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Affinity and folding properties both influence the selection of antibodies with the selectively infective phage (SIP) methodology

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Abstract We investigated which molecules are selected from a model library by the selectively infective phage (SIP) methodology. As a model system, we used the fluorescein binding single-chain Fv fragment FITC-E2, and from a 3D-model, we identified 11 residues potentially involved in hapten binding and mutated them individually to alanines. The binding constant of each mutant was determined by fluorescence titration, and each mutant was tested individually as well as in competitive SIP experiments for infectivity. After three rounds of SIP, only molecules with K_D values within a factor of 2 of the tightest binder remain, and among those, a mutant no longer carrying an unnecessary exposed tryptophan residue is preferentially selected. SIP is shown to select for the best overall properties of the displayed molecules, including folding behavior, stability and affinity.

Key words: Selectively infective phage; Single-chain Fv fragment; Antibody engineering; Antibody affinity; Phage display

1. Introduction

The selectively infective phage technology [1–11] selects for binding events by directly coupling infection of *E. coli* by filamentous phages to the association of two interacting molecules. This method thus completely avoids solid phase binding of phages and their elution. Selective infection is achieved by breaking up the gene-3-protein (g3p), which is needed for the infection process, into two fragments. The C-terminal domain, part of the phage coat, is fused to one of the interacting molecules, in our case a single-chain Fv fragment (scFv) of an antibody. The resulting phage is completely non-infectious [12–15]. This is in contrast to phage display [16], where infective phages are used.

For infection, the phage is missing both the N-terminal domains N1 (involved in penetration of the bacterial membrane) [15,17] and N2 (involved in docking to the F-pilus) [15,18] of g3p. These are supplied together in the form of a separate soluble adapter protein, fused to the other interacting molecule. In our case, the antigen fluorescein was chemically coupled to an engineered cysteine at the C-terminus of N2 (Fig. 1).

Previous experiments [7] with an anti-fluorescein scFv have shown that the infection event is strictly dependent on the coupling of fluorescein to the adapter protein. No infection is observed if the adapter does not carry antigen, demonstrating that the N-terminal domains need to be physically coupled to the phage (via the antibody-antigen interaction) and do not simply permeabilize the bacterial membrane. The dependence of infection on N1-N2 adapter concentration shows a concentration optimum. Presumably, at high concentrations, adapter binds to both pilus and phage and thereby blocks the infection process.

In this study, we have investigated the dependence of the infection efficiency on the dissociation constant of an antibody-antigen pair. As a model system, we have used the anti-fluorescein antibody FITC-E2 [19], which binds fluorescein with a dissociation constant K_D of about $7.5 \cdot 10^{-10}$ M. To create a panel of closely related antibodies with different binding constants, we built a model of the scFv fragment and identified potential antigen binding residues as those amino acids whose side chains are close to the two-fold molecular axis, relating V_H and V_L. Residues with side chains pointing into the binding pocket were individually mutated to alanines, and the binding constants determined by fluorescence spectroscopy. The infectivity of the resulting phages was then determined in individual SIP experiments, and they were tested in competitive experiments over one and three rounds.

2. Materials and methods

2.1. Molecular modeling

A molecular model for the lambda V_L domain of the antibody FITC-E2 was built on the basis of the structure of the Fab fragment KOL (75% sequence identity at the amino acid level, 81% similarity, PDB file 2fb4, 1.9 Å resolution) and 2ig2 (3.0 Å res.). The structures of the Bence-Jones protein LOC (71% identity, 80% similarity, file 1bjm, 2.4 Å res.) and the light chain dimer MCG (73% identity, 79% similarity, file 1mcb, 2.7 Å res.) were used for comparison. The V_H domain was modeled according to the structures of the Fab fragment of antibody structures 2fbj (64% identity, 73% similarity, 1.95 Å res.), while structures 1igc (73% identity, 77% similarity, 2.6 Å res.) and 2gfb (72% identity, 75% similarity, 3.0 Å res.) were used for comparison.

With a length of 18 amino acids, the CDR3 loop of FITC-E2 V_H was longer than any found in the PDB database (1ikf, 1dfb, 2fb4 are 17 aa, 1opg is 16 aa, 1fai is 15 aa). The structure of a loop of this length cannot be predicted reliably and is presumably flexible. However, the overall direction of these long loops is determined by the presence or absence of the Arg-H94/Asp-H101 salt bridge, which is present in FITC-E2. Therefore, the conformation of the first and last six amino acids of the loop were taken from the structure 1dfb, the 6 amino acids at the tip filled in by a conformational search, and the entire loop was allowed to relax by a molecular dynamics annealing. All modeling was performed using the InsightII 95/Discover 95 software packages (Biosym/MSI, San Diego).

2.2. Molecular biology

Site directed mutagenesis was carried out with the SIP fCKC-E2,

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encoding the w.t. scFv FITC-E2 [7], by the method of Kunkel [20], and confirmed by sequencing. The mutated scFv gene was recloned as a SfiI-SfiI cassette into the expression vector pAK400 [21], and recloned into the SIP vector.

2.3. Expression and purification of scFv fragments

The soluble scFv fragments were purified from *E. coli* SB536 [22], harboring the pAK400-based secretion vectors. The cells (2 l) were grown at 25°C, induced at an OD_{550} of 0.5, grown for a further 3.5 h, centrifuged and resuspended in 15–20 ml IMAC buffer (20 mM HEPES, 150 mM NaCl, pH 7.0) on ice, and passed three times through a French Press. The suspension was centrifuged (48 000×*g*, 4°C) and the supernatant was passed through a 0.22 μm sterile filter. The protein was purified on a Ni^{2+}-NTA column (Qiagen) with a step gradient and eluted at 100 mM imidazole. It was then concentrated, with a buffer change to 20 mM MES, pH 6.0, 50 mM NaCl. The remaining impurities were removed on a Sepharose-SP column (Pharmacia) in the same buffer, using a NaCl step gradient, where the scFv samples eluted at 130–175 mM NaCl. The final protein concentration was determined from the OD_{280} according to Gill and von Hippel [23].

2.4. Fluorescence titration

The dissociation constant of fluorescein was determined by measuring the fluorescence spectra from 495 to 530 nm (excitation at 485 nm), using a constant amount of fluorescein (usually 3.4 nM) and variable scFv concentrations, in a 2 ml cuvette at 18°C. The dissociation constant was determined by the following formula:

$$F_{corr} = F_0 + (F_\infty - F_0)\cdot\frac{1}{[Flu_{corr}]}\left(\frac{[scFv_{tot}]\cdot f_A + [Flu_{corr}] + K_D}{2} - \sqrt{\left(\frac{[scFv_{tot}]\cdot f_A + [Flu_{corr}] + K_D}{2}\right)^2 - [scFv_{tot}]\cdot f_A\cdot[Flu_{corr}]}\right).$$

Here, $[Flu_{corr}]$ is the total fluorescein concentration, which becomes automatically corrected for dilution [24]. This is achieved by directly substituting $[Flu_{corr}] = [Flu_{ini}]\cdot([scFv_{stock}] - [scFv_{tot}])/[scFv_{stock}]$, which adjusts it for the added volume from the titration with scFv, starting from the initial value $[Flu_{ini}]$, where $[scFv_{stock}]$ is the concentration of the stock solution. F_{corr} is the fluorescence at the intensity maximum (512 nm), corrected for dilution effects, F_0 is the fluorescence in the absence of scFv, and F_∞ is the final value at high $[scFv]$ which was found to be insignificantly different from zero and was thus set to zero. The term $[scFv_{tot}]$ is the total concentration of scFv in the cuvette at each experimental point, calculated from OD_{280} of the stock solution. The above formula thus fits the observed fluorescence intensity, corrected for dilution, directly as a function of one independent variable, $[scFv_{tot}]$, and two adjustable parameters, the dissociation constant K_D and the fraction of active protein f_A. When f_A was independently determined prior to the fluorescence titration, and $[scFv_{tot}]$ therefore precisely corresponded to the active concentration, a value of 1.0 for f_A was obtained from the fit, demonstrating the robustness of the method.

2.5. SIP experiments

The preparation of adapter protein and coupling with fluorescein to give N1-N2-Flu has been described previously [7]. Phages were prepared from *E. coli* XL1-Blue by PEG precipitation. The phage concentration was determined by ELISA, after directly coating the phages [7]. Functional (antigen binding) phage ELISAs were carried out by coating with fluorescein isothiocyanate coupled to bovine serum albumin (BSA-FITC), and detecting bound phages with anti-M13 serum, as described previously [7].

Individual SIP experiments were carried out by incubating 10 μl of a 0.34 μM solution of N1-N2-Flu in TBS buffer with 1 μl phage suspension (between 10^{11} and 10^{14} phages, obtained from PEG-precipitated overnight culture) overnight at 4°C. To this mixture, 100 μl of exponentially growing *E. coli* XL1-Blue (OD_{550} = 0.8 to 0.95) were added and incubated 1 h at 37°C and plated on 2YT plates with cam (25 mg/l) and tet (15 mg/l).

For competitive SIP experiments, approximately equal amounts of phages were mixed, and 1.5 ml *E. coli* were used. For one-round experiments, 31 clones were sequenced to identify the selected mutants. For three-round experiments, after adding bacteria to phages and incubation for 1 h, two aliquots of 600 and 700 μl were diluted into 50 ml of 2YT, 1% glucose, 2% glycerin, 50 mM $MgCl_2$, 25 mg/l cam, while 50 and 100 μl were plated to determine the number of infection events. The liquid culture was grown overnight, phages were obtained from the supernatant and PEG-precipitated twice. These phages were then incubated with adapter for the next round. After the third round, cells were plated, and 44 clones were sequenced.

3. Results and discussion

A molecular model of the antibody FITC-E2 [19] was built on the basis of the PDB structures of the antibodies KOL and J539. Because of the long CDR-H3, an uncertainty in the CDR-H3 structure results, but the residues pointing toward the two-fold molecular axis, and thus to the presumed antigen binding pocket, can clearly be identified (Fig. 2). In CDR-H2, we individually mutated Arg-H53, Leu-H56 and His-H58, in CDR-H3 Arg-H95, Trp-H100C, His-H100E, Phe-H100F and Tyr-H100G, while in CDR-L1 Asn-L31 and in CDR-L3 Trp-L91 and Asp-L93 were mutated, always to alanine (see Table 1 for nomenclature).

The resulting proteins were produced by secretion to the

Table 1
Summary of K_D determination and SIP experiments of alanine scan mutants of the antibody FITC-E2

Mutant[a]	K_D (nM)	Activity[b]	Frequency after 1-round SIP[c]				Frequency after 3-round SIP
			exp A	exp B	exp C	Σ(A−C)	
w.t. FITC-E2	0.75	0.86	2	0	0	2	4
Arg-H53-Ala	0.63	0.83	5	3	0	8	6
Leu-H56-Ala	0.74	0.84	3	0	1	4	1
His-H58-Ala	8.9	0.29	0	0	0	0	0
Arg-H95-Ala	no bind. detec.	–	0	0	0	0	0
Trp-H100C-Ala	0.71	0.89	3	0	0	3	30
His-H100E-Ala	1.6	0.91	1	1	2	4	3
Phe-H100F-Ala	2.9	0.70	2	0	2	4	0
Tyr-H100G-Ala	3.1	0.67	2	0	0	2	0
Asn-L31-Ala	0.74	0.87	2	1	0	3	0
Trp-L91-Ala	0.71	0.07	0	0	0	0	0
Asp-L93-Ala	0.93	0.80	1	0	0	1	0

[a]Numbering scheme according to Kabat, where CDR residues inserted after position 100 carry letters, such as in 100G. Thus, Tyr-H100G-Ala means that tyrosine, present at position 100G of the heavy chain (H), has been changed to alanine.
[b]Coefficient f_A determined from fluorescence titration. It relates active protein to total purified protein, determined from OD_{280}.
[c]Three independent experiments.

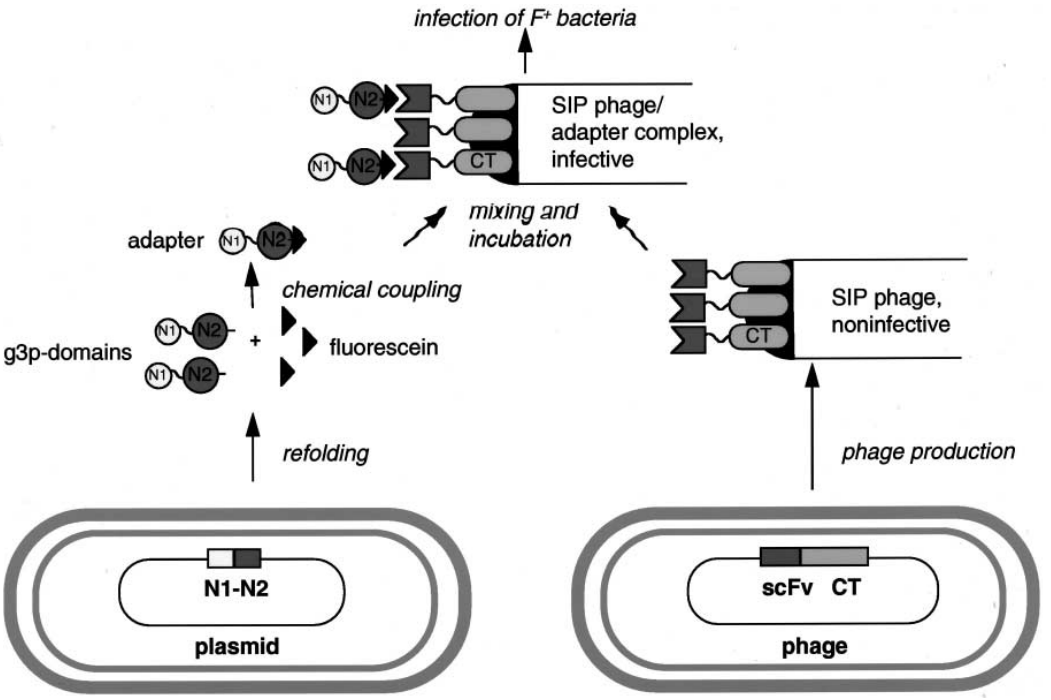

Fig. 1. Principle of SIP. The adapter protein was prepared by refolding N1-N2 from inclusion bodies and chemically coupling fluorescein to an engineered cystein at the C-terminus of the protein [7]. The non-infective phage becomes infective when mixed with adapter in vitro, provided the antigen is recognized by the antibody. After incubating adapter and phage, bacteria are added and plated on selective media to select for cam^R, carried by the phage.

E. coli periplasm as soluble and functional scFv, containing a C-terminal his-tag, and purified by IMAC, followed by ion-exchange chromatography. The dissociation constant of fluorescein was determined by fluorescence titration, using a constant amount of fluorescein to which scFv was added in small portions, while measuring the resulting fluorescence quenching. Using a correction term for active protein, excellent agreements with the theoretical curve (correlation coefficients of 99.99%) were obtained for all mutants (Fig. 3), except Arg-H95, for which no quenching at all was found. This mutant was also the only one which did not give a significant phage ELISA signal in antigen binding assays, while all others were similar within experimental error.

The resulting K_D values are summarized in Table 1. It can be seen that a large number of mutations, which are all on the periphery of the molecule, have almost no influence on binding, suggesting that the binding site is deep in the pocket. The antibody is remarkably resistant against mutation, with 5 mutations giving rise to the same sub-nanomolar affinity of the w.t. One mutation, Arg-H95-Ala completely eliminates binding beyond detection. His-H58-Ala leads both to a 10-fold reduction in binding constant and to a low amount of functional protein (30%) in the preparation of homogeneous, soluble scFv. This suggests the presence of two different molecular species in solution, one with reduced affinity, the other with no affinity at all. The mutation Trp-L91-Ala also leads to only a low amount of functional protein (7%) in the preparation of homogeneous, soluble scFv, but the protein which is functional has essentially an unaltered binding affinity from w.t. While the phages carrying this mutated scFv also give a normal antigen binding phage ELISA signal, they differ from the others in that they cannot be inhibited with free fluorescein (data not shown).

Interestingly, all proteins were obtained in comparable amounts upon expression of native protein and purification from *E. coli*. This suggests that the mutations Arg-H95-Ala, His-H58-Ala, Trp-L91-Ala do not appear to make the protein particularly unstable, insoluble or aggregation prone. Similarly, all phages are produced at comparable levels.

The w.t. and the 11 alanine-scan mutants were then tested in individual SIP experiments. Individual SIPs were prepared, and the infection events were measured as a function of adapter concentration. In all cases, except for Arg-H95-Ala, His-H58-Ala, Trp-L91-Ala, an optimum curve similar to the w.t. [7] was observed (data not shown). The number of phages were adjusted prior to the infection experiment according to an ELISA in which phages were coated and detected with an anti-M13 antiserum. However, this method is not accurate enough to eliminate small errors in phage concentration, and therefore, it is impossible to interpret any small differences in absolute infection rates.

The three mutants which gave low percentages of active protein upon testing purified, soluble scFv (His-H58-Ala, Arg-H95-Ala and Trp-L91-Ala) also gave very much lower infectivities, indicating that the scFv fragment on the phage is also folded only to a small percentage. However, His-H58-Ala and Trp-L91-Ala did lead to infection, when about 100-fold higher phage concentrations were used (data not shown). In contrast, for Arg-H95-Ala, infections never exceeded the level of the background.

We then investigated all mutants in competitive experiments. First, the w.t. and the 11 mutant phages were mixed at approximately equimolar concentrations, and DNA from single colonies was sequenced after one round of SIP, i.e. after adding adapter protein (28 nM final concentration) and bacteria to the mixture. This experiment was carried out three

times independently, and Table 1 gives the results how often each mutant was found. It can be seen that all mutants are found at comparable levels, but the three mutations with a large decrease in affinity and/or folding defects (His-H58-Ala, Arg-H95-Ala and Trp-L91-Ala) are immediately lost. On the other hand, this experiment also shows that the discrimination between closely similar binding constants is very subtle under these conditions, if all are very tight binders, and requires more than one round of SIP.

Thus, we carried out an experiment over three competitive rounds. In order to avoid accidental loss of phages in the first round, high numbers of phage were used in the first round ($5 \cdot 10^{10}$ phages for each species). After three rounds, Trp-H100C-Ala was enriched, with w.t. Arg-H53-Ala, Leu-H56-Ala, and His-H100E-Ala still present. Thus, all selected scFvs are within a factor 2 of the best binding constant. Nevertheless, the binding constant cannot be the only criterion, since Asn-L31-Ala and Asp-L93-Ala were lost, even though both have sub-nanomolar dissociation constants. Because of the relatively low infectivities of the phages, underrepresented phages can be lost fast in a competitive experiment, and we cannot exclude that the original mixture was not equimolar.

The competitive selection experiments over three rounds enormously amplify small differences in selective advantage. It is likely that a combination of molecular properties is selected, including affinity, folding efficiency, stability or any difference in toxic effect on the producing host. Thus, it is unlikely that binding affinity is the sole criterion of selection. Nevertheless, the only molecules surviving three rounds of SIP were those with a K_D of 1.6 nM or better with good folding properties. The molecule getting enriched appears to be Trp-H100C-Ala. Modeling suggests that this tryptophan is exposed and apparently does not contribute to binding since its mutation to alanine has no effect. It is possible that this exposed tryptophan very slightly disfavors expression on phage, even though this difference is not measurable by phage ELISA (data not shown), and it becomes apparent only after three rounds of SIP.

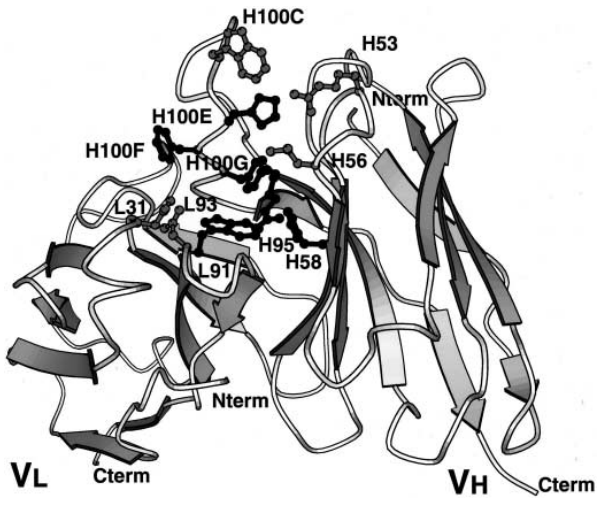

Fig. 2. Model of the fluorescein binding antibody FITC-E2. Residues mutated in this study are shown with their side chains. Those residues are shown in white whose mutation into alanine does not change the binding constant within experimental error, those which have a two- to four-fold higher K_D are shown in grey and those with a serious defect in black. The numbering shown is according to Kabat, with H for heavy and L for light chain.

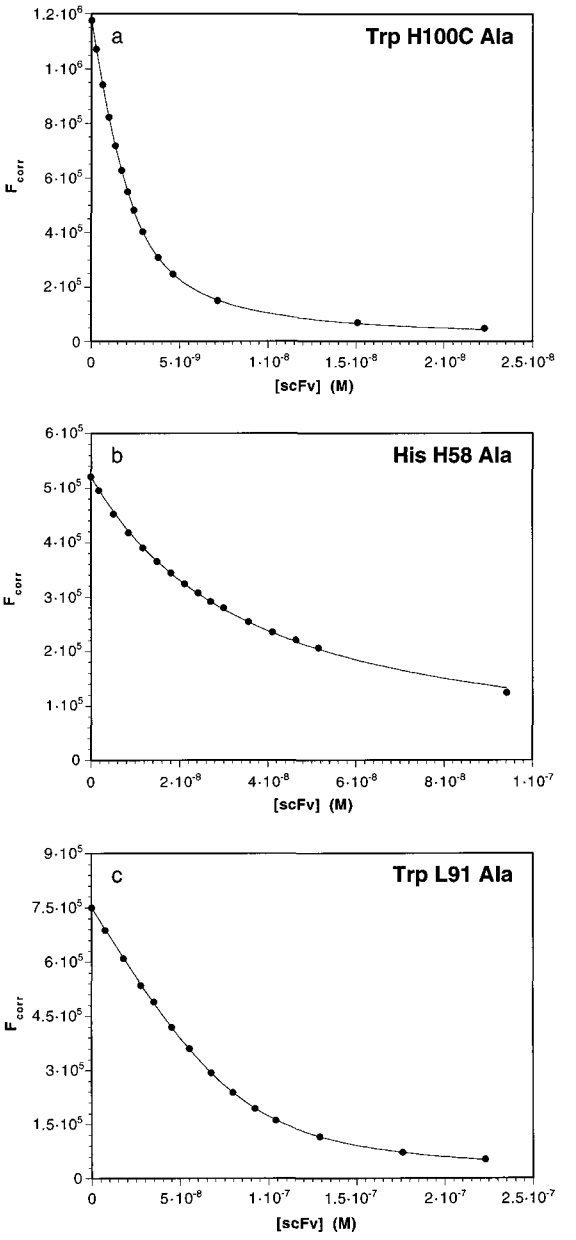

Fig. 3. Three selected fluorescence titration curves for K_D determination. The fit shown is according to the formula in Section 2, and the extracted parameters are summarized in Table 1.

Further molecular properties, which are currently in the process of being determined, such as the association and dissociation rate, may also influence the selection process. However, it is clear that the overall molecular properties will determine the winner in competitive SIP experiments. In previous experiments using several anti-HIV peptide Fab fragments [6], the selection for affinity and kinetic properties have been investigated. Similar to the experiments described here, the selection of antibodies with a dissociation constant of 10^{-9} M over those with one of 10^{-8} M was apparent, but any potential differences in functional expression and the folding properties of the Fab fragments were not tested quantitatively.

In conclusion, SIP appears a very powerful technique for selecting against poor binders and poor folders in a single round. Since, in contrast to phage display, there is no solid phase binding involved at any step, no complications result from poor elution of the best binders and from different recoveries of tight binders. Thus, small differences can be more easily amplified over several rounds and the best 'overall' molecule identified, such as Trp-100C-Ala here. SIP thus appears to be very promising for screening of libraries which contain tight binders, and to be especially suited for molecular optimization.

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