A comparison between response curves obtained for the dimeric variant and its monovalent counterpart clearly shows that the binding of the dimeric version is characterized by a higher equilibrium response and slower off-rate kinetics (Figure 5). The apparent affinity (K_D) of the dimeric di- $Z_{TaqS1-1}$ affibody was calculated to approximately 8 nM, corresponding to a threefold increase in affinity compared with the monomeric counterpart. However, in affinity determinations of interactions where avidity effects are present, the obtained numbers are dependent on the density of immobilized target protein and will therefore vary depending on experimental conditions. Nevertheless, the obtained increase in apparent affinity for the dimeric construct shows upon the potential of further engineering of affibody moieties to obtain, for example, multimeric constructs of high binding avidities.

Discussion

The constructed hierarchical library of the *Taq* DNA polymerase binding affibody $Z_{Taq4:8}$ involved a re-randomization of six positions in helix 2 using NN(G/T) degenerate codons including all 20 amino acids covered by 32 possible codons. Although the library only covered approximately 1.5% of all possible genetic variants, it corresponds to a more than 10^{10} -fold better sampling of helix two variants than in the original Z-lib1 library from which the two parental affibodies $Z_{Taq4:1}$ and $Z_{Taq4:8}$ initially were selected (Nord *et al.*, 1995, 1997).

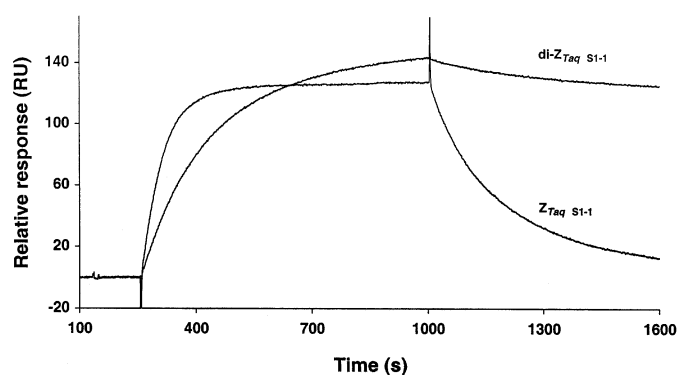


Fig. 5. Effect of dimerization of the $Z_{TaqS1-1}$ affibody. Overlay plot of sensorgrams obtained after injection of $Z_{TaqS1-1}$ -ABD or di- $Z_{TaqS1-1}$ -ABD affibody fusion proteins (40 nM of both constructs) over a sensor chip surface containing immobilized *Taq* DNA polymerase. Note the slower off-rate kinetics seen for the dimeric di- $Z_{TaqS1-1}$ -ABD construct indicating the influence of avidity effects in the binding.

Closely related sequences with a strong resemblance to the initially selected binders were found in the selected clones. A strong selection for glycine was observed at position 24 for all but one sequenced second generation variants. The positioning at the beginning of helix two could possibly suggest a structural role for this residue in these *Taq* DNA polymerase specific variants, rather than being involved in the actual binding interaction.

The affibody with the highest observed affinity (clone $Z_{TaqS1-1}$) was efficiently enriched using a low concentration of soluble target during selections. In this variant, four of the six randomized positions are occupied by amino acids previously observed at the corresponding positions in either of the two *Taq* DNA polymerase binding affibodies $Z_{Taq4:1}$ and $Z_{Taq4:8}$ previously isolated from the naive Z-library (Nord *et al.*, 1997). This suggests that the two initially selected *Taq* DNA polymerase binding affibodies as well as the newly selected and affinity matured variants bind to the same site on the target. Thus, during selections, *Taq* DNA polymerase binding determinants present in the unaltered helix one could have contributed to a directed selection of variants, resulting in a selection advantage over variants binding to other parts of the target mediated by helix two only. This notion is supported by results from affinity recovery of recombinant *Taq* DNA polymerase from crude *Escherichia coli* lysates in which both the $Z_{Taq4:8}$ and the $Z_{TaqS1-1}$ affibodies have been used as affinity ligands. Using both ligands, a *Taq* DNA polymerase degradation product is co-purified together with the full-length protein, indicating that both ligands recognize an 'epitope' located within this fragment (Nord, K., Gunneriusson, E., Uhlin, M. and Nygren, P.-Å., manuscript in preparation).

In this study, three different selection protocols were used, based on either having the target in solution (two different concentrations) or immobilized onto paramagnetic particles. Interestingly, the $Z_{TaqS1-1}$ variant, shown to have one of the highest affinities of the variants tested, was present among clones isolated using both strategies. However, a more selective enrichment (8/10) of this variant was seen using the target in solution, relative to when an immobilized target was used (1/10). This suggests that the soluble selection strategy was favourable for efficient selection of stronger binders from a background of lower affinity variants. Of the variants analysed, the five with the highest affinities were all isolated using this

strategy. The obtained increase in affinity of those affibody variants was found to primarily be a result from slower off-rate kinetics. These results are in accordance with results from affinity maturation of antibody fragments using similar selection principles (Schier *et al.*, 1996a).

Although affibodies with affinities in the micromolar range (K_D) can be readily selected from medium sized naive libraries of the Z-domain (Nord *et al.*, 1997; Hansson *et al.*, 1999) in some applications ligands of higher affinities could prove important. In this work, the affinity of a *Taq* DNA polymerase-specific affibody previously selected from a naive library of the Z-domain derived from staphylococcal protein A was increased 15-fold after a single round of affinity maturation directed to only one of the α -helices taking part in the interaction. This strategy resulted in a panel of related second generation variants with K_D values in the range of 30–50 nM. Interestingly, the affinity between the ancestral wild-type Z domain and its binding partner Fc of IgG has earlier been determined by several groups and K_D values between 10 and 40 nM have been reported (Nilsson *et al.*, 1994; Jendeborg *et al.*, 1995; Braisted and Wells, 1996; Jansson *et al.*, 1998). The fact that the dimeric version of the $Z_{TaqS1-1}$ showed cooperative binding effects also suggests that higher multimeric forms of selected affibodies could be constructed to make this novel class of ligands attractive reagents in different applications. Taken together, the results demonstrate that it is possible to isolate variants of the IgG binding Z domain, directed to an entirely different target, without loss of binding strength.

Acknowledgements

We thank Professor Torleif Hård for valuable discussions. This work was founded through grants from the NUTEK Immunotechnology Programme, The Swedish Foundation for Strategic Research (SSF), The Swedish Natural Science Research Council (NFR) and the NUTEK Centre for Bioprocess Technology (CBioPT).

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Received January 7, 1999; revised June 7, 1999; accepted June 18, 1999