Affinity maturation of a TNFα-binding Affibody molecule by Darwinian survival selection

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The introduction of different methodologies for the construction and screening of complex protein libraries has provided powerful means in protein engineering for the development of molecules with desired traits. A challenge faced in many situations is to adapt a given methodology for efficient and rapid identification of the most interesting variants present in a library. In the present study, the concept of Darwinian selection based on a growth advantage for clones having the desired trait has been investigated. Using a β-lactamase-based PCA (protein fragment complementation assay), affinity maturation of a TNFα (tumour necrosis factor α)-binding Affibody molecule with an initial 2 nM affinity for the target has been performed. Initial characterization of the PCA system, based on the affinity-driven reconstitution of β-lactamase activity in the periplasm of cells harbouring a library member showing affinity for a co-expressed target protein, showed that the system was responsive to promoter induction level, interaction affinity and applied selection pressure. Using combinatorial protein engineering principles, a 10^7 library of second-generation Affibody molecules was constructed and subjected to selection of improved variants by library growth in liquid culture. The results show that, after a pre-selection step on semi-solid medium to eliminate non-binding variants, present in the majority, two rounds of selection in liquid culture resulted in an enrichment for binders showing up to 8-fold higher affinity for the TNFα target than the ancestral variant. Biosensor analyses showed that the major factor for the improved affinity could be attributed to reduced off-rate constants.

Introduction

Systems capable of providing a clonal link between genotype and phenotype and used to select specific affinity proteins from large complex libraries of binder candidates can roughly be divided into two classes: display and non-display systems. A main difference between these classes is that in display systems, the protein library members are typically exposed for possible interaction *in vitro* with a target selector molecule, whereas in non-display systems, the interactions between library members and the target takes place inside a cell. Phage display and variants thereof have, since it was published almost a quarter of a century ago [1], been the most widely used display technique with numerous publications of successful projects (reviewed in [2]). More recently described are cell-surface-display systems utilizing yeast [3], *Escherichia coli* [4] or *Staphylococcus carnosus* [5] as host organisms and different methods based on mRNA [6,7] or DNA display [8]. The use of intracellular non-display systems such as the yeast two- or three-hybrid systems or PCAs (protein fragment complementation assays) [9] have mostly concentrated on the discovery and analysis of interactions between pairs of natural proteins [10–13]. However, novel systems utilizing the concept of PCA in different host cells for the selection of affinity proteins from large combinatorial libraries have also been described [14–18]. Common to the PCA systems is that an affinity-driven reconstitution of the activity of a genetically split reporter protein whose two subfragments are translated in fusion with a target protein and the individual library members respectively is used to select the library member variants showing affinity towards the chosen target. Although a variety of different read-outs for PCA are possible, including FRET (Förster resonance energy transfer) [19], fluorescence [20] and luminescence [21], only reporter activities providing cell survival capability have been reported for selection trials. These include the use of mDHFR (mouse dihydrofolate reductase) as a reporter enzyme in both *E. coli* and yeast, where selection is based on an acquired resistance to trimethoprim [22], or the use of TEM-1 β-lactamase, where selection is based on resistance to Amp (ampicillin) [16,18].

Key words: Affibody affinity protein, affinity maturation, β-lactamase, protein fragment complementation assay (PCA), tumour necrosis factor α (TNFα).

Abbreviations used: ABP, albumin-binding protein; Amp, ampicillin; Cml, chloramphenicol; HSA, human serum albumin; IPTG, isopropyl β-D-thiogalactoside; PCA, protein fragment complementation assay; Taz, tazobactam; Tc, tetracycline; TNFα, tumour necrosis factor α; TSB+Y, tryptic soy broth supplemented with yeast extract.

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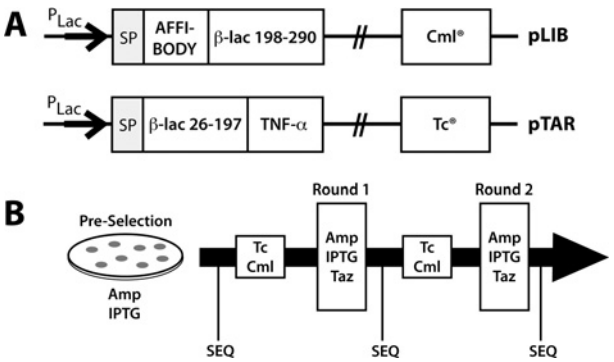

Figure 1 General description of vectors and the selection process

(**A**) Schematic representation of expression cassettes of the two vectors used in the present study. The library vector (pLIB, Cml-resistance marker) codes for Affibody proteins expressed as fused to the N-terminus of the amino acids 198–290 fragment of β-lactamase. The target vector (pTAR, Tc-resistance marker) codes for the amino acids 26–197 fragment of β-lactamase as fused to the N-terminus of the target protein (TNFα in the present study). Both vector products are under the control of inducible *lac* promoters (P_{Lac}) and directed to the periplasm by OmpA signal peptides (SP). (**B**) Overview of the selection strategy applied. A pre-selection on semi-solid medium supplemented with Amp was followed by two rounds of selection in liquid medium supplemented with both Amp and Taz. After each selection round, 96 clones were sequenced (SEQ) and, in between each selection round, overnight culture was performed in medium without PCA selection pressure (Tc and Cml only) in order to propagate the populations for a subsequent selection round.

We have described previously a β-lactamase-based PCA system [18] for the selection of Affibody molecules in which the target protein was co-expressed in the periplasm of *E. coli* as C-terminally fused to the first part (amino acids 26–197) of β-lactamase via a 15-residue-long linker with library members N-terminally fused via a similar linker to the latter part of the enzyme (amino acids 198–290) (Figure 1A). Both vector products were directed to the periplasm of *E. coli* by an OmpA signal peptide [23] and the inducible expression of both fusion proteins was under the control of a *lac* promoter. Using a naïve Affibody molecule library of 10^9 complexity, several TNFα (tumour necrosis factor α)-binding clones could be selected using a plate-based survival selection methodology [18]. However, an alternative and attractive format for simple selection, possible for systems based on cell survival, would be to perform the selection in liquid medium by co-culturing the entire library, relying on Darwinian principles of survival of the fittest as criteria for enrichment of desired variants by an expected capability for faster growth in an appropriate selective medium.

In the present study, we have investigated further the potential of the β-lactamase-based PCA system for use in selections related to protein engineering of binding proteins. The concept of competitive growth in selection medium as a selection format was explored for the affinity maturation of an Affibody molecule showing an initial moderate affinity for TNFα. The influence on the system performance from the induction level, interaction affinity and selection pressure was initially systematically investigated, leading to an optimized procedure resulting in the identification of variants showing an 8-fold increase in affinity for the TNFα target.

Materials and methods

Bacterial strains, chemicals and enzymes

E. coli strains RR1ΔM15 [*supE*44 *lacY*1 *lacZ* *ara*-14 *gal*K2 *xyl*-5 *mtl*-1 *leuB*6 *proA*2 Δ(*mrcC-mrr*) *recA*$^+$*rpsL*20 *thi*-1 *lambda*$^-$ F$'$ (*lacI*^{q}*lacZ*ΔM15)] [24] and BL21(DE3) [*B dcm ompT hsdS*($r_B^-m_B^-$) *gal*] were used for all work, as indicated. All chemicals were purchased from Sigma–Aldrich, and all enzymes were from New England Biolabs (NEB) unless indicated otherwise.

Pre-selection trials

Restricted PCR products coding for Z_{wt}, $Z_{TNF\alpha:185}$ and $Z_{TNF\alpha:2}$ Affibody molecules were inserted between the XhoI and SalI restriction sites in the pPCA-LIB vector [18], and correct clones were verified by sequencing. Library vectors were transformed to *E. coli* cells of strain RR1ΔM15 already harbouring pPCA-TARGET-TNF-α vector, and double-vector-carrying individuals were selected on Cml (chloramphenicol)- and Tc (tetracycline)-supplemented semi-solid medium. A single positive clone of each was selected and propagated by overnight culture supplemented with antibiotics as above, and 1 ml aliquots from all cultures were frozen for starter culture inoculation purposes.

Induction level trial

Portions of approx. 7×10^8 cells from an overnight culture of *E. coli* strain RR1ΔM15 harbouring TNFα target and $Z_{TNF\alpha:185}$ library vector grown in TSB+Y (tryptic soy broth supplemented with yeast extract) supplemented with Tc and Cml were inoculated into 20 ml of TSB+Y in nine 50 ml conical tubes. All nine cultures were supplemented with 25 μg/ml Amp and 50 ng/ml Taz (tazobactam) and, in addition, IPTG (isopropyl β-D-thiogalactoside) was added to each culture to a final concentration of 0, 5, 10, 20, 50, 100, 200, 500 or 1000 μM. Cultures were incubated at 37 °C on a horizontally shaking table at a speed of 150 rev./min. Samples from each culture were withdrawn about every 30 min and the D_{600} was determined.

Affinity discrimination trial

Approx. 10^8 cells from each of the three library–target vector combinations described above were mixed, and a small volume of the cell mixture was transferred on to plates and incubated overnight before 96 of the resulting colonies were sequenced regarding their Affibody molecule

insert to determine the composition of the mixture. The cell mixture culture was used at a final D_{600} of 0.07 to inoculate two 20 ml cultures in TSB+Y in 50 ml conical tubes. One was supplemented with 20 μg/ml Cml and 10 μg/ml Tc and incubated at 37 °C with horizontal shaking at 150 rev./min overnight. The other was supplemented with 250 μg/ml Amp, 50 ng/ml Taz and 20 μM IPTG and was incubated in a similar manner, but only for 200 min. After completion of cultivations, cell densities were measured, and small volumes were transferred to plates in order to receive separate colonies for sequencing purposes before 96 clones were collected from each plate and sequenced.

Construction of an affinity maturation library

Two partly complementary (by 15 nt) oligonucleotides were supplied by MWG Biotech: one was 87 nt long and the other was 85 nt long, the latter containing seven NNK degenerate codons as described in Figure 4. In order to receive double-stranded DNA fragments, 75 pmol of each oligonucleotide was mixed in 100 μl of 1× Phusion polymerase PCR buffer (Finnzymes) in a PCR tube also containing 300 μM each of dATP, dTTP, dCTP and dGTP. Using a thermocycler, the reaction mixture was heated to 98 °C before 1 μl (2 units) of Phusion polymerase (Finnzymes) was added. The reaction were subjected to a normal PCR thermocycling scheme, except that the annealing temperature, starting at 60 °C, was lowered decrementally for each cycle. The quality of the DNA was checked on an agarose gel, and the entire reaction mixture was purified using a PCR-clean-up spin column (QIAquick®, Qiagen). To obtain full-length restriction site-flanked fragments, 60 ng of the purified product was used as template in a 1 ml PCR volume also containing forward and reverse primers adding XhoI and NheI restriction sites respectively. PCR mixtures were purified as above. Approx. 25 μg each of the purified PCR product and the vector pPCA-LIB were doubly restricted using 150 units each of XhoI and NheI restriction enzymes. After restriction, a PCR-clean-up spin column was used to purify the PCR fragments, the vectors were purified by electrophoresis using a 0.7% agarose gel, and bands of correct size were cut out and gel-purified (JETquick kit, Genomed). Approx. 0.7 μg of the digested PCR product was ligated (T4 DNA ligase) to 2.5 μg of digested pPCA-lib vector in a 200 μl reaction mixture incubated for 3.5 h at room temperature (22 °C). DNA-binding magnetic beads (Chemagic PCR Pure Kit, Chemagen) were used to purify the ligation, and products were eluted in a total volume of 180 μl. To estimate the library size and quality, 1 μl of the purified ligation was transformed to 50 μl of chemically competent RR1ΔM15 cells. After 1 h of cultivation at 37 °C with shaking at 100 rev./min, 100 μl aliquots of different dilutions were transferred to semi-solid medium supplemented with 20 μg/ml of Cml and incubated overnight at 37 °C. The next morning, colonies formed were counted, and Affibody molecule-encoding inserts of 96 colonies were individually colony-PCR-propagated, and the resulting products were used as templates for DNA sequencing purposes. The remaining purified ligation was ethanol-precipitated and dissolved in 42 μl of pure water. The concentrated ligation products were electroporated into *E. coli* strain RR1ΔM15 cells in 20 transformations, each involving 2 μl of ligation mixture and 100 μl of competent cells. All transformants were pooled and transferred to a 250 ml flask, and 25 ml of SOC (super optimal broth with catabolite repression) medium [25] added. After 1 h of cultivation at 37 °C with shaking at 100 rev./min, 100 μl aliquots of different dilutions were transferred to semi-solid medium supplemented with 20 μg/ml Cml to determine the total number of transformants (approx. 10^7). The remaining culture was transferred to a 5 litre flask and 1 litre of TSB+Y supplemented with 20 μg/ml Cml was added. The resulting culture was left to grow overnight at 37 °C with shaking at 100 rev./min. The next morning, plasmids were prepared from a total of 5 ml of the culture. A total of 5 μg of the purified library vectors were in five transformations electroporated into *E. coli* strain RR1ΔM15 cells already harbouring pPCA-TARGET-TNFα vector. All transformants were pooled, and, after 1 h of cultivation at 37 °C with shaking at 100 rev./min, 100 μl aliquots of different dilutions were transferred to semi-solid medium supplemented with 20 μg/ml Cml and 10 μg/ml Tc to determine the total number of transformants (approx. 5×10^8). To the remaining culture, medium supplemented with 20 μg/ml Cml and 10 μg/ml Tc was added to a final volume of 100 ml. After incubation overnight at 37 °C with shaking at 150 rev./min, cells were pelleted, the supernatant was removed and medium supplemented with 15% (v/v) glycerol was added to a final cell density of 7×10^9 cells/ml. Aliquots of 2 ml of double-transformed cells concentrated in this way were frozen at −80 °C.

PCA selection of TNFα-binding Affibody molecule clones

A 2 ml aliquot of double-plasmid-carrying library cells was thawed and cultured overnight in medium without any selection pressure and approx. 7×10^8 cells from this culture were transferred to a large (140-mm-diameter) semi-solid medium selection plate supplemented with 30 μg/ml Amp, 10 μg/ml Tc, 10 μg/ml Cml and 1 mM IPTG. After 20 h of incubation at 37 °C, the colonies on the selection plate were harvested using a scraper and a total of 5 ml of TSB+Y, the recovered cells were moved to a 50 ml conical tube and supplemented with 15 ml of medium, Tc and Cml to final

concentrations of 10 and 20 μg/ml and cultivated at 37 °C and 150 rev./min for 9 h. At this time, the D_{600} had reached 5, and one 100 μl aliquot of the culture was transferred to semi-solid medium for colony sequencing purposes and 1 ml aliquots of the culture were frozen as above.

For the first selection step in liquid medium, a sample containing approx. 10^9 pre-selected cells was thawed and cultured in medium without selection pressure overnight. This culture, at a D_{600} of 0.09, was used to inoculate 20 ml of TSB+Y supplemented with 50 μg/ml Amp, 50 ng/ml Taz and 50 μM IPTG in a 50 ml conical tube. The cultivation was performed for 465 min with the tube being incubated at 37 °C with horizontal shaking at 150 rev./min, before 1 ml aliquots were frozen and a portion transferred to semi-solid medium for sequencing of colonies.

The second selection round was performed essentially as the first; again, a sample containing approx. 10^9 cells originating from the first round was thawed and first cultured in medium without selection pressure overnight. This culture was used at a D_{600} of 0.09 for inoculation of medium composed as above, but with 100 μg/ml Amp.

Analyses of selection steps

The Affibody molecule inserts of 96 clones each from the three selection steps were individually PCR-propagated, and the product was sequenced. The outcome from all steps were, together with $Z_{TNF\alpha:185}$ and the 'raw' library, cultivated separately in 50 ml conical tubes and TSB+Y supplemented with 50 μg/ml Amp, 50 ng/ml Taz and 50 μM IPTG. Roughly every 30 min, a sample was taken from each culture and the cell density was measured.

Protein production and purification

In separate PCRs, the Affibody molecule DNA sequences of 17 selected clones were amplified, together with the ancestral $Z_{TNF\alpha:2}$, using a forward primer adding a NotI restriction site and a reverse primer adding an AscI restriction site. Resulting fragments were sequentially digested and ligated into AscI- and NotI-cleaved and gel-purified pAff8c [26] expression vector. This vector codes for a product with N-terminal fusions of both a His_6 tag and an ABP (albumin-binding protein) tag. Products from the ligation step were transformed individually to *E. coli* cells of strain RR1ΔM15, PCR-screened for the insert of the correct size and sequenced. Plasmids prepared from cultures of clones with verified sequences were transformed to cells of strain BL21(DE3). Cultivation and automated purification under denaturing conditions with the use of the N-terminal His_6 tag were carried out, essentially as described previously [27]. For biosensor analysis purposes, the buffer of a 1 ml aliquot from each of the purified denatured samples was changed to HBS-EP [0.01 M Hepes (pH 7.4), 0.15 M NaCl, 3 mM EDTA and 0.005 % surfactant P20] using low-protein-binding centrifugal filter (VWR) microcentrifuge columns with a molecular-mass cut-off of 10 kDa. The correct protein size and purity of eluate were checked by SDS/PAGE (4–12 % gels) and protein concentration was determined using the absorbance values at 280 nm.

Analysis of selected binder candidates

All analyses were performed on a BIAcore® 2000 (Biacore AB) instrument, and proteins were covalently coupled to a CM-5 sensorchip surface with use of the amine coupling chemistry. For an initial selection of clones, approx. 2 μM solutions of all 17 Affibody molecule-binder candidates were sequentially injected over an HSA (human serum albumin)-coated surface at a flow rate of 10 μl/min. This was followed directly by an injection of TNFα (500 nM trimer) (Prospec).The response from a previously activated and ethanolamine-deactivated sensorchip surface was subtracted from all measurements to minimize systematic errors. With the use of Biacore evaluation software, the response profiles for all 17 candidates were determined, and five of them were selected for further studies based primarily on low dissociation rates. All five selected clones were, together with $Z_{TNF\alpha:2}$, immobilized individually by standard amine coupling chemistry to CM-5 sensorchip surfaces to a low density, and, at a flow rate of 20 μl/min, eight different concentrations (300 pM–300 nM) of TNFα were injected in duplicate until a steady-state response was reached. Using curve-fitting software (GraphPad Prism), equilibrium values in RU (relative units) were analysed by the four-parameters equation:

$$Y = (R_{max} \times X)/(K_d \times X) + NS \times X + C$$

where C and NS are constant and concentration-dependent unspecific binding values. The dissociation phase kinetics of all five analysed variants together with the original $Z_{TNF\alpha:2}$ clone were normalized and plotted against time (s), with the time set to zero at the end of the TNFα injection. The dissociation phases were evaluated according to a bivalent analyte interaction model using BIAeval 3.2 software (Biacore AB).

Results

Initial trials

The potential of the periplasmic β-lactamase-based PCA system for use in affinity maturation experiments based on competitive growth was first analysed by a series of model experiments. Assuming that differences in the growth rates of individual cells correlate to the number of functionally complemented β-lactamase molecules within each cell, then both the concentrations of the interaction pair members and the affinity of the interaction should be parameters to

which the system should be responsive. Overexpression could potentially lead to a saturation situation that theoretically would give similar growth properties to all clones in a library for which the molar periplasmic concentrations of the interacting moieties substantially exceed a given dissociation constant (K_d) of the interaction between the target and the library member.

To investigate such effects related to expression levels of PCA system components, a model system based on an affinity pair consisting of a TNFα target and $Z_{TNF\alpha:185}$, an Affibody molecule of sub-nanomolar affinity for TNFα described previously [28], was used. Cells containing this PCA pair were cultured in the presence of nine different concentrations of the IPTG inducer as well as β-lactam antibiotics in order to analyse the correlation between growth rate and induction levels. The analysis showed that, after an initial phase of approx. 150 min, during which the growth rates of all cultures were similar, the use of an IPTG concentration of 100 μM or higher seemed to saturate the system, since growth rate differences were very small (Figure 2A). On the other hand, the use of IPTG concentrations of 10 μM or lower resulted in very low growth rates. Interestingly, in a window of inducer concentrations corresponding to approx. 10–50 μM IPTG, significant differences in growth rates were observed between the different cultures, with increasing growth rates seen in concordance with higher IPTG concentrations (Figure 2A). Towards the end of this experiment (after 325 min of growth), the cell density of the culture grown at the highest IPTG concentration was 3-fold higher compared with the culture grown at the lowest IPTG concentration. Taken together, these results indicate that a saturation effect was indeed present at higher inducer concentrations, but also that the system showed a dynamic responsiveness to the rate of expression in a window of intermediate inducer concentrations. Importantly, this in turn indicated that, if the expression level could be uniformly regulated to any of these intermediate levels for all members in a library culture, and thus adjusted to subsaturation conditions, a growth rate dependent on affinity rather than expression rate should be expected.

To investigate this, a model PCA library was prepared involving a clonal mixture (1:3:1 proportions, as determined by DNA sequencing) of three different affinity protein pairs: the two Affibody molecules $Z_{TNF\alpha:185}$ [28] and $Z_{TNF\alpha:2}$ (P.-Å. Löfdahl and P.-Å. Nygren, unpublished work), and the wild-type Z domain (Z_{WT}), showing high, medium and insignificant affinity respectively for the co-expressed TNFα target protein. After an overnight co-culturing of the clonal mixture, using medium supplemented only with Cml and Tc, corresponding to the library (pLIB) and target vector (pTAR) resistance markers respectively, the relative proportions between the three clones was shown to be essentially

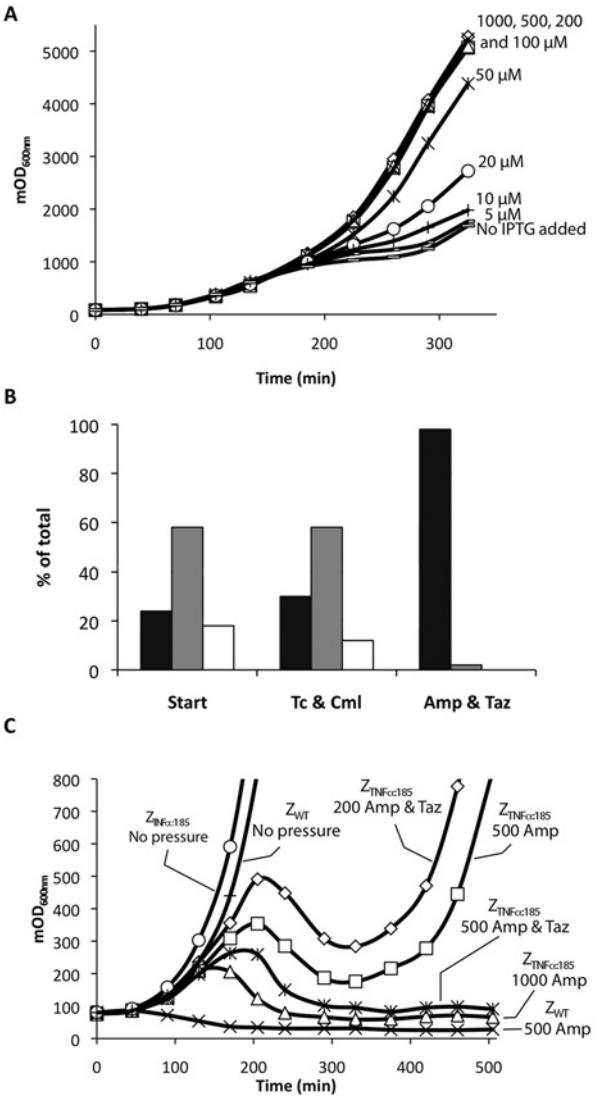

Figure 2 System parameter investigations

(**A**) Induction level effects. To investigate effects on cell growth related to applied induction levels of PCA system components, an affinity pair consisting of TNFα target and the sub-nanomolar binder $Z_{TNF\alpha:185}$ was cultivated in medium supplemented with Amp, Taz and different concentrations of IPTG ranging from zero to 1 mM, as indicated. Determinations of the cell density measured as the D_{600} (mOD_{600nm}) were performed every ~30 min. (**B**) Affinity dependence. A clonal mixture (1:3:1 proportions, as determined by DNA sequencing) of three different affinity protein pairs, showing high ($Z_{TNF\alpha:185}$ in black), medium ($Z_{TNF\alpha:2}$ in grey) and insignificant (Z_{WT} in white) affinity respectively for the co-expressed TNFα target protein, was subjected to liquid culture in medium with (Amp & Taz) or without (Tc & Cml) PCA selection pressure. Shown are the relative clonal composition of the cultures before and after the cultivations. (**C**) Selection pressure level effects. Two different model PCA pairs, consisting of TNFα together with either $Z_{TNF\alpha:185}$ or Z_{WT} (non-binding control), were subjected to liquid culture growth in medium of different compositions corresponding to different selection pressures or non-selection conditions as indicated, and the culture D_{600} (mOD_{600nm}) values monitored (see the text for details).

unchanged (Figure 2B). In contrast, a dramatic change in clonal proportions was observed when an aliquot of the same clonal mixture instead was cultured in PCA selection medium containing 20 μM IPTG inducer, 250 μg/ml Amp and 50 ng/ml of the β-lactamase inhibitor Taz. Here, in a sample collected after 200 min of growth, no less than 94 out of 96 sequenced colonies (98%) were found to be the high-affinity variant $Z_{TNF\alpha:185}$ and the remaining two were the medium-affinity clone $Z_{TNF\alpha:2}$. None of the clones analysed was of the Z_{WT} variant type (Figure 2B). This showed that growth of the clonal mixture in selection medium had resulted in an efficient enrichment of the high-affinity pair, over both the medium-affinity and non-affinity counterparts.

Furthermore, a series of experiments were performed where the effect on cell growth from applying different antibiotic pressures were investigated. Here, clones containing PCA pairs consisting of the TNFα target together with either the high-affinity clone $Z_{TNF\alpha:185}$ or the non-binding Z_{WT} variant were investigated. The behaviour for both pairs in medium containing only Tc and Cml was characterized by rapid growth, reaching cell densities (D_{600}) above 1 within 3 h (Figure 2C). However, when applying an Amp concentration of 200 μg/ml together with Taz, a more complex behaviour of the clone containing the high-affinity variant $Z_{TNF\alpha:185}$ was observed where the cell density first increased for approx. 3 h, reaching a D_{600} peak of approx. 0.4, after which it entered a phase of approx. 2.5 h during which the cell density decreased by approx. 50%, followed by a phase where the cell density again increased (Figure 2C). The appliance of a higher selection pressure using higher Amp concentrations (500 or 1000 μg/ml) resulted in both lower D_{600} peak values being reached and earlier observation of the peaks. Using the very high Amp concentration of 1000 μg/ml resulted in an early and very low peak cell density, followed by a cell density decrease period after which the culture never recovered. This behaviour was also seen for the clone containing the Z_{WT} variant using any Amp concentration. The results from the use of an alternative PCA pair consisting of the TNFα target and the medium-affinity clone $Z_{TNF\alpha:2}$ were similar to that observed for the high-affinity $Z_{TNF\alpha:185}$ variant, but with the difference that, for a given selection pressure, a lower and earlier-appearing peak value was observed (results not shown). This analysis showed that after an initial 'lag phase', the behaviour of the different cultures was significantly affected by the applied selection pressure, and that a higher affinity between target and binder correlated to a more pronounced resistance to cell lysis.

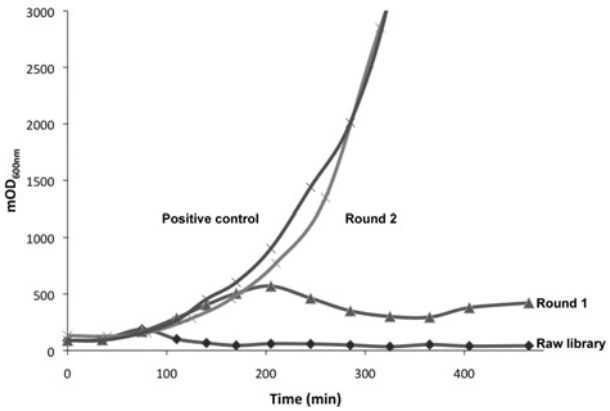

Figure 3 Monitoring of library selections in liquid medium

Cell density (mOD_{600nm}) is plotted against time for library cultures from the indicated different stages of the affinity maturation selection in liquid medium supplemented with 50 μg/ml Amp and 50 ng/ml Taz. The positive control corresponds to a culture of cells containing the high-affinity PCA pair of TNFα and $Z_{TNF\alpha:185}$, grown under identical conditions.

Selections from a complex affinity maturation library

Supported by the results from the model experiments, an effort was made to use the β-lactamase PCA system for an authentic affinity maturation of the $Z_{TNF\alpha:2}$ Affibody molecule showing medium affinity to TNFα. The sequence of this Affibody molecule shows partial homology with the high-affinity variant denoted $Z_{TNF\alpha:185}$, with three identities and one similarity among the six randomized positions in helix 2, whereas their sequences within helix 1 differed significantly (Figure 4). Hence, for the construction of the affinity maturation library based on the sequence of the variant $Z_{TNF\alpha:2}$, it was decided to fully re-randomize seven positions in the first helix using NNK codons, and locking the sequence of the second helix sequence as it was. To prepare library cell aliquots ready for selection trials, a prepared pool of library vectors was transformed into cells already harbouring the PCA target vector expressing TNFα. The quality of the resulting library, with a size of 10^7 members, was investigated by DNA sequencing of 96 random library vector clone inserts, and no unexpected anomalies were found.

In a first trial to use the raw library for selection of binders with improved affinities to the TNFα target, library aliquots were inoculated directly into selection medium containing different concentrations of Amp (50–100 μg/ml), 50 μM IPTG and supplemented or not with Taz and the cell density was monitored (Figure 3). Using this procedure, a very low growth rate of the culture was observed independently of the applied selection pressure. This possibly reflected that the frequency of target binding variants in the constructed library was very low, and that the behaviour of the culture was dominated by the vast majority of clones not capable of surviving under the applied selective pressure (results not shown). Therefore a pre-selection step was implemented involving the plating of

Clone name	Sequence *	Freq. P-1-2 (%) †	Affinity K_D (nM)	Off-rate k_d (s^{-1}) ‡
	1 10 20 30 40 50			
	• • • • • •			
$Z_{TNFα:2}$	VDNKFNKERAAATGEIANLPNLNALQFRAFIISLWDDPSQSANLLAEAKKLNDAQAPK		1.93	32.3 x 10^{-3}
$Z_{TNFα:185}$	--------LGW-IG--GT-----HQ-FR---L--W----------			
LIB §	VDNKFNKEXXXAXXEIXXLPNLNALQFRAFIISLWDDPSQSANLLAEAKKLNDAQAPK			
$Z_{TNFα:P1}$	--------CSS-IG--GG----------	0-3-3	0.23	8.2 x 10^{-3}
$Z_{TNFα:P2}$	--------VGW-FG--GA----------	0-3-0	0.28	6.1x 10^{-3}
$Z_{TNFα:P3}$	--------DAW-LG--AT----------	1-0-3	0.34	9.1 x 10^{-3}
$Z_{TNFα:P4}$	--------VGS-VG--GE----------	0-3-3	0.52	3.7 x 10^{-3}
$Z_{TNFα:P5}$	--------SGE-LG--GE----------	6-25-8	0.84	10.9 x 10^{-3}
$Z_{TNFα:P6}$	--------ISF-LG--GG----------	0-4-33	ND	ND
$Z_{TNFα:P7}$	--------CMS-VG--GQ----------	0-3-0	ND	ND
$Z_{TNFα:P8}$	--------SFG-VG--IA----------	0-5-3	ND	ND
$Z_{TNFα:P9}$	--------EMA-IG--IG----------	0-3-3	ND	ND
$Z_{TNFα:P10}$ ‖	-------DCYA-IS--YA----------	0-3-0	ND	ND
$Z_{TNFα:P11}$	--------RFG-IT--SG----------	0-3-0	ND	ND
$Z_{TNFα:P12}$ ‖	-------DYLD-FH--VG----------	0-0-8	ND	ND
$Z_{TNFα:P13}$	--------RFE-LT--SM----------	0-0-5	ND	ND
$Z_{TNFα:P14}$	--------YRL-IG--GV----------	0-0-3	ND	ND
$Z_{TNFα:P15}$	--------LGW-LG--AI----------	1-0-0	ND	ND
$Z_{TNFα:P16}$	--------VAL-VG--AN----------	0-1-0	ND	ND
$Z_{TNFα:P17}$	--------LAF-SG--GL----------	1-0-3	ND	ND
$Z_{TNFα:P18}$	--------ATK-LG--GN----------	0-0-3	ND	ND

Figure 4 Deduced amino acid sequences of the selected clones

ND, not determined *Amino acid sequences in one-letter-code of Affibody molecule proteins. Horizontal lines indicate identity with the parental $Z_{TNFα:2}$ clone. †Frequency in percentage of clonal occurrence, from the pre-, first and second selection rounds respectively. ‡The k_d value shown is the k_{d1} parameter obtained in BIAeval 3.2 using a bivalent analyte evaluation model. §Library randomization strategy; positions marked X were randomized using NNK codons. ‖These two clones contain E8D mutations, probably originating from errors in the degenerated oligonucleotides.

library cells (approx. 7×10^8 cells) on semi-solid medium supplemented with 1 mM IPTG, 10 μg/ml Tc, 10 μg/ml Cml and 30 μg/ml Amp (Figure 1B). The selection pressure in this step was deliberately held low since the aim was only to eliminate non-binding library clones before subsequent selection in liquid medium. Surviving cells were collected by scraping and were propagated overnight in non-selection medium containing Cml and Tc only. For a first PCA selection round in liquid culture, approx. 10^9 cells from after the pre-selection were inoculated into a selection medium containing Amp and Taz, and grown for approx. 8 h, with samples withdrawn after every 40 min for measurements of the D_{600} (Figure 3). For a second round of PCA selection, an aliquot of cells (approx. 10^9 cells) collected after 8 h in the first round was first propagated overnight without selection pressure and subsequently used to inoculate fresh selection medium of the same composition as used in the first round and the culture was again monitored for 8 h (Figure 3). During the first selection round, the library culture behaviour closely resembled what had previously been observed when growing the clone corresponding to the high-affinity pair TNFα–$Z_{TNFα:185}$ under a high PCA selection pressure (200 μg/ml Amp) (Figure 2C). During the second PCA selection round, the library culture showed an even higher growth capacity under the applied selection pressure (Figure 3). After approx. 220 min of growth, an aliquot was withdrawn for plating and DNA sequencing for comparison with samples collected from the raw library, from the pre-selection plate step and from cells collected after selection round one. Not surprisingly, all clones sequenced from the raw library were different. After the pre-selection step, two clones were found more than once. One of them (clone P5) was found in four copies (6% of total). After the first selection round in liquid medium, 47 different clones were found, and, of them, ten clones were found multiple times. The clone P5 was again the most abundant, being found in 19 copies (25% of total) (Figure 4). An analysis of the sequences of clones withdrawn at different stages in the process showed that the raw library as expected displayed an unbiased character, whereas a shift towards a more pronounced convergence could be observed as a result of each step in the selection process with the first round of selection in liquid culture contributing the most (Figure 5).

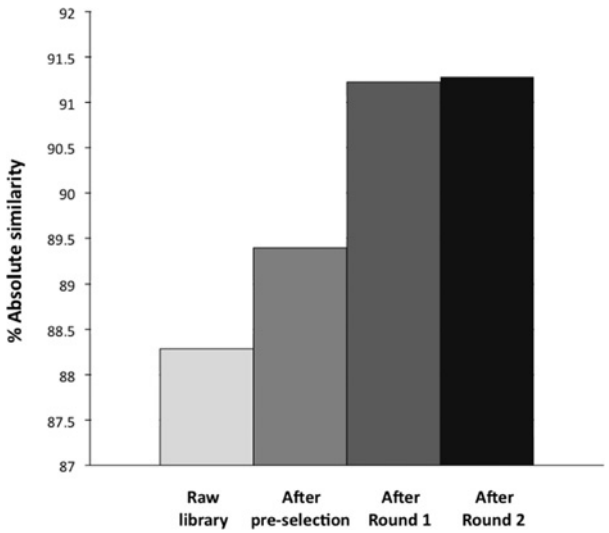

Figure 5 Monitoring library clone sequence similarity during stages of the selection process

The computer software Align X (Invitrogen) was used to compare the average absolute pairwise similarity between deduced Affibody molecule sequences present in pools derived from the different steps in the selection process. An average value of 88% would correspond to a minimum level of similarity, representing a situation where all analysed sequences differ from all the other analysed sequences at all seven randomized positions in the 58 residue protein (7/58 = 0.12). The value obtained for a set of sequences derived from the raw library was very close to this number, but after each new selection step, the population became more homogenous with the largest single contribution in this respect coming from the first selection round performed in liquid medium.

Affinity determination of selected clones

A total of 18 Affibody molecule binder candidates were selected for further study on the basis of their frequency of appearance and/or sequence homologies. A first biosensor-based affinity ranking was performed using the candidates recombinantly produced in *E. coli* as His_6–ABP-tagged proteins and non-covalently immobilized on to HSA-coated sensorchip surfaces. From binding kinetics profiles obtained after TNFα injections, five clones were chosen for further studies and affinity determination, based on their seemingly high on-rate and/or low off-rate characteristics. The five selected variants, as well as the ancestral affinity clone $Z_{TNF\alpha:2}$, were separately covalently coupled to sensorchip surfaces and equilibrium responses were recorded as different concentrations of TNFα were injected. From these values, the apparent dissociation constants (K_d) towards TNFα were calculated and found to be in the range 0.23–0.84 nM for the affinity-matured clones and 1.93 nM for the $Z_{TNF\alpha:2}$ variant. Thus the PCA-based affinity maturation had generated binders with up to an 8-fold improved affinity for the TNFα target (Figure 4). An analysis of the dissociation phase of the interactions indicated that all five variants analysed had lower off-rate kinetics than the original $Z_{TNF\alpha:2}$ variant (Figures 4 and 6).

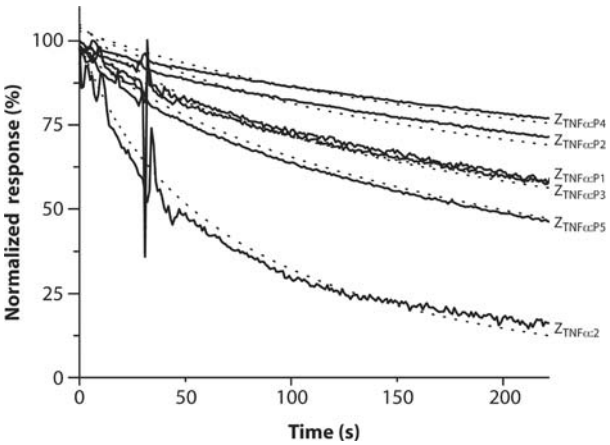

Figure 6 Dissociation rate analysis of selected clones using biosensor analysis

The dissociation phases kinetics obtained after analyte injections at 300 nM of all five analysed variants together with the original $Z_{TNF\alpha:2}$ clone were normalized and plotted against time (s) (solid lines). The parental clone $Z_{TNF\alpha:2}$ (bottom trace) is showing considerably higher off-rate kinetics than the PCA-selected variants (the five top traces). The slowest dissociation kinetics are shown by the clone $Z_{TNF\alpha:P4}$. Taking into account the ability of TNFα to self-associate into multimeric forms, the dissociation phases were evaluated according to a bivalent analyte interaction model using BIAeval 3.2 software (Biacore AB) from which the dissociation rate constants for the first binding step (k_{d1}) are reported in Figure 4.

Discussion

The results of the present study demonstrate the possibility of using a β-lactamase-based PCA system also for selections where there is a crucial need to discriminate between clones with high and comparable affinities for the target. Starting with an Affibody molecule showing moderate affinity towards TNFα (2 nM) we are able, from an affinity maturation library consisting of approx. 10^7 clones, to select binders with a close to 10-fold increase in affinity. The binding strength obtained is thus in parity with the best Affibody molecule binder towards this target described previously [28].

We have, in a previous study [18], discussed the use of the β-lactamase inhibitor Taz [29] in selections, and found it to be a valuable additive when performing selections on semi-solid medium (agar) owing to this compound's ability to almost completely prevent the formation of 'satellite opportunist' colonies and thus the survival of false-positive clones. On agar, these unwanted colonies appear in the vicinity of β-lactamase-producing colonies because of a local depletion of Amp originating from leakage of active enzyme to the medium. In the present study, Taz was added to the liquid selection medium, since, applying the reasoning behind this local β-lactamase-based depletion of Amp during selections on solid medium to liquid medium, a similar depletion could be due to the lower viscosity of the medium,

and the stirring of the cultures instead turns the situation into a global and homogeneous Amp depletion, resulting in all individual members of the library being challenged by a gradually lowered selection pressure. Under these conditions, Taz should be a valuable addition to prevent β-lactamase activity in the selection medium, since this compound, in contrast with Amp, is not degraded by β-lactamase activity.

In our previous study, vectors encoding the library of Affibody molecules were delivered to cells harbouring the TNFα target vector by phage infection [18]. Also in the present study, this gene-delivery principle was initially tested in a similar manner. However, in selection trials monitored by frequent cell density measurements, a lower rate of growth of such cultures compared with a culture of cells harbouring the same vectors introduced by electroporation could be noticed (results not shown). We believe this difference in behaviour is due to contamination by infection-competent helper phage in these cultures, originating from the mixture of phagemids and helper phage inevitably produced during the conversion of the library format using this method. Recently, cell lines especially designed for packing of phagemids were described, capable of providing all necessary phage proteins as encoded from a plasmid devoid of phage packaging signals [30]. Such technology would be very interesting for future PCA library constructions, since problems with helper phage contaminations by definition could be circumvented.

A possible explanation for the mechanism behind the temporal cell density during growth of library and model clones under selection pressure could be a relatively low rate of transfer of Amp (and most probably Taz) through the *E. coli* outer membrane [31]. According to such reasoning, an equilibrium between cell division and lysis must be present at the first plateau point. After this point, the periplasmic antibiotic concentration may become so high that lysis of cells dominates, after which the culture eventually comes to a point when the cell population is so enriched for highly PCA-active cells and the medium is so Amp-depleted owing to β-lactamase activity and thermal breakdown that division of cells again takes over. To elucidate this hypothesis further, it would be very interesting to follow the antibiotic concentration and/or the β-lactamase activity in the medium during the culture growth.

After the first selection step in liquid medium, the sequences of a total of 77 clones were determined and 47 different clones were found with the clone P5 found 19 times. All clones selected for affinity and kinetics evaluation showed a higher affinity towards TNFα than the previously selected clone $Z_{TNF\alpha:2}$. The kinetic data indicated a bias towards lower off-rate kinetics as a reason for the improved affinity (Figure 6). This is interesting since, in contrast with display systems, where typically the selection involves a considerable number of washing steps that selects for variants having low off-rate kinetics, PCA could be described as a system based on binding equilibrium. It could be speculated that a reason for this could be that a certain stability of the complexes formed is favourable for the substrate turnover process. A further development of the PCA system could be to use alternative strains or promoters, potentially allowing a more precise and reproducible control over low-level expression conditions, which could be expected to promote the selection of high-affinity pairs. For example, strains deficient in lactose permease and arabinose transport and metabolism functions may in conjunction with the use of arabinose operon promoters (pBAD) provide systems that allow for a fine-tuned induction control [32]. The production and periplasmic secretion of the target protein of interest is an integrated part of the β-lactamase PCA concept, and some proteins or fragments thereof may be more difficult than others to produce in sufficient quantities and/or export in soluble forms to the *E. coli* periplasm. In situations where such problems occur, alternative promoter and signal peptide choices, altered induction or growth conditions (e.g. medium composition, culture temperature) or the use of strains with certain engineered characteristics may provide solutions. For example, strains deficient in particular proteases or strains that provides extra protein-folding factors or rare codon tRNAs may be investigated [33,34]. As the β-lactamase PCA principle is not dependent on a particular choice of *E. coli* host, the large experience and technology toolbox available for this prokaryotic host has the potential to provide solutions to many situations, holding promise for the PCA technology to be of broad use for the investigations of protein–protein interactions [35].

Funding

The Swedish Research Council (VR) is acknowledged for financial support [grant number 50548301].

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Received 2 October 2009/13 January 2010; accepted 1 February 2010
Published as Immediate Publication 1 February 2010, doi:10.1042/BA20090274