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# Phage antibodies against an unstable hapten: oxygen sensitive reduced flavin

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**Abstract It is difficult to raise antibodies against haptens and antigens that are unstable under the physiological conditions of the serum. Here we have used a phage antibody library to isolate antibody fragments against oxygen sensitive reduced flavin, by selection of the phage under anaerobic and reducing conditions at pH 5 and a pre-elution step with the oxidized flavin. The binding of the reduced hapten to one of the antibody fragments was characterised by time-resolved polarised fluorescence, and shown to be highly specific for the reduced flavin.**

*Key words:* Phage display; Single-chain Fv; Reduced flavin; Time-resolved fluorescence

## 1. Introduction

The use of large repertoires of antibody fragments displayed on phage has allowed the isolation of antibodies of different binding specificities without the need for immunization (for a review see [1]), including those that are difficult to raise by immunization, for example, human self-antigens [2,3] or highly conserved intracellular proteins [4]. Here we have explored the use of this technology for isolation of antibodies binding to compounds that are unstable under physiological condtions.

Flavin, a cofactor in many redox enzymes and electron transfer proteins (for a review see [5]), appears to be an excellent model. The flavin molecule can exist in oxidized, one-electron-reduced and two-electron-reduced states. The reduced states are not stable under aerobic conditions. At physiological pH (7.2) two-electron-reduced flavin is present as a mixture of two ionization states (the p$K_a$ of the N(1) of $Fl_{red}$ is 6.7 (see Fig. 1)). Because two-electron-reduced flavin differs from oxidized flavin in its conformational and electronic properties [6], antibodies can discriminate between both forms [7,8], and it is possible to prepare mouse monoclonal antibodies to the two-electron-reduced flavin after immunisation with oxygen-stable analogues [7].

However, using phage technology, it should be possible to make antibodies against two-electron-reduced flavin directly, by undertaking the selection process under anaerobic and reducing conditions at a non-physiological pH. We describe the use of a 'single pot' phage library of synthetic scFv fragments [4] to isolate antibody fragments against the reduced flavin, and time-resolved polarized fluorescence to characterise flavin binding to the selected fragments and to gain insight in the flexibility of bound flavin [7].

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*Abbreviations:* BSA, bovine serum albumine; ELISA, enzyme-linked immunosorbent assay; $Fl_{ox}$, *N*(10)-5′-carboxybutylflavin; $Fl_{red}$, *N*(10)-5′-carboxybutyl-1,5-dihydroflavin; PBS, phosphate-buffered saline; scFv, single chain Fv fragment; $\phi$, rotational correlation time.

## 2. Materials and methods

### 2.1. Phage selection

A single pot human synthetic phage-antibody scFv library [4] was used for phage selection. The library was rescued with M13K07 helper phage [9] (Pharmacia) and phage particles were purified using polyethylene glycol (PEG) [10].

Immunotubes (Nunc; Maxisorb, 75×12 mm) were coated overnight at room temperature with 250 μg/ml *N*(10)-5′-carboxybutylflavin ($Fl_{ox}$, gift from J. Santema) conjugated to BSA ($Fl_{ox}$-BSA, 8 $Fl_{ox}$ molecules per BSA molecule) in PBS and blocked with 2% skimmed milk powder in PBS for 2 h at 37°C. After washing 3 times with PBS the empty tube was transferred to an argon atmosphere in an anaerobic chamber (Miller Howe Ltd., UK). $Fl_{ox}$ was reduced to *N*(10)-5′-carboxybutyl-1,5-dihydroflavin ($Fl_{red}$) by 10 mM sodium dithionite in 100 mM sodium phosphate, 150 mM NaCl, pH 5.0 ($NaP_i$/NaCl pH 5.0). All buffers were deaerated by flushing with argon before transfer into the anaerobic chamber.

For the first round of selection $10^{13}$ transducing units of phage were PEG precipitated. The pellet was transferred to the anaerobic chamber and resuspended in 4 ml $NaP_i$/NaCl pH 5.0 containing 1 mM sodium dithionite and 2% skimmed milk powder. The phages were incubated in the $Fl_{red}$-coated tube for 2 h during which period the tube was manually rotated repeatedly. After each round of selection the tube was washed 20 times with $NaP_i$/NaCl pH 5.0, 0.1% Tween 20, 0.1 mM sodium dithionite and 20 times with $NaP_i$/NaCl pH 5.0, 0.1% dithionite. The library was subjected to four rounds of panning. In the first three rounds the phage were eluted with 1.5 ml of 0.1 mM $Fl_{red}$ (a solution of $Fl_{ox}$ in $NaP_i$/NaCl pH 5.0 was deaerated, transferred to the anaerobic chamber and reduced with dithionite). Eluted phage were amplified as described by Marks et al. [11]. In the fourth round two tubes coated with $Fl_{red}$-BSA were panned with phage from the third round. One tube was eluted with 0.1 mM $Fl_{red}$, the other was pre-eluted with 0.1 mM carefully deaerated $Fl_{ox}$ followed by elution with 0.1 mM $Fl_{red}$.

### 2.2. Phage ELISA

Single ampicillin-resistant colonies of *E. coli* TG1 infected with eluted phage from the fourth round were assayed for binding to $Fl_{red}$ and $Fl_{ox}$ in an ELISA. Two ELISAs were performed simultaneously, one under aerobic conditions ($Fl_{ox}$-ELISA), the other in the anaerobic chamber ($Fl_{red}$-ELISA). After coating two plates with BSA-$Fl_{ox}$, the '$Fl_{red}$-ELISA plate' was transferred to the anaerobic chamber. For both ELISAs all steps were carried out in $NaP_i$/NaCl pH 5.0. For the $Fl_{red}$-ELISA 0.1 mM sodium dithionite was present in all steps to reduce flavin and remove traces of oxygen. After the last wash step the $Fl_{red}$-ELISA plate was removed from the anaerobic chamber and both plates were developed simultaneously.

Binding of phage-antibody to antigen was detected with mouse


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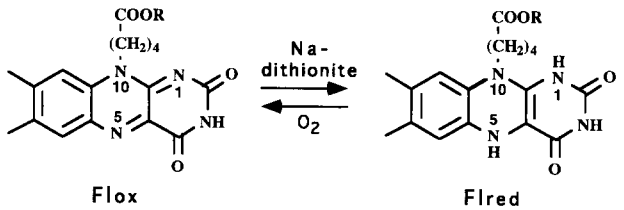


Fig. 1. Structures of $Fl_{red}$ and $Fl_{ox}$. R = H for free flavins, R = BSA for conjugates.

polyclonal anti-M13 antibody and alkaline phosphatase-conjugated rat-anti-mouse antibody (Jackson Immuno Research Laboratories Inc.). Readings were taken by substracting the $A_{620}$ from the $A_{405}$ after 1 h incubation at room temperature. Clones were considered specific for $Fl_{red}$ (or $Fl_{ox}$) if the difference in absorbance reading between corresponding wells on the two ELISA plates was more than 0.4 (on a scale of 1.8). Clones with a difference of less than 0.4 were considered cross-reacting.

*2.3. Expression, purification and sequence analysis*

Clone $\alpha Fl_{red}5$ was subcloned into pUC119SfiNotMycHis using standard recombinant DNA techniques. Expression as a soluble scFv and purification were performed as described by Griffiths et al. [12].

For sequencing, clone $\alpha Fl_{red}5$ was transferred from TG1 into DH5α. DNA purified with Qiagen Plasmid Midiprep (Qiagen Inc.) was used as template in sequencing reactions. Sequencing reactions with fluorescent dATP (AutoRead Sequencing Kit, Pharmacia Biotech) were carried out according to the manufacturer's instructions using standard M13 primers. The sequencing reactions were analysed on an A.L.F. DNA Sequencer (Pharmacia Biotech).

*2.4. Analysis of affinity and specificity*

Affinity constants for $Fl_{red}$ and $Fl_{ox}$ were determined by time-resolved total fluorescence and fluorescence anisotropy in $NaP_i$/NaCl pH 6.0 and 7.5.

3 μM $Fl_{red}$ was titrated anaerobically with scFv concentrations ranging over a 0–5-times molar excess of scFv as compared to $Fl_{red}$. 0.05 μM $Fl_{ox}$ was titrated with a 0–6-times molar excess of scFv. Measurements, data analysis by the maximum entropy method and calculation of the order parameters were performed as previously described [7]. The fractions of free and bound flavin in solution were calculated from the decrease in the free $Fl_{red}$ contribution in the rotational correlation time spectra. Affinity constants were obtained by fitting data to:

$$H_{bound} = (A_0 + H_0 + K_d - ((A_0 + H_0 + K_d)^2 - 4H_0A_0)^{1/2})/2$$

where $H_0$ corresponds to the total hapten concentration and $A_0$ to the total scFv concentration.

The affinity constant for $Fl_{ox}$ (in PBS) was determined by competitive ELISA as described in [13].

## 3. Results

*3.1. Selection for hapten binding*

The goal of this study was the generation of antibody fragments against oxygen sensitive reduced flavin. After coating with (oxidized) 5′-carboxybutyl-N(10)-flavin-BSA ($Fl_{ox}$-BSA, Fig. 1) an immunotube was transferred to an anaerobic chamber where the conjugated flavin was reduced to 1,5-dihydro-5′-carboxybutyl-*N*(10)-flavin ($Fl_{red}$, Fig. 1). The selection was performed at pH 5, where N(1) is protonated ($pK_a$ = 6.7). For selection of $Fl_{red}$ binders, the phage library [4] was subjected to rounds of affinity selection with elution of the bound phage by $Fl_{red}$. However, at the end of the fourth (and final) round, one of the two tubes of bound phage was pre-eluted with $Fl_{ox}$, then eluted with $Fl_{red}$ (see Section 2). This proved essential to remove cross-reactive phages since the pre-eluted fraction contained no cross-reactive phages whereas in the other fraction about 50% of the phages were cross-reactive.

DNA sequence analysis of the clones giving the best signal to $Fl_{red}$ revealed that at least three different clones had been selected. One of the clones ($\alpha Fl_{red}5$) comprised the VH segment DP-32 [14], with CDR3 of 12 amino acids (GWVNVKVSKNPL). The library we used has an unmutated light chain as described by Hoogenboom et al. [15].

*3.2. Characterization of scFv fragments*

The clone $\alpha Fl_{red}5$ was further characterised as a soluble scFv fragment. To facilitate purification, $\alpha Fl_{red}5$ was subcloned into the pUC based vector pUC119SfiNotMycHis (lacking the gene III insert but harbouring a hexahistidine tag for purification and a cmyc tag for detection) for soluble expression. This scFv was purified with a yield of 600 μg/l.

Binding of $Fl_{red}$ to $\alpha Fl_{red}5$ was studied by time-resolved polarized fluorescence. The rate of depolarization of emitted light reflects the rotation of the probe on a nanosecond time scale. The rotational correlation times (ϕ), calculated from such experiments, can be used to quantify binding and to obtain information about the flexibility of the fluorescent probe. Reduced flavin was titrated with $\alpha Fl_{red}5$ at pH 6 where the N(1) of $Fl_{red}$ is protonated (as in the selection) and at pH 7.5 where N(1) is negatively charged. In both cases, the $Fl_{red}$ fluorescence increased upon binding to $\alpha Fl_{red}5$, presumably because the constraint induced by binding to the antibody restricts the number of accessible conformational states of $Fl_{red}$ [16].

A typical example of a distribution of rotational correlation times, obtained after analysis of time-resolved polarized fluorescence decays, is shown in Fig. 2A. Free $Fl_{red}$ (curve a) shows a main contribution at 0.07 ns. The contribution at 0.14 ns is ascribed to self-association of $Fl_{red}$ molecules [7]. Upon addition of $\alpha Fl_{red}5$ the motional freedom of $Fl_{red}$ becomes more restricted, as is reflected in a contribution ($\phi_{int}$) of 1–3 ns. This value reflects flexible binding of $Fl_{red}$ in the antigen binding site (examples are shown in Fig. 2A, curves b,c). Affinity constants for $Fl_{red}$ were calculated from the decrease in the free $Fl_{red}$ contribution in the rotational correlation time spectra (Table 1).

Tumbling of the whole scFv ($\phi_{scFv}$) appears to be slower than the time scale of observation, and results in a contribution at infinitely long time ($\phi_\infty$) (Fig. 2A, curves b,c). This was unexpected as the rotational correlation time of a scFv molecule can be calculated according to Visser et al. [17] as 10–20 ns. It appears that this discrepancy may be due to association of scFv molecules by hydrophobic interactions in the presence of salt, as in experiments performed in the absence of NaCl $\phi_{scFv}$ shifts to about 20 ns (data not shown).

Binding of $\alpha Fl_{red}5$ to $Fl_{ox}$ was also studied at pH 6.0 and 7.5 by time-resolved polarized fluorescence. No binding could be detected under the experimental conditions used in fluor-

Table 1
Affinity constants ($K_d$) and order parameters ($S$) for binding of $\alpha Fl_{red}5$ to $Fl_{red}$ and $Fl_{ox}$

| pH | $K_d$ ($Fl_{red}$) (μM) | $K_d$ ($Fl_{ox}$) (μM) | $S$ ($Fl_{red}$) |
|---|---|---|---|
| 7.5 | 0.6 ± 0.06 | 100 ± 10[a] | 0.39 ± 0.05 |
| 6.0 | 4.0 ± 0.4 | | 0.61 ± 0.11 |

[a]pH 7.2.

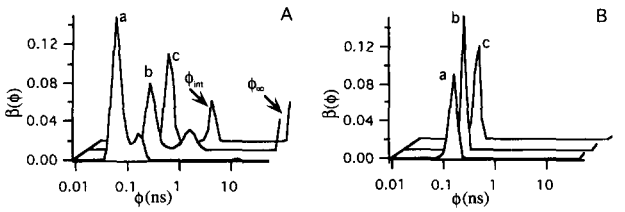


Fig. 2. Rotational correlation time distributions of free and $\alpha Fl_{red}5$-bound $Fl_{red}$ and $Fl_{ox}$ at pH 6.0. (A) Curve a, free $Fl_{red}$; curve b, $Fl_{red}$:$\alpha Fl_{red}5$=1:0.6; curve c, $Fl_{red}$:$\alpha Fl_{red}5$=1:2.4. (B) Curve a, free $Fl_{ox}$; curve b, $Fl_{ox}$:$\alpha Fl_{red}5$=1:2.3; curve c, $Fl_{ox}$:$\alpha Fl_{red}5$=1:4.0.

escence experiments (Fig. 2B), suggesting that the affinity of $\alpha Fl_{red}5$ for $Fl_{ox}$ is low. Competitive ELISA had to be used to determine an affinity constant (Table 1). This experiment was performed in PBS at pH 7.2, since the ionization state of $Fl_{ox}$ is identical at pH 6.0 and 7.5.

## 4. Discussion

By use of phage display technology we were able to make antibodies with high binding specificity to haptens that are unstable under physiological conditions, and this may obviate the need for synthesis of stable analogues. The antibody fragment $\alpha Fl_{red}5$ was highly specific for unstable $Fl_{red}$ compared with stable $Fl_{ox}$ (by a factor of 25–160-fold, depending on the pH). In this case the binding specificity was fashioned in two stages; firstly during the selection process by binding of the phage to $Fl_{red}$ under anaerobic and reducing conditions; and secondly by pre-elution of the bound phage with $Fl_{ox}$ to remove cross-reactive phages.

The binding affinity of scFv $\alpha Fl_{red}5$ for $Fl_{red}$ (Table 1) is similar to those of other antibodies isolated from this library [4]. Although phage antibodies were selected (at pH 5.0) against $Fl_{red}$ protonated at N(1), the affinity of $\alpha Fl_{red}$ for $Fl_{red}$ is higher at pH 7.5 (Table 1). In principle, this could reflect pH-dependent changes in conformation and/or charge in either the hapten or the antibody. Upon increasing the pH, the hapten adopts a negative charge on the pyrimidine nucleus of the flavin resulting in a redistribution of electron density [18] whereas the geometry of the hapten is similar in both ionization states [19]. As indicated by the order parameters (Table 1), flexibility of $Fl_{red}$ in the antigen binding site is more restricted at the lower pH. This decrease in flexibility combined with an increase in affinity is counter-intuitive. We suppose that the enhancement of electrostatic interactions is at the expense of other interactions that anchor the flavin to the antibody.

The success of this strategy to select antibodies against haptens that are unstable under the physiological conditions of the serum suggests further possibilities. For example, it may allow the selection of antibodies against conformations of proteins unstable at physiological pH, or against haptens stabilised by co-solvents or in the presence of reactive chemical species (potentially useful for the generation of catalytic antibodies), provided that the infectivity of the phage can be maintained.

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