Affinity maturation of a TNF- α binding affibody molecule by Darwinian survival selection

Running title:

Affinity maturation using β -lactamase PCA

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Abbreviations used: messenger ribonucleic acid, mRNA; deoxyribonucleic acid, DNA; protein fragment complementation assay, PCA; Förster resonance energy transfer, FRET; mouse dihydrofolate reductase, mDHFR; ampicillin, Amp; tumour necrosis factor alpha, TNF- α ; Isopropyl β -D-1-thiogalactopyranoside, IPTG; tetracycline, Tc; chloramphenicol, Cml; Immobilized metal ion affinity chromatography, IMAC; albumin binding protein, ABP; surface plasmon resonance, SPR; tryptic Soy Broth media supplemented with yeast extract, TSB+Y; polymerase chain reaction, PCR; super optimal broth with catabolite repression, SOC; molecular weight cut-off, MWCO; sodium dodecyl sulphate polyacrylamide gel electrophoresis, SDS-PAGE; HSA, Human Serum Albumin

ABSTRACT

The introduction of different methodologies for construction and screening of complex protein libraries has provided powerful means in protein engineering for 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 Protein Fragment Complementation Assay (PCA), an affinity maturation of a TNF- α binding affibody molecule of 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 showed that after a pre-selection step on semi-solid media to eliminate non-binding variants, present in majority, two rounds of selection in liquid culture resulted in an enrichment for binders showing up ten-fold higher affinity to the TNF- α target than the ancestral variant. Biosensor analyses showed that the major factor for the improved affinity could be attributed to reduced off-rate constants.

KEYWORDS (5 words): protein fragment complementation assay (PCA), β -lactamase, affinity maturation, affibody affinity proteins, tumour necrosis factor- α

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 has 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 [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 like the yeast two or three-hybrid systems or protein fragment complementation assays (PCAs) [9] and have mostly been 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 selection of affinity proteins from large combinatorial libraries have also been described [14-18]. Common for 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 [19], fluorescence [20] and luminescence [21], only reporter activities providing cell survival capability have been reported for selection trials. These include the use of mouse dihydrofolate reductase (mDHFR) as reporter enzyme in both *E. coli* and yeast and where selection is based on an acquired resistance to trimethoprim [22] or the use of TEM-1 beta-lactamase where selection is based on resistance to ampicillin [16, 18].

We have earlier described a β -lactamase-based PCA system [18] for 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 (aa 26-197) of β -lactamase via a 15 residues long linker with library members N-terminally fused via a similar linker to the latter part of the enzyme (aa 198-290) (Fig. 1). Both vector products were directed to the periplasm of *E. coli* by an Omp A signal peptide [23] and the inducible expression of both fusion-proteins was under control of a *lac* promoter. Using a naïve affibody molecule library of 10^9 complexity, several TNF- α binding clones could be selected using 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 media 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 of faster growth in an appropriate selective media.

In the present study, we have further investigated 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 media as selection format was explored for the affinity maturation of an affibody molecule showing an initial moderate affinity to TNF- α . The influence on the system performance from induction level, interaction affinity and selection pressure was initially systematically investigated, leading to an optimized procedure resulting in the identification of variants showing a ten-fold increase in affinity for the TNF- α target.

RESULTS

Initial trials

The potential of the periplasmic β -lactamase based protein fragment complementation assay (PCA) system for use in affinity maturation experiments based on competitive growth was first analyzed 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 enzyme 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 to. Over-expression 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 TNF- α target and $Z_{\text{TNF}\alpha:185}$, a previously described affibody molecule of sub-nanomolar affinity to TNF- α [24] was used. Cells containing this PCA pair were cultured in the presence of nine different concentrations of the IPTG inducer as well as beta-lactam antibiotics in order to analyze the correlation between growth rate and induction levels. The analysis showed that after an initial phase of about 150 minutes during which the growth rate of all cultures were similar, the use of an IPTG concentration of 100 μM or higher seemed to saturate the system since growth rates differences were very small (Fig. 2a). On the other hand, the use of IPTG concentrations of 10 μM or lower resulted in very slow growth rates. Interestingly, in a window of inducer concentrations corresponding to approximately 10-50 μM of IPTG, significant differences in growth rates were observed between the different cultures, with increasing growth rates seen in concordance with higher IPTG concentrations (Fig. 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 three-fold higher compared to the culture grown at the lowest IPTG concentration. Taken together, the results indicated 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 mix (20/60/20 proportions, as determined by DNA sequencing) of three different affinity protein pairs; the two affibody molecules $Z_{\text{TNF}\alpha:185}$ [24] and $Z_{\text{TNF}\alpha:2}$ (Löfdahl and Nygren, unpublished), and the wild type Z domain (Z_{WT}), showing high, medium and

insignificant affinity, respectively, to the co-expressed TNF- α target protein. After an overnight co-culturing of the clonal mix, using medium supplemented only with chloramphenicol (Cml) and tetracycline (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 unchanged (Fig. 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 a low 20 μ M of IPTG inducer, 250 μ g/ml of ampicillin and 50 ng/ml of the β -lactamase inhibitor tazobactam. Here, in a sample collected already after 200 minutes of growth no less than 94 out of 96 sequenced colonies (98%) were found to be the high affinity variant $Z_{\text{TNF}\alpha:185}$ and the remaining 2 % was the medium affinity clone $Z_{\text{TNF}\alpha:2}$. None of the analyzed clones were of the Z_{WT} variant type (Fig. 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.

Further, 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 TNF- α target together with either the high affinity clone $Z_{\text{TNF}\alpha:185}$ 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 ($\text{OD}_{600\text{nm}}$) above one within three hours (Fig 2c). However, when applying an ampicillin concentration of 200 μ g/ml together with tazobactam, a more complex behaviour of the clone containing the high affinity variant $Z_{\text{TNF}\alpha:185}$ was observed where the cell density first increased for approximately three hours reaching an $\text{OD}_{600\text{ nm}}$ peak of approximately 0.4, after which it entered a phase of approximately 2.5 hours during which the cell density decreased by approximately 50%, followed by a phase where the cell density again increased (Fig. 2c). The appliance of a higher selection pressure using higher ampicillin concentrations (500 or 1000 μ g/ml) resulted in both that lower $\text{OD}_{600\text{ nm}}$ peak values was reached and that the peaks were observed earlier in time. Using the very high ampicillin concentration of 1000 μ g/ml resulted in an early and very low cell density peak value 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 ampicillin concentration. The results from the use of an alternative PCA pair consisting of the TNF- α target and the medium affinity clone $Z_{\text{TNF}\alpha:2}$ were similar to that observed for the high affinity $Z_{\text{TNF}\alpha:185}$ variant, but with the difference that for a given selection pressure a lower and earlier appearing peak value was observed (data 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.

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 $Z_{\text{TNF}\alpha:2}$ affibody molecule showing medium affinity to TNF- α . The sequence of this affibody molecule shows partial homology to the high affinity variant denoted $Z_{\text{TNF}\alpha:185}$, with three identities and one similarity among the six randomized positions in helix 2, whereas their sequences within helix 1 differed significantly (Table 1). Hence, for the construction of the affinity maturation library based on the sequence of the variant $Z_{\text{TNF}\alpha:2}$ it was decided to fully re-randomize seven positions in the first helix using NN(G/T) 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 to cells already harbouring the PCA target vector expressing TNF- α . 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 TNF- α target, library aliquots were directly inoculated into different selection media containing different concentrations of Amp (50-100 $\mu\text{g/ml}$), 50 μM of IPTG and w/o addition of tazobactam and the cell density was followed (Fig. 3, curve a). Using this procedure, a very slow growth rate of the culture was observed independent 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 (data not shown). Therefore, a pre-selection step was implemented involving the plating of library cells (approx. 7×10^8 cells) on semi-solid media supplemented with 1 mM IPTG, Tc, Cml and Amp (30 $\mu\text{g/ml}$) (Fig. 1). 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 propagated over night in non-selection medium containing Cml and Tc only. For a first PCA selection round in liquid culture approximately 10^9 cells from after the pre-selection were inoculated into a selection medium containing Amp and tazobactam, and grown for ca. 8 hours, with samples withdrawn after every 40 minutes for OD_{600 nm} measurements (Fig. 3, curve b). For a second round of PCA selection, an aliquot of cells (approx. 10^9 cells) collected after 8 hours in the first round was first propagated over night under no selection pressure and subsequently used to inoculate fresh selection medium of the same composition as used in the first round and the culture again monitored for 8 hours (fig. 3, curve c). 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 TNF- α / $Z_{\text{TNF}\alpha:185}$ under a high PCA selection pressure (200 $\mu\text{g/ml}$ amp) (Fig. 2c). During the second PCA selection round, the library culture showed an even

higher growth capacity under the applied selection pressure (Fig. 3, curve d). After approximately 220 minutes of growth, an aliquot was withdrawn for plating and DNA sequencing for comparison to 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 2 clones were found more than once. One of them (clone no. P5) was found in four copies (6% of total). After the first selection round in liquid media 47 different clones were found and of them 10 clones were found multiple times. The clone no. P5 was again the most abundant and found in 19 copies (25% of total) (Table 1). 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 (Fig. 4).

Affinity determination of selected clones

A total of 18 affibody molecule binder candidates were selected for further studies based on 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 His₆-ABP (albumin binding protein)-tagged proteins and non-covalently immobilized onto human serum albumin (HSA)-coated sensor chip surfaces. From binding kinetics profiles obtained after TNF- α injections five clones were chosen for further studies and affinity determination, based on their seemingly fast on-rate and/or slow off-rate characteristics. The five selected variants, as well as the ancestral affinity clone Z_{TNF α :2}, were separately covalently coupled to biosensor chip surfaces and equilibrium responses were recorded as different concentrations of TNF- α were injected. From these values the apparent dissociation constant (K_D) towards TNF- α were calculated and found to be in the range 0.23 to 0.84 nM for the affinity matured clones and 1.93 nM for the Z_{TNF α :2} variant. Thus, the PCA-based affinity maturation had generated binders with up to a ten-fold improved affinity for the TNF- α target (Table 1). An analysis of the dissociation phase of the interactions indicated that all five analyzed variants had slower off-rate kinetics than the original Z_{TNF α :2} variant (Fig. 5 and Table 1).

DISCUSSION

The results presented here demonstrate the possibility to use 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 TNF- α (2 nM) we are able, from an affinity maturation library consisting of about 10^7 clones, to select binders with an almost tenfold increase of affinity. , The obtained binding strength is thus in parity with the hitherto described best affibody molecule binder towards this target [24].

We have in a previous study [18] discussed the use of the β -lactamase inhibitor tazobactam [25] in selections, and found it to be a valuable additive when performing selections on semi-solid media (agar) due 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 beta-lactamase producing colonies due to a local depletion of ampicillin originating from leakage of active enzyme to the media. In the present study tazobactam was added to the liquid selection medium, since a transfer of the reasoning around a local β -lactamase based depletion of ampicillin during selections on solid media to liquid media could due to the lower viscosity of the media and the stirring of the cultures instead turn the situation into a global and homogeneous ampicillin depletion resulting in that all individual members of the library are challenged by a gradually lowered selection pressure. Under these conditions tazobactam should be a valuable addition to prevent β -lactamase activity in the selection medium, since this compound in contrary to ampicillin is not degraded by β -lactamase activity.

In our previous study vectors encoding the library of affibody molecules were delivered to cells harbouring the TNF- α target vector by phage infection. Also in the present study, this gene delivery principle was initially tested in a similar manner. However, in selection trials monitored by continuous cell density measurements a slower rate of growth of such cultures compared to a culture of cells harbouring the same vectors introduced by electroporation could be noticed (data not shown). We believe this difference in behaviour is due to contamination by infection competent helper phage in these cultures, originating from the mix of phagemids and helper phage inevitably produced during conversion of 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 [26]. Such technology would be very interesting for future PCA library constructions since problems with helper phage contaminations per 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 slow

rate of transfer of ampicillin (and most probably tazobactam) through the *E. coli* outer membrane [27]. 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 media is so ampicillin-depleted due to β -lactamase activity and thermal breakdown that division of cells again takes overhand. To elucidate this hypothesis further, it would be very interesting to follow the antibiotic concentration and/or the beta-lactamase activity in the media during the culture growth.

After the first selection step in liquid medium the sequences of totally 77 clones were determined and 47 different clones were found with the clone no. P5 found 19 times. All clones selected for affinity and kinetics evaluation showed a higher affinity towards TNF- α than the previously selected clone $Z_{\text{TNF}\alpha:2}$. The kinetic data indicated a bias towards slower off-rate kinetics as a reason for the improved affinity (Fig. 5). This is interesting since in contrast to display systems, where typically the selection involves a considerable number of washing steps that selects for variants having slow 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 formed complexes is favourable for the substrate turnover process.

MATERIALS AND METHODS

Bacterial strains, chemicals and enzymes

Escherichia coli strains RR1 Δ M15 [*supE44 lacY1 lacZ ara-14 galK2 xyl-5 mtl-1 leuB6 proA2 Δ (mrcC-mrr) recA⁺rpsL20 thi-1 lambda⁻ F' (lacI^qlacZ Δ M15)*] [28] and BL 21 (DE3) [*B dcm ompT hsdS(r_B⁻m_B⁻) gal*] were used for all work, as indicated. All chemicals were delivered from Sigma-Aldrich and all enzymes from New England Biolabs (NEB) if nothing else is indicated.

Pre-selection trials

Restricted PCR product coding for Zwt, Z_{TNF α :185} and Z_{TNF α :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 RR1 Δ M15 already harbouring pPCA-TARGET-TNF- α vector and double vector carrying individuals were selected on Cml and Tc supplemented semi-solid media. A single positive clone of each were selected and propagated in an over-night culture supplemented with antibiotics as above and aliquots from all cultures were frozen for starter culture inoculation purposes.

Induction level trial

Portions of about 7×10^8 cells from an overnight culture of *E. coli* of strain RR1 Δ M15 harbouring TNF- α target and Z_{TNF α :185} library vector grown in TSB+Y media supplemented with Tc and Cml were inoculated to 20 ml of TSB+Y media in nine 50 ml conical tubes. All nine cultures were supplemented with 25 μ g/ml Amp and 50 ng/ml tazobactam and in addition to this IPTG was added to each culture to a final concentration of 0, 5, 10, 20, 50, 100, 200, 500 or 1000 μ M. Cultures were incubated at 37°C on a horizontally shaking table at a speed of 150 rpm. Samples from each culture were withdrawn about every 30 min and OD₆₀₀ determined.

Affinity discrimination trial

About 10^8 cells from each of the three library-target vector-combinations described above were mixed and a small volume of the cell-mixture were transferred to plates, incubated over-night and the next morning 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 to a final OD₆₀₀ of 0.07 inoculate two TSB+Y media 20 ml cultures in 50 ml conical tubes, one supplemented with 20 μ g/ml Cml and 10 μ g/ml Tc incubated at 37°C horizontally shaking at 150 rpm over-night, the other supplemented with 250 μ g/ml Amp, 50 ng/ml Tazobactam and 20 μ M of IPTG were 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 after what 96 clones collected from each plate were sequenced.

Construction of affinity maturation library

Two partly complementary (15 nucleotides) oligonucleotides were ordered (MWG Biotech AG); one 87 nucleotides long and one 85 nucleotides long, the latter containing seven NNK degenerate codons as described. In order to receive double stranded DNA fragments, 75 pmoles of each oligo were mixed in a 100 μ l 1x Phusion polymerase PCR buffer (Finnzymes) reaction in a PCR tube also containing 300 μ M each of dATP, dTTP, dCTP and dGTP. With use of a thermocycler the reaction was heated to 98°C when 1 μ l (2U) of Phusion polymerase (Finnzymes) was added. The reaction were subjected to a normal PCR thermo-cycling scheme except for that the annealing temperature, starting at 60°C was decrementally lowered for each cycle. The quality of the DNA was checked on an agarose gel and the entire reaction was purified using a PCR-cleanup spin column (QIAquick®). To receive full-length restriction site-flanked fragments 60 ng of the purified product was used as template in a 1 ml PCR reaction also containing forward and reverse primers adding XhoI and NheI restriction sites respectively, PCR reactions were purified as above. About 25 μ g each of the purified PCR product and the vector pPCA-LIB were doubly restricted using 150 U each of XhoI and NheI restriction enzymes. After restriction a PCR-cleanup spin column was used to purify the PCR fragments, the vector were purified by electrophoresis by the use of a 0.7% agarose gel, band of correct size were cut out and gel-purified (JETquick kit, Genomed). Approximately 0.7 μ g of the digested PCR product was ligated (T4 DNA ligase) to 2.5 μ g digested pPCA-lib vector in a 200 μ l reaction incubated for 3.5 h at room temperature. DNA binding magnetic beads (Chemagic PCR Pure Kit, Chemagen) were used to purify the ligation and product was eluted in a total volume of 180 μ l. To estimate the library size and quality 1 μ l of the purified ligation was transformed to 50 μ l of chemically competent RR1 Δ M15 cells. After 1 h of cultivation at 37°C and 100 rpm shaking, aliquots were transferred to semi-solid media supplemented with 20 μ g/ml of Cml and incubated overnight at 37°C. The next morning formed colonies were counted and affibody molecule encoding inserts of in total 96 colonies were individually colony PCR propagated and the resulting products used as templates for DNA sequencing purposes. Remaining purified ligation was ethanol precipitated and dissolved in 42 μ l of pure water. The this way concentrated ligation was electroporated to *E. coli* strain RR1 Δ M15 cells in 20 transformations, each involving 2 μ l ligation mixture and 100 μ l of competent cells. All transformations were pooled, transferred to a 250 ml flask and 25 ml of SOC media [29] added. After 1 h of cultivation at 37°C and 100 rpm shaking, aliquots were transferred to semi-solid media supplemented with 20 μ g/ml of Cml to determine the total number of transformants (about 10⁷). The remaining culture was transferred to a 5 l flask and 1 l of TSB+Y supplemented with 20 μ g/ml of Cml was added. The resulting culture was left to grow over night at 37°C and 100 rpm and next morning plasmids were prepared from a total of 5 ml of the culture. A total of 5 μ g of the purified library vectors were in 5 transformations electroporated to *E. coli* strain RR1 Δ M15 cells already harbouring pPCA-TARGET-TNF- α vector. All

transformations were pooled and after 1 h of cultivation at 37°C and 100 rpm shaking, aliquots were transferred to semi-solid media supplemented with 20 μ g/ml of Cml and 10 μ g/ml of Tc to determine the total number of transformants (about 5×10^8). To the remaining culture media supplemented with 20 μ g/ml of Cml and 10 μ g/ml of Tc were added to a final volume of 100 ml. After incubation over-night at 150 rpm and 37°C cells were pelleted, supernatant removed and media supplemented with 15% of glycerol added to a final cell density of 7×10^9 cells/ml. Aliquots of in this way concentrated double plasmid carrying cells were frozen at -80°C.

PCA-selection of TNF- α binding affibody molecule clones

An aliquot of double plasmid carrying library cells were thawed and cultured overnight in media without any selection pressure and about 7×10^8 cells from this culture were transferred to a large (140 mm diameter) semi-solid media selection plate supplemented with 30 μ g/ml Amp, 10 μ g/ml Tc, 10 μ g/ml Cml and 1 mM of IPTG. After 20h of incubation at 37°C the colonies on the selection plate were harvested using a scraper and in total 5 ml of TSB+Y media, the recovered cells were moved to a 50 ml conical tube and supplemented with: 15 additional ml of media, Tc and Cml to a final concentration of 10 respectively 20 μ g/ml and cultivated at 37°C and 150 rpm for 9h at what time OD_{600nm} had reached 5 after what a portion of the culture were transferred to semi-solid media for colony sequencing purposes and aliquots of the culture were frozen as above. For the first selection step in liquid media a sample containing about 10^9 preselected cells was thawed and cultured in media without selection pressure overnight, this culture were used to an OD_{600nm} of 0.09 inoculate a 20 ml TSB+Y media supplemented with 50 μ g/ml Amp, 50 ng/ml tazobactam and 50 μ M of IPTG in a 50 ml conical tube. The cultivation was performed for 465 min with tube 150 rpm horizontally shaking at 37°C where after aliquots were frozen and a portion transferred to semi-solid media for sequencing of colonies. The second selection round was performed essentially as the first, again a sample containing about 10^9 cells originating from the first round was thawed and first cultured in media without selection pressure overnight and this culture was used to obtain an OD_{600nm} of 0.09 for inoculation of media composed as above but with a concentration of Amp of 100 μ g/ml.

Analyses of selection steps

The affibody molecule inserts of 96 clones each from the three selection steps were individually PCR propagated and the product sequenced. The outcome from all steps were also together with Z_{TNF α :185} and the “raw” library separately cultivated in 50 ml conical tubes and TSB+Y media supplemented with 50 μ g/ml Amp, 50 ng/ml tazobactam and 50 μ M of IPTG. About every 30 min a sample was taken from each culture and the cell density measured (Fig. 4).

Protein production and purification

In separate PCR reactions the affibody molecule DNA sequences of 17 selected clones were together with the ancestral Z_{TNF α :2} amplified using a forward primer

adding a NotI 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 [30] expression vector. This vector codes for a product with N-terminal fusions of both a hexahistidyl (His₆) tag and an ABP (Albumin Binding Protein) tag. Products from the ligation step were individually transformed to *E. coli* cells of strain RR1 Δ M15, PCR screened for insert of 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 use of the N-terminal His₆ tag were carried out, essentially as described earlier [31]. For biosensor analysis purposes, the buffer of an 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, 0.005% surfactant P20) using low protein binding VWR Centrifugal Filter (VWR) micro centrifuge columns with a MWCO of 10 kDa. Correct protein size and purity of eluate were checked on SDS-PAGE gels and protein concentration determined using the 280 nm absorbance.

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 chip surface with use of the amine coupling chemistry. For an introductory selection about 2 μ M dilutions of all 17 affibody molecule binder candidates were with a flow-rate of 10 μ l/min sequentially injected over a HSA (Human Serum Albumin) immobilized surface. This was directly followed by an injection of TNF- α (500 nM trimer) (Prospec) the response from a previously activated and ethanolamine deactivated chip surface were subtracted from all measurements to minimize systematical errors. With use of Biacore evaluation software the response profiles for all 17 candidates were determined and five of them were selected for further studies primarily based on slow dissociation rates. All five selected clones were together with Z_{TNF α :2} individually immobilized to CM-5 chip surfaces to a low density and at a flow rate of 20 μ l/min 8 different concentrations (300 pM to 300 nM) of TNF- α were in duplicate injected until a steady-state response was reached. With the use of a curve fitting software (GraphPad Prism) the 4 parameters equation $Y=(R_{\max} * X)/(K_D * X) + NS * X + C$, where C and NS are recognized as constant and concentration dependent unspecific binding respectively were fitted to the equilibrium RU values.

ACKNOWLEDGEMENTS

The Swedish Research Council (VR) is acknowledged for financial support.

TABLES

Table 1. Listing of deduced amino acid sequences of the selected clones.

Clone name	Sequence ¹						Freq. ² P-1-2 (%)	Affinity K _D (nM)	Off- rate k _d (s ⁻¹)
	1	10	20	30	40	50			
Z _{TNFα} :2	VDNKFNKERAAATGEIANLPNLNALQFRAFIISLWDDPSQSANLLAEAKKLNDQAQPK							1.93	6.7x10 ⁻³
Z _{TNFα} :185	-----LGW-IG--GT----HQ-FR---L-W-----								
LIB ³	VDNKFNKEXXXXXXEIXXLPNLNALQFRAFIISLWDDPSQSANLLAEAKKLNDQAQPK								
Z _{TNFα} :P1	-----CSS-IG--GG-----						0-3-3	0.23	1.6x10 ⁻³
Z _{TNFα} :P2	-----VGW-FG--GA-----						0-3-0	0.28	1.1x10 ⁻³
Z _{TNFα} :P3	-----DAW-LG--AT-----						1-0-3	0.34	1.7x10 ⁻³
Z _{TNFα} :P4	-----VGS-VG--GE-----						0-3-3	0.52	0.9x10 ⁻³
Z _{TNFα} :P5	-----SGE-LG--GE-----						6-25-8	0.84	2.6x10 ⁻³
Z _{TNFα} :P6	-----ISF-LG--GG-----						0-4-33	ND	
Z _{TNFα} :P7	-----CMS-VG--GQ-----						0-3-0	ND	
Z _{TNFα} :P8	-----SFG-VG--IA-----						0-5-3	ND	
Z _{TNFα} :P9	-----EMA-IG--IG-----						0-3-3	ND	
Z _{TNFα} :P10	-----DCYA-IS--YA----- ⁴						0-3-0	ND	
Z _{TNFα} :P11	-----RFG-IT--SG-----						0-3-0	ND	
Z _{TNFα} :P12	-----DYLD-FH--VG----- ⁴						0-0-8	ND	
Z _{TNFα} :P13	-----RFE-LT--SM-----						0-0-5	ND	
Z _{TNFα} :P14	-----YRL-IG--GV-----						0-0-3	ND	
Z _{TNFα} :P15	-----LGW-LG--AI-----						1-0-0	ND	
Z _{TNFα} :P16	-----VAL-VG--AN-----						0-1-0	ND	
Z _{TNFα} :P17	-----LAF-SG--GL-----						1-0-3	ND	
Z _{TNFα} :P18	-----ATK-LG--GN-----						0-0-3	ND	

(1) Amino acids sequences in one-letter-code of affibody molecule proteins. Horizontal lines indicate identity to the parental Z_{TNFα-2} clone.

(2) Frequency in percent of clonal occurrence, from the pre, first and second selection round respectively.

(3) Library randomization strategy, positions in red marked X were randomized using NN(G/T) codons

(4) These two clones contain E8D mutations, probably originating from errors in the synthesized oligonucleotides.

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Figure 1. Expression cassette design and selection process scheme. (A) Schematic representation of expression cassettes of the two vectors used in this study. The library vector (pLIB, Cml resistance marker) codes for affibody proteins expressed as fused to the amino-terminus of the aa 198-290 fragment of β -lactamase. The target vector (pTAR, Tc resistance marker) codes for the aa 26-197 fragment of β -lactamase as fused to the amino-terminus of the target protein (in this study TNF- α). Both vector products are under the control of inducible *lac* promoters (P_{Lac}) and directed to the periplasm by Omp A 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 tazobactam. After each selection round, 96 clones were sequenced and in between each selection round over-night culturing was performed in medium without PCA selection pressure (Tc and Cml only) in order to propagate the populations for a subsequent selection round.

Figure 2. System parameter investigations. (A) **Induction level effects.** To investigate effects related to applied induction levels of PCA system components, an affinity pair consisting of TNF- α target and the sub-nanomolar binder $Z_{\text{TNF}\alpha:185}$ was cultivated in media supplemented with Amp, tazobactam and different concentrations of IPTG (given in the legend). Determinations of the cell density measured as OD_{600nm} were performed about every 30 min. (B) **Affinity dependence.** A clonal mix (20/ 60/20 proportions, as determined by DNA sequencing) of three different affinity protein pairs, showing high ($Z_{\text{TNF}\alpha:185}$), medium ($Z_{\text{TNF}\alpha:2}$) and insignificant (Z_{WT}) affinity, respectively, to the co-expressed TNF- α target protein was subjected to liquid culture in media with (Amp & Taz) or without (Tc & Cml) selection pressure. The diagram shows the relative clonal composition of the cultures before and after the cultivations. (C) **Selection pressure level effects.** Two different model PCA pairs, consisting of TNF- α and $Z_{\text{TNF}\alpha:185}$ or Z_{WT} (non-binding control), respectively were subjected to liquid culture growth in media of different compositions corresponding to different selection pressures or non-selection conditions and the culture OD_{600 nm} values monitored (see text for details).

Figure 3. Monitoring of liquid medium library selections. In the diagram the cell density vs. time of library cultures from different stages of the affinity maturation selection in liquid medium supplemented with 50 μ g/ml Amp and 50 ng/ml of tazobactam is plotted. Curve (a): raw library; Curve (b): Round 1 selection (using library cells from after the initial pre-selection step); Curve (c): Round 2 selection using cells from after round 1; Curve (d): Growth under identical conditions of a culture of cells containing PCA vectors encoding the high affinity pair of TNF- α and Z_{TNF α :185}.

Figure 4. Monitoring library clone sequence similarity during stages of the selection process. The computer software Align X (Invitrogen) was used to compare the absolute amino acid similarity of all affibody molecule sequences extracted from each step during the selection process. The average is plotted, and since only seven out of 58 aa were randomized an absolute similarity value of 88% would indicate a total dissimilarity in the randomized positions. The value of the unprocessed library was very close to this number, but after each new selection step the population became more homogenous with the largest single contribution coming from the first step performed in liquid media.

Figure 5. Dissociation rate analysis of selected clones. The dissociation phase kinetics of all five analyzed variants together with the original $Z_{TNF\alpha:2}$ clone were normalized and plotted versus time (s). In the diagram the time is set to zero at the end of the TNF- α injection. The respective dissociation phase curves were curve-fitted to the parameters of an exponentially decaying function in order to obtain the off-rate values (see Table 1). As can be judged from the figure all selected clones had considerable slower off-rate kinetics than the parental $Z_{TNF\alpha:2}$ variant.