Flavobodies

Generation and redox properties of flavin-binding antibodies

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Generation and redox properties of flavin-binding antibodies

Yvonne E. Bruggeman

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The cover is an autostereogram which means that a three-dimensional picture is hidden in the pattern. The pattern consists of antibody displaying phage particles; the hidden picture represents a flavin molecule. The phage particles and in particular the flavin molecule play an important role in this thesis.
The autostereogram was made by Hans Kövi (Graphix Visions).

Stellingen

1. De mogelijkheid om antilichamen te verkrijgen tegen instabiele antigenen kan bijdragen aan de ontwikkeling van betere overgangstoestandsanaloga en dus leiden tot katalytische antilichamen met een hogere katalytische efficientie.
(Dit proefschrift)

2. De door Thorn *et al* gebruikte methode om de versnellingsfactor van katalytische antilichamen uit te drukken leidt tot een overschatting van deze parameter.
(Thorn et al, Nature 373, 228-230, 1995)

3. Het bewijs voor het bestaan van enzymen die Diels-Alder reakties katalyseren is verre van overtuigend.
(Laschat, Angew. Chem. 108, 313-315, 1996; Oikawa et al, J. Chem. Soc. Chem. Commun. 1321-1322, 1995)

4. Het door de tabaksindustrie (Philip Morris) verspreide bericht dat meeroken geen gevaar voor de gezondheid oplevert, berust op onderzoek dat uitgevoerd is naar de risico's op longkanker en mag dan ook niet betrokken worden op gezondheidsrisico's voor meerokers in het algemeen. Bovendien is dit onderzoek verricht door een door de tabaksindustrie gefinancierde onderneming en moet derhalve de integriteit van de onderzoekers in twijfel getrokken worden.
(Smith and Phillips, British Medical Journal, 313, 929-933, 1996)

5. Consumenten zullen met moderne biotechnologie geproduceerde voedingsmiddelen pas dan accepteren als deze aantoonbare voordelen, bijvoorbeeld wat betreft voedingswaarde, smaak of prijs, bieden boven "klassieke" voedingsmiddelen.

6. Het is zinloos om een vaccin te ontwikkelen tegen cocaïne of een andere drug, omdat de verslaafde na deze symptoombestrijding op zoek zal gaan naar alternatieve geestverruimende middelen en derhalve meer gebaat zal zijn bij een sociale of psychologische bestrijding (oorzaakbestrijding).
(Rocío et al, Nature 378, 727-730, 1995)

7. Het nuttigen van "light" producten nodigt uit tot de consumptie van grotere hoeveelheden voedsel en leidt daardoor juist tot een hogere energie-opname.
(van Dokkum, Chemisch Magazine, 439-443, 1992)

8. In het huidige wetenschappelijke systeem is het beter om hypotheses te publiceren verspreid over meerdere artikelen en zo de citatie-index op te voeren, dan om in één goed artikel tot een definitieve oplossing van een wetenschappelijk probleem te komen.

9. In plaats van het aanstellen van bursaal AIO's als maatregel om het wachtgeldprobleem uit de weg te ruimen, kunnen universiteiten beter overgaan tot het instellen van numeri fixi voor promovendi.

10. Religie, atheïsme en bijgeloof zijn allen gebaseerd op geloof.

11. Schaatsen is de enige bezigheid waarbij men met de handen op de rug tot grote prestaties kan komen.

Stellingen behorende bij het proefschrift:

Flavobodies. Generation and redox properties of flavin-binding antibodies

Yvonne E. Bruggeman
Wageningen, 5 maart 1997

Voorwoord

Een promotie wordt beschouwd als de eerste werkelijke proef van wetenschappelijk kunnen. In mijn ogen is dat niet alles: promoveren was voor mij een sprong in het diepe waarbij het uiteindelijk weer bereiken van de oppervlakte mij heeft geleerd dat in principe alles mogelijk is als je maar genoeg uithoudingsvermogen hebt. Tijdens de zwemtocht naar de oppervlakte zwommen een aantal personen mee en velen staken onderweg een helpende hand toe.

Riet, afgelopen jaren ben jij nauw bij het onderzoek betrokken geweest. Onze samenwerking heeft wat ups en downs gekend, maar uiteindelijk is het daar alleen maar beter van geworden. Jij bent iemand die kritiek zeer ter harte neemt en er ook daadwerkelijk wat mee doet en dat bewonder ik zeer. Van jouw nauwgezette manier van werken en wetenschappelijke instelling heb ik, als iemand met niet al teveel oog voor detail, veel geleerd. Arjen, jij zorgde voor prima "antilichaamtechnologische faciliteiten" die onontbeerlijk waren voor de totstandkoming van dit proefschrift. Bedankt hiervoor, en zeker ook voor de steun en het "meedenken" de afgelopen jaren. Colja, jij werd promotor toen het onderzoek al in de eindfase was gekomen. Toch wist je snel de draad op te pakken en al mijn epistels (zeer snel) van nuttig commentaar te voorzien, hetgeen, zeker in de laatste schrijfmaanden, katalyserend heeft gewerkt. Professor Veeger, u gaf mij de kans dit promotie-onderzoek te beginnen. Hiervoor wil ik u nog graag bedanken.

Ronald Schoenmakers, Anja Boogert, Huub Kreuwel, Jan Engelbertink en Doeke Hoekstra, jullie hebben in het kader van afstudeervakken/stages allemaal een flink eind meegezwommen. Veel van jullie werk is terug te vinden in dit boekje. De samenwerking met jullie vond ik een van de leukste aspecten aan mijn promotie-onderzoek.

An important turn in the research project was when I jumped into the world of phage. This was possible due to the hospitality and help of Dr. Greg Winter, Centre of Protein Engineering, MRC, Cambridge, UK. Greg, I am grateful for your hospitality and support. I also would like to thank Pete Jones who was my tutor in antibody phage display. Pete, thank you for your practical support en for many, many helpful E-mails.

Das vierte Haupstück dieser Doctorarbeit war nie zustande gekommen ohne die Hilfe von Dr. Annemarie Honegger und Gastfreundschaft von Professor Andreas Plückthun, Universität Zürich, Schweiz. Annemarie, gerne möchte ich mich bei dir bedanken für die Mühe die du dich damals gegeben hast einer ausgezeichneter "Modelling" Arbeit zu bewerkstelligen.

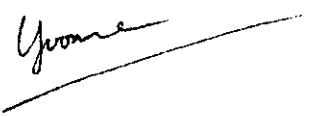
Ook dichter bij huis heb ik veel hulp ondervonden. Allereerst op het "fluorescentie-vlak". Deze letterlijk en figuurlijk schitterende techniek vormt een substantieel onderdeel van dit proefschrift. Ton Visser, Arie van Hoek, Eward Pap en Petra van den Berg, zonder jullie hulp was dit niet gelukt! Winfried Mulder en Masha Sergeeva, jullie waren, helaas te

kort, deel van het katalytische antilichamenteam. Bedankt voor de fijne samenwerking. Michel Eppink, bedankt voor je hulp bij het visualiseren van de antilichaammodellen. Een goed voorbeeld hiervan is te zien in hoofdstuk 4. Jillert Santema, bedankt voor je hulp bij de flavinesynthese. Laura Ausma, bedankt voor je hulp bij vele niet-wetenschappelijke karweitjes op de vakgroep.

Ook buiten de vakgroep Biochemie kwam ik graag. Vooral op het Laboratorium voor Monoklonale Antistoffen (LMA), waar ik een groot deel van mijn tijd heb doorgebracht. Alle LMA-medewerkers, bedankt voor de fijne samenwerking. Rikus Pomp, jou wil ik graag met name bedanken, vooral voor je hulp en vakmanschap die het monoklonale antilichaamwerk tot een succes maakten. Als ik experimenten wilde doen die wat minder prettig roken, maakte ik graag gebruik van de zuurkast van Maurice Franssen op Organische Chemie. Maurice, bedankt voor je gastvrijheid en ook je "synthetische" adviezen tijdens het eerste jaar van mijn promotie-onderzoek. Op de vakgroep Microbiologie deed ik een poging tot antilichaamproductie op grote schaal. Hans Scholten, bedankt voor je hulp bij de fermentatie-experimenten.

Inhoudelijke bijdragen zijn vaak duidelijk genoeg. Niet zichtbaar in dit boekje, maar voor mij onmisbaar, waren de gesprekken, weekendjes weg, etentjes, schaatstochten etc. met familie en vrienden. Luc, Anne, Evert, René, Wendelmoet, Elles, Jan Willem, Tanja, Marc, Mirjam, Ellen, bedankt! Luc, de relativerende gesprekken met jou over het afronden van dit proefschrift hebben flink bijgedragen aan de uiteindelijke vlotte afronding. Papa en mama, jullie hebben mij altijd gestimuleerd "eruit te halen wat er in zat" en mij daarbij onvoorwaardelijk gesteund.

Romke, jij hebt het hele traject meegezwommen en dat was voor mij van onschatbare betekenis. Vooral het laatste, hectische jaar heb je het zwaar te verduren gehad en je zult wel opgelucht ademhalen dat ik uiteindelijk de oppervlakte van de "promotiepool" bereikt heb. Bedankt voor alles.



Contents

Chapter 1

Introduction

1. *Monoclonal antibodies*

1.1. *Introduction to antibodies*

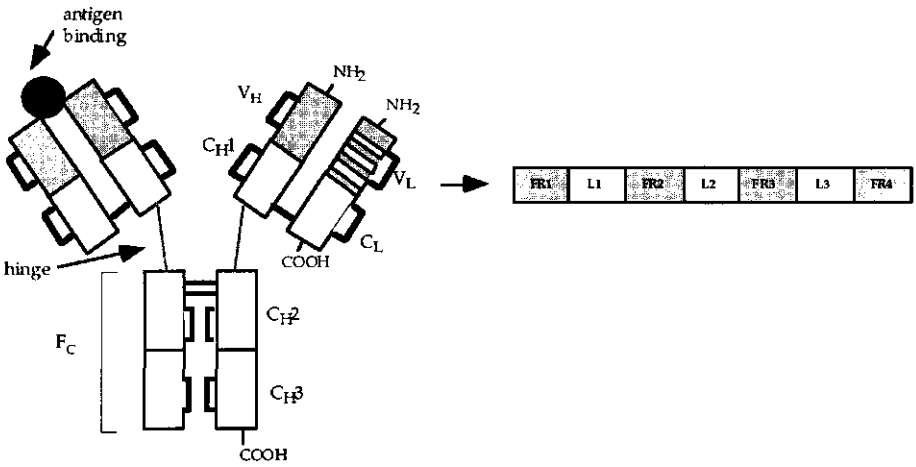
Basically, antibodies are proteins that have the ability to form specific, stable complexes with other molecules, the antigens (= *anti*body *gen*erator). Antibodies (or immunoglobulins, Igs) are produced and secreted by B lymphocytes. In their natural role secreted antibodies circulate in the blood and serve as the effectors of humoral immunity by searching out and neutralizing or eliminating antigens. Natural antigens are usually immunogenic macromolecules of protein or carbohydrate composition, or a combination of these. Smaller molecules such as peptides, steroids and other small organic molecules (called haptens) are only immunogenic if first covalently attached to a carrier protein.

In principle, each B lymphocyte produces only one specific antibody. However, when an animal is immunized its serum is still polyclonal, i.e. contains many different antibody specificities. Such a heterogeneous population does not allow the investigation of the molecular properties of individual (monoclonal) clones nor is it possible to continuously produce unlimited amounts of antibody. In 1975 Köhler and Milstein described a method for the production of monoclonal antibodies (mAbs) from B lymphocytes immortalized by fusion to a tumour cell line [1] (further described in 1.4.1). This technique, the hybridoma technology, was a major breakthrough for which they received the Nobel prize. More recently a new technology has been developed, based on the production of antibodies in bacteria (see 1.4.2) and on the surface of filamentous bacteriophage, that allows the generation of monoclonal antibodies totally *in vitro* thus circumventing the need for animal immunization (see 1.4.3).

Nowadays antibodies and in particular monoclonal antibodies have been used in many biotechnological applications: as catalytic antibodies (see 3), in the search for specific binders (for detection and identification) and in therapy (for example in tumor imaging) [2,3].

1.2. *Antibody structure*

The antibody (IgG) is a Y shaped molecule with two arms (the F_{ab} fragments) joined by a flexible hinge to the stem (F_c fragment) (Fig. 1.1.A). The tips of the arms bind to the antigen and the F_c fragment is responsible for triggering effector functions (like complement activation). Each antibody molecule consists of two identical heavy (H) chains and two



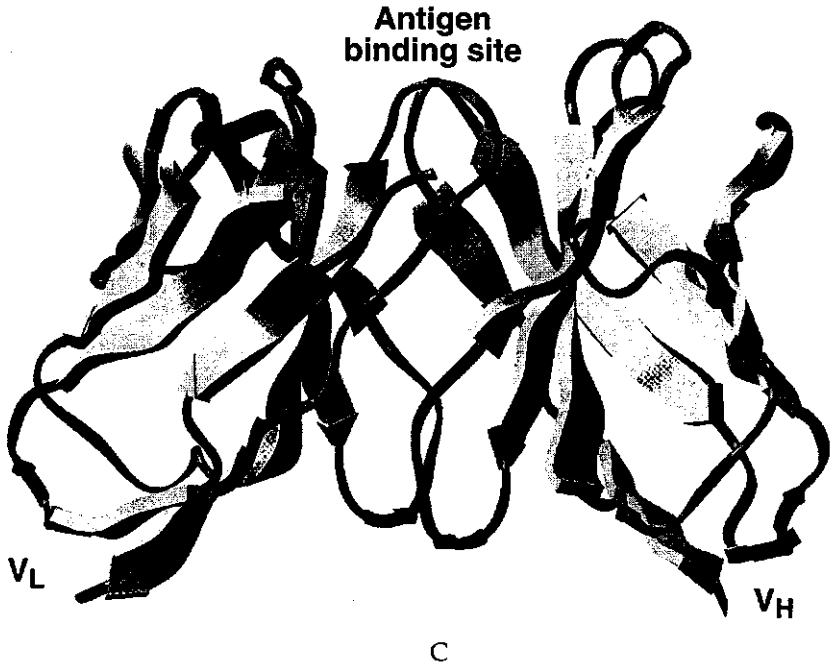


Fig. 1.1. A) Schematic overview of an IgG showing the domains of the heavy and light chains (thick black lines reflect disulfide bridges), B) Framework (FR) and complementarity determining regions (CDRs) of V_L.C) the antigen binding domain (V_H +V_L) consisting of the CDR loops interspacing the β-strands of this domain (drawn by Mike van Suylekom, Unilever Research Laboratorium).

identical light (L) chains. Each chain has an N-terminal variable domain (V_L and V_H on the L and H chain, respectively) that together form the antigen-binding site (F_v). The C terminal parts of each chain consist of constant (C) domains: C_L for the L chain and C_H1, C_H2, C_H3 and sometimes C_H4 for the H chain). There are two L chain classes (κ and λ) and five H chain classes (α, γ, δ, ε and μ). The various Ig isotypes have different biological activities, for example IgE (that has an ε heavy chain) is involved in triggering allergic reactions (for extensive information see [4]).

1.2.1. The antigen-binding site

The antigen-binding site of an antibody is formed by six polypeptide loops: three from V_L (L1, L2 and L3) and three from V_H (H1, H2 and H3) (Fig. 1.1.B). The sequence variability within the V_H and V_L domains is mainly concentrated in these hypervariable loops [5], which form the actual antigen-binding site. The loops are referred to as complementarity-determining regions (CDRs). The remainder of the variable domains are the framework (FR) regions. These well-conserved FR regions form anti-parallel β-strands that together form a β-sheet sandwich. The CDR loops are built on top of this β-sheet sandwich, each loop connecting two β-strands. The V_L and V_H domains associate non-covalently to form a β-barrel structure, placing the CDRs close to each other at the amino-terminal end of the V_L-V_H dimer (Fig. 1.1.C).

Chothia and co-workers [6] have established the concept of "canonical families" for five of the six CDR loops (H1, H2, L1-L3). Canonical loops are defined on the basis of their length and the position of canonical residues in the loop and in the framework. These canonical residues dictate CDR conformation through hydrogen bonding, packing with other CDR loops or part of the framework regions and torsional preferences. Unfortunately, not all loops are canonical and not all loops classified as "canonical" obey the canonical rules [7]. The H3 loop always is an exception because of its highly variable length, sequence and structure. Several residues outside the antigen-binding site have also been shown to be important for the conformation of CDR loops [8] and V_L-V_H interactions may have some effect on the conformation of the CDR loops [9].

There are three different types of surface topographies for antigen-binding sites based on analysis of antibody X-ray structures: cavity, groove and planar. Small size antigens (haptens) give rise to a cavity topography, intermediate size antigens (peptides, DNA, carbohydrates) to grooves and the larger proteins to planar binding sites. There is no relation between a particular combination of CDR lengths and the type of surface topography. Generally, the rough topography of the binding site will be dictated by the backbone conformation, while the specificity for the epitope (antigenic derminant) will largely be determined by the side chains [10].

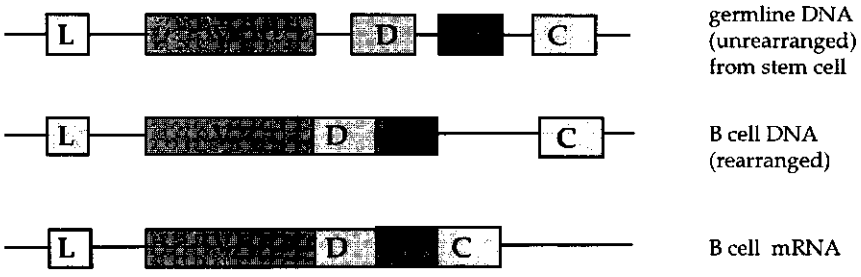


Fig. 1.2. Simplified overview of the formation of the V_H domain. L: leader sequence; V: V_H gene segment; D: D gene segment; J: J_H gene segment; C: constant domain gene segment.

1.2.2.1. *Formation of the V domains*

The immune system is capable of generating a large repertoire of different antibodies (~ 10^7 for a single mouse or ~10^{12} for a human being), from a relatively small number of gene segments (the germline segments). This is achieved by a combinatorial process in which the complete gene for the protein is produced through recombination of a number of gene segments, each of which is drawn from a pool of moderate size. The V_H domain is assembled by the recombination of three gene segments: V_H, D and J_H (see Figure 1.2). This region includes the first and the second CDR of the H chain. The third loop is formed by all the three segments: the end of V_H, D and the beginning of J_H. The V_L domains are formed by a combination of two gene segments V_L and J_L. As in the V_H domain, the V_L segment codes for the first two CDRs and the third CDR is formed by the end of V_L and the beginning of J_L. The enormous diversity generated this way is further augmented by a phenomenon known as junctional flexibility. This is based on the imprecise joining of sequences and leads to many non-productive rearrangements, but it also generates several productive combinations, thereby increasing antibody diversity [4,11].

The primary antibodies produced by gene recombination are capable of recognizing all antigens with at least moderate affinity. Cells producing these antibodies are selected by encounter and binding of antigen, and they are triggered to differentiate to short-lived plasma cells that secrete antibody and to long-lived memory cells that persist in lymph nodes, spleen, and bone marrow. The V genes coding for the selected antibodies displayed on memory cells are subject to somatic hypermutation, leading to antibodies of improved binding affinity after further selection with antigen. Thus, repeated immunization leads to "affinity maturation" of the response.

1.2.2.2. Modelling of the antigen-binding site

To understand the molecular basis of antibody specifity, high resolution X-ray crystallographic or NMR structures of free and antigen-bound antibody are required. If such a structure is not available, advantage can be taken of the highly conserved and well-studied structures of antibody V domains in order to generate models of antibodies. Antibody modelling has attracted increased interest over the past few years, owing to an explosion in the number of published antibody sequences [12], a gradual increase in availability of well-resolved crystal structures [13] and the availability of fast work stations.

The approaches taken to modelling of the antigen-binding site can be divided in two groups: knowledge based and *ab initio*. In the first approach antibody crystal structures from which CDRs are selected on the basis of length and/or sequence are used as templates to construct models. Advantages of knowledge-based approaches are that starting structures are known to exist in nature and that less computer time is needed compared to *ab initio* methods. The main limitation of this approach is the number of available antibody structures. The second approach in antibody modelling is based on *ab initio* methods that generate all possible loops by conformational search methods (see for example [14]). The drawback of this approach is that the number of loop conformations increases in size exponentially as the length of the loop increases. Therefore this method is only suitable for short loops. *Ab initio* methods fail to make use of the valuable information available in the structural database [13] and, consequently, are expensive in computer time.

To date, the knowledge-based and *ab initio* approaches, when used alone, have had only limited success [15]; they are not routinely able to construct all six CDRs with a high level of accuracy. Martin and co-workers [16] have combined the two approaches in a computer program (AbM [17]) that overcomes the limited size of the knowledge base by using conformational search methods but does not ignore the information available in the structural data base. This program is a "black box" program (i.e. sequences of heavy and light chains have to be imported and then the program generates the antibody model) and works fairly well when CDR H1, H2, L1-L3 belong to canonical families and H3 is short [15]. Interactive modelling programs, based on a combination of a knowledge based and an *ab initio* approach, are also available (for example [18]). These programs allow the user more freedom but also require more experience and knowledge of the user. When constructing an antibody-antigen model, possible conformational differences in the free and bound forms of the antibody must be considered as well as the flexibility of the antigen. In general haptens are more rigid than peptides or proteins and therefore easier to deal with.

The validity of antibody models must be confirmed by binding (e.g. ELISA, fluorescence or surface plasmon resonance [19]) or mutagenesis studies. Reliable models can provide valuable information for

betterunderstanding antibody-antigen interactions, antibody catalysis [20], to improve binding by mutagenesis, to "humanize" antibodies for therapy [21] and even for the complete *de novo* design of new antibodies [10,22].

1.3. Antibody-antigen interactions

Perhaps the most important function of antibody molecules is to specifically combine with the corresponding antigen to form an antibody-antigen complex. The attractive and repulsive forces involved in antibody-antigen interactions are the same as for other non-covalent protein-ligand interactions: on the one hand hydrogen bonds, Van der Waals interactions, electrostatic interactions and hydrophobic interactions, and on the other hand steric repulsion forces caused by a non-optimal complementarity. The better the complementarity between antibody and antigen, the lower the repulsive forces. Antibody affinity is the summation of these attractive and repulsive forces. Affinity is a thermodynamic measurement of the strength of the non-covalent interaction between an antigenic determinant and the homologous antibody binding site and can be described as the dissociation constant K (ranging from 10^{-5} to 10^{-10} M^{-1} for antibodies) or as the standard free energy change $\Delta G_o'$ for binding:

$$K = k_{diss} / k_{ass} = [Ab\text{-}Ag]/[Ab].[Ag]$$

$$\Delta G_o' = -RT \ln K$$

The term affinity indicates the strength of an interaction between one site of the antibody and one site of the antigen. However, full size antibodies have more binding sites, that can each complex to an antigen. In case the antigen is multivalent or linked to a surface, large networks are formed. In contrast to affinity, avidity is an operational term expressing the total strength of an antibody-antigen interaction and depends, therefore, not only on affinity but also on multivalency of antibody and antigen and other non-specific factors. Basically, the measurement of antibody affinity depends upon the determination of free and antibody-bound antigen or free and antigen-bound antibody at equilibrium. Such determinations are usually performed over a range of antibody or antigen concentrations. Affinities can be determined using various techniques such as Enzyme-Linked Immunosorbent Assay (ELISA) [23], fluorescence spectroscopy [24-26] or surface plasmon resonance [27].

1.3.1. Molecular basis of antibody-antigen interactions

The H chain seems to play a dominant role in antigen-binding. However, there are also examples of antibodies in which the L chain is predominantly involved in antigen-binding. The minimum number of

CDRs used in binding, even the smallest antigens, is four. Conversely, antibodies binding larger antigens do not necessarily use all six CDRs. An analysis of known structures shows that L3 and H3 are always used and play a dominant role in ligand binding, the contact with other CDRs and with the opposite domain [28,29]. There is a surprising lack of interaction between L2 and smaller antigens or haptens (for overview see [29]).

Despite the "lock and key" appearance of antigen-antibody complexes, both antibodies and ligands are dynamic entities that undergo structural fluctuations [30]. Conformational adjustments may even be required for binding. "Induced fit" can be achieved by side chain shifts or rotations, CDR conformational changes and even V_H and V_L domain shifts. Where significant conformational changes occur in the antibody, H3 has always been involved so far [30].

New insights reveal that solvent molecules also help to mediate antibody-antigen interactions by forming hydrogen bonds, increasing the packing density and contributing to charge complementarity [31]. So most of the ordered water molecules in the free antigen-binding site are retained upon complex formation (instead of transfer to bulk solvent). Recruitment of additional water molecules has also been observed. Both effects restrict the entropic contribution to binding energy but this is compensated by a more favorable enthalpy. The location of water molecules becomes more certain with the higher resolution of the X-ray structures nowadays.

1.4. Antibody technology

1.4.1. Hybridoma technology

In 1975 a method was described for making cell lines that secrete a single species of antibody (monoclonal antibody) with the desired specificity to antigen [1]. This technology is based on the fusion of spleen cells from an immunized animal with myeloma cells resulting in hybrid myeloma or hybridoma cells. Myeloma cells are tumour cells that have the ability to grow *in vitro* and were altered in such a way that they do not express antibodies themselves. Thus, the hybridoma cell obtains its immortality from the myeloma cell and capacity to produce antibodies from the spleen cell.

The first step in hybridoma technology is animal (usually mouse) immunization. Immunization is essential to derive hybridomas secreting high-affinity antibodies. Upon the first immunization step antibodies of low affinity are produced (usually IgMs). This involves proliferation of cells drawn from the repertoire already available at the time of immunization (10^7-10^8 clones in a mouse). After a second immunization high affinity antibodies (mainly IgGs) are formed, due to hypermutation of the genes used in the first stage. The actual immunization protocol depends on the nature of the antigen and the mouse strain used.

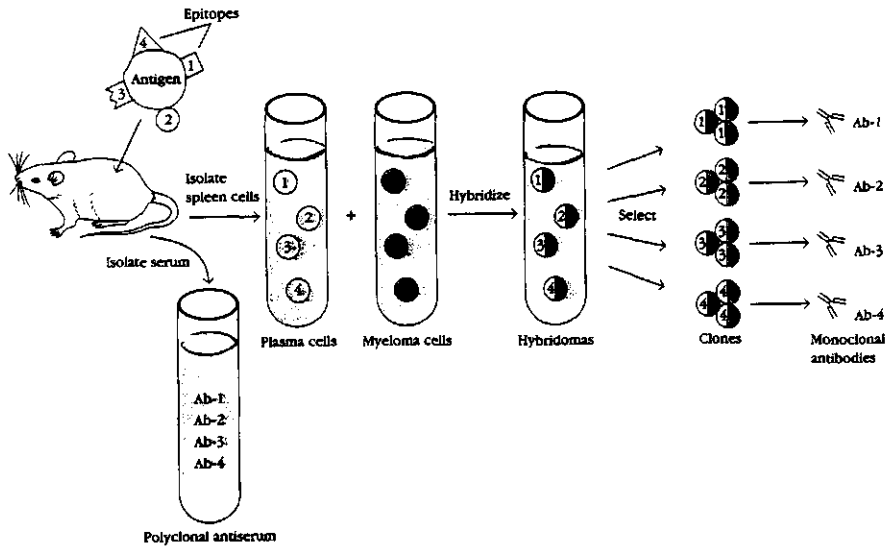


Fig. 1.3. Overview of hybridoma technology (see text for details).

Spleen cells of the hyperimmunized mouse are then fused with myeloma cells using polyethylene glycol or electrofusion. Unfused spleen cells die because they lack the ability to grow *in vitro*. Unfused myeloma cells are eliminated by blocking the synthesis of the essential metabolite guanosine monophosphate (GMP) using azaserine or aminopterin. In normal cells this problem can be circumvented in an alternative pathway via the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) that converts hypoxanthine in GMP. Myeloma cells lack HGPRT and will therefore die in a selective medium. Spleen cells, however, possess this enzyme and provide it to the hybridoma cell that can thus survive in this selective medium (Fig. 1.3).

After fusion, clones are screened for antigen-binding by assays such as the Enzyme-Linked Immunosorbent Assay (ELISA). Positive clones are produced as stable monoclonal clones by cloning them under limiting dilution conditions.

Hybridoma technology has led to a wealth of murine monoclonal antibodies with predefined specificity. However, the technique depends on animal immunization followed by culturing mammalian cells which is both time-consuming and expensive. Moreover, hybridoma cells are not easily accessible for genetic engineering which precludes the use of mutagenesis experiments in order to improve or study binding properties or catalytic activity of antibodies. Furthermore, it is difficult to produce human monoclonal antibodies (needed for therapy and *in vivo* diagnostics), partly for ethical reasons.

1.4.2. Antibody expression in E.coli

Due to the developments in recombinant DNA technology it is now possible to produce antibodies in *E.coli*. Production in *E.coli* has several attractive features: genetic manipulations are quite simple and well-established, growth is rapid, fermentation is relatively simple and the transformation efficiency of *E. coli* is probably better than of any other micro-organism. However, it is not possible to express full size antibodies in *E.coli* because bacteria are not capable of glycosylating the F_c part of the antibody. Glycosylation seems to contribute to stability and is essential for biological effector functions [32]. As the antigen-binding parts of an antibody are usually not glycosylated, these fragments can be produced in *E.coli* in their natural form and can be used for purposes for which no effector function is needed: as catalytic antibodies, for studying antibody-antigen interactions or fusion with enzymes or toxins.

1.4.2.1. Choice of fragment

The smallest antibody fragment containing the complete antigen-binding site is the F_v fragment composed of the variable domains V_L and V_H (Fig. 1.4). The relative affinity of V_L and V_H for each other depends on the particular sequence of the antibody [33]; the domains may dissociate in one antibody, while others do not have this problem. The reversible dissociation of the F_v fragment can be counteracted in several ways: chemical cross-linking [34], engineering of disulfide bonds into the fragment [34,35] or covalently linking V_L and V_H by a genetically encoded peptide linker (resulting in a single chain F_v fragment, scF_v) [36]. The first method requires obtaining the F_v fragment in the first place and is therefore not generally applicable. The second method, comprising genetic engineering of disulfide bonds, overcomes this problem but suffers the drawback that the positions to engineer these bonds are not universal for all antibodies. The latter method is the most popular due to its universal applicability. A variety of peptide linkers has been tested [37] and the most frequently used one has the sequence $(Gly_4Ser)_3$ [38]. NMR evidence suggests that this linker is a flexible entity, which does not change the structure of the variable domains [39]. Closest to the naturally occurring antibodies is the F_{ab} fragment consisting of a covalently associated light chain and a part of the heavy chain (Fig. 1.4). The constant domains substantially contribute to the interactions between the heavy and the light chain and thus increase the stability.

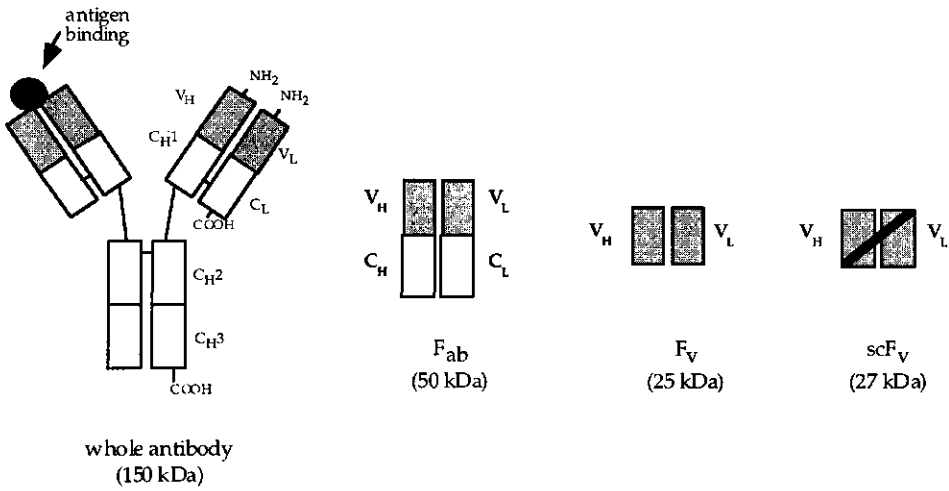


Fig. 1.4. Overview of the antibody fragments.

1.4.2.2. Folding, secretion and purification of antibody fragments from E.coli

At first antibodies could only be produced in *E.coli* as cytoplasmic inclusion bodies [40,41]; refolding experiments led only to a small percentage of correctly folded antibody. Moreover, experiments with a number of fragments have shown that the refolding procedure has to be adapted to the particular fragment under study [37]. Additionally, correctly folded molecules must be separated from soluble, but incorrectly folded material.

In 1988 two publications appeared at the same time describing the functional expression of antibody fragments [42,43]. The expression system described was the result of attempts to reproduce in *E.coli* the folding and assembly pathway of antibodies in eukaryotic cells. In the eukaryotic plasma cell, the two chains of an antibody are separately transported from the cytoplasm to the lumen of the endoplasmic reticulum (ER). This transport requires an amino-terminal signal sequence, which is cleaved off after or during the translocation event by a signal peptidase to produce the mature protein. In the lumen of the ER protein folding, formation of disulfide bonds and association of individual chains to form the functional antibody take place. These steps must be mimicked in the bacterial cell. The crucial hypothesis in the design of the secretory system for antibody fragments was that protein transport from the reducing environment of the cytoplasm to the oxidizing periplasm is functionally equivalent to the transport of a protein to the lumen of the ER of the eukaryotic cell. This was achieved by equipping heavy and light chains, obtained from hybridoma cells, with signal sequences that direct both

chains to the periplasm. In the periplasm both chains were correctly folded and assembled and the intramolecular disulfide bonds, that appeared to be crucial for the antibody stability [35], were formed. This approach led to the formation of functional antibody fragments with the same affinity constants as the whole antibody or its proteolytic F_{ab} fragments.

Usually, for purification the antibody fragment is selectively liberated from the periplasm as a functional and soluble protein by EDTA treatment combined with osmotic shock [44] (subcellular fractions termed "cytoplasmic" contain membrane-associated periplasmic antibody material that is pelleted together with the spheroblasts [45]). However, under conditions of prolonged high level expression and at elevated expression temperature, the outer membrane of *E.coli* becomes permeable [46] and the fragment can be isolated from the culture medium [44]. Secretion into the medium may be useful for analytical work but for large scale preparations it is generally considered more practical to prepare the protein from the whole cell or the periplasmic fraction. Purification of the recombinant antibody fragments is facilitated by the incorporation of genetically encoded carboxyl-terminal peptide tags, for example a hexahistidine tag for Immobilized Metal Chelate Chromatography (IMAC) [47]. Nowadays, several hundreds of milligrams of antibody can be produced using high-cell density fermentation of E.coli [48,49] but this is not common practice in most laboratories since sophisticated fermentor systems and clones producing reasonable amounts of antibody fragments are required

Despite this success, it appears that only part of the secreted antibody folds correctly in the periplasm and the remainder of the secreted protein is deposited as insoluble material [50]. The yield of the folding process depends primarily on the primary sequence [51], but also on temperature and growth physiology [51] and the type of fragment used [52]. It appears that aggregation, which is a side reaction of folding, limits the folding yield [51]. Empirically the simplest method to improve the folding yield is to lower the growth temperature of the cells to about room temperature [52,53]. The distribution of folding intermediates between pathways of folding and aggregation is probably more favourable at lower temperature [53]. However, the most effective way to improve folding may be to change the primary sequence of the antibody. Knappik and Plückthun introduced mutations in turns of the antibody loops which reduce the formation of aggregates and diminished the lysis of the bacterial cells [51]. However, the mechanism of how these mutations accomplish their effects is not yet known and therefore this approach must be optimized for each particular antibody sequence.

In contrast to natural occurring antibodies, F_{ab}, F_v and scFv fragments lack multivalency, which is a very effective means of increasing the functional affinity (avidity) to a surface or a polymeric antigen. This problem can be solved by further protein-engineering of the recombinant antibody fragments: either by using a linker that is too short to allow

pairing between the two domains on the same chain, forcing the domains to pair with the complementary domains of another chain resulting in a bivalent "diabody" [54] or by linking scFv fragments by small amphiphatic helices resulting in bivalent [55] and tetravalent [56] miniantibodies. The production of whole functional antibodies in *E.coli* remains impossible. However, if applications are envisaged, where glycosylation would be vital, the stepwise engineering of the binding site may still be performed in *E.coli*, and the final version of the antibody may then be expressed in mammalian cells [57], yeast [58], insect cells [59,60], fungi or plants ("plantibodies"), although glycosylation is different in each system [61].

1.4.3. Antibody phage display

Despite the fact that expression of antibodies in *E.coli* has overcome the need for culturing hybridoma cells and genetic manipulation of antibodies is much facilitated, animals still "invent" the antibody molecules. Antibody expression in *E.coli* proved to be the key step to circumvent even this. Recombinant DNA technology has enabled the immortalization of antibody heavy and light chain V-genes, instead of immortalizing B-cells, for production of monoclonal antibodies. These genes can be displayed on the surface of filamentous bacteriophage allowing mimicking of immune selection [62] and nowadays antibodies can be selected totally *in vitro* .

1.4.3.1. Principles of phage display

In the first instance, filamentous phage have been used for the display of peptides [63]. Nowadays antibody fragments can be displayed on phage [62] as well as as functional enzymes and other proteins such as receptors, protease inhibitors and DNA binding proteins (for review see [64]). The single-stranded DNA genome of non-lytic filamentous bacteriophage (such as M13 or fd) is coated with approximately 2700 copies of the major coat protein, pVIII, forming a thin flexible cylinder around the enclosed genome. Three to five copies of the minor coat protein, pIII, are located at the tip of the phage particle (for a extensive description see [65]) (Fig. 1.5.A). Although both pIII and pVIII can be used for display, both leaving the phage infective, fusions to pIII are preferred for antibody display to prevent strong avidity effects [66].

The pIII protein has two domains. The amino-terminal domain, which extends from the phage particle as a knob-like structure, binds to the bacterial F-pilus to infect male *E.coli* bacteria. The carboxy-terminal domain anchors pIII into the phage coat. Antibody fragments are fused to the amino-terminus of pIII (Fig. 1.5.B), which is, during phage assembly, located in the periplasm of their bacterial cell where the antibody fragments can fold and assemble. Bacteria infected by phage become resistant to further infection and therefore phage, like the B cell, each carry only a single antibody species.

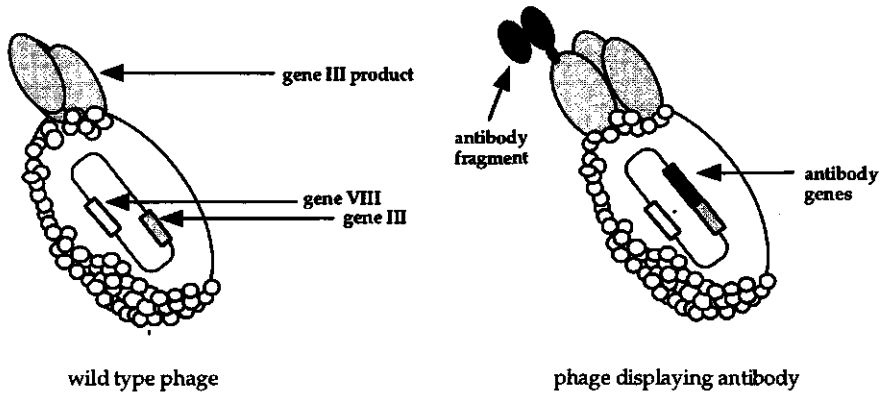


Fig. 1.5 Wild type (A) and antibody (F_V fragment) displaying phage (B).

To achieve display of antibody fragments, V-genes are genetically fused to gene III (coding for protein III) either in a phage vector or in a phagemid vector. Phagemids are plasmid vectors, harbouring bacteriophage gene III, that can be packaged into phage particles, but require the use of helper phage [67]. Phage vectors should lead to three to five copies of the antibody fragments on each phage (assuming no proteolysis), but with phagemids the pIII coat protein of the helper phage competes for incorporation into the phage particle, resulting in an average display of less than a single fusion protein per particle [66]. When an amber stop codon is introduced between the antibody gene and gene III it is possible to mimic the switch from surface display to secretion of soluble antibody from plasma cells. When phage are grown in a *sup*E suppressor strain of *E.coli*, this amber codon is read as a glutamine and the antibody is displayed on top off the phage. In non-suppressor strains the amber codon is read as a stop codon and soluble protein is secreted into the bacterial periplasm [68].

1.4.3.2. Antibody phage display libraries

Antibody genes can be amplified from hybridoma or B cells using the polymerase chain reaction (PCR). The key step towards expression of whole repertoires of V-genes (libraries) came from the use of "universal" PCR primers, suitable for amplifying a range of heavy and light chain V-genes, and the cloning of amplified DNA directly into expression vectors [69]. The resulting libraries are combinatorial, i.e. amplified heavy and light chain V genes are randomly recombined irrespective of their pairing *in vivo* . Libraries can be either biased or naive (Fig. 1.6). In biased libraries the repertoires of V_H and V_L genes are amplified from the spleen mRNA of hyper-immunized mice [70]. Immunization enriches the mRNA for V_H

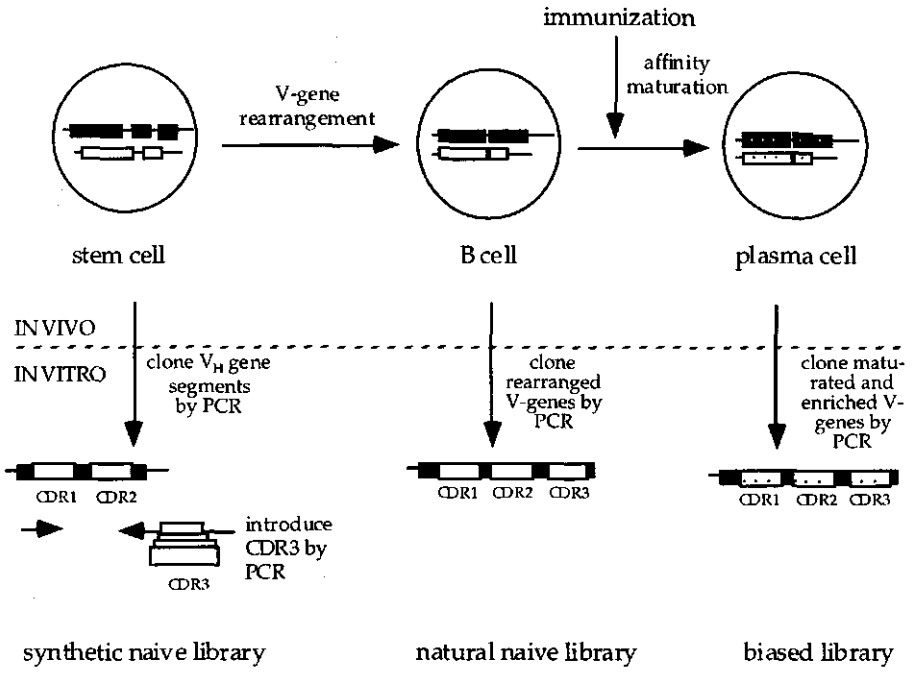


Fig. 1.6. Antibody production *in vitro* and *in vivo* and an overview of the libraries that can be generated *in vitro*. Mutations, introduced by affinity maturation, are represented as dots.

and V_L genes encoding binding activities [71,72]. Libraries from immunized sources provide high affinity antibodies (Kd $\leq 10^{-9}$ M) to the immunogen without the need to construct hybridomas. However, animal immunization is required and this results in a different library for each antigen [70,73].

It is possible to by-pass immunization using naive repertoires (i.e. V-genes derived from a non-immunized source). However, the probability of isolating a clone from such a repertoire is dependent on the size and diversity of the library: larger libraries should lead to the isolation of antibodies against more epitopes and also to antibodies exhibiting higher affinities [74]. The first naive libraries (repertoire size ± 10^7), derived from rearranged V-genes (see 1.2.2.) and therefore called natural libraries (Fig. 1.6), produced only a few different specificities of moderate affinity [75,76].

The diversity of the V-genes can be extended by a synthetic approach using unrearranged V-gene segments from stem cells, encoding the first two CDRs, as building blocks; from human individuals repertoires of 51 human V_H segments [77], 40 V_κ [78] and 30 V_λ [79] gene segments have been cloned. *In vivo* the third CDR is generated from the recombination of these segments with the D and J segments for the heavy chain and with J segments for the light chains, in this way multiplying the diversity. *In vitro* this process is mimicked by creating a synthetic CDR3 using PCR

with diverse oligonucleotide primers (Fig. 1.6). These synthetic naive libraries (repertoire size of ~10^8 clones) have yielded antibodies of different specificity and moderate affinities (10^5-10^7 M) [80,81].

However, the limiting factor in the generation of large phage library is the transfection efficiency of bacteria; in practice this limits the library size to 10^7-10^9 clones [66], although by several hundreds of transformations a very large library (1.4×10^{10}) that led to antibodies with sub-nanomolar affinities has been obtained [82]. These transformations could be circumvented by a "combinatorial infection" approach [83]. Bacteria harbouring a "donor" heavy chain repertoire on a plasmid are infected with an acceptor light chain repertoire on phage. The two chains were combined on the same (phage) replicon within the bacterium by Cre catalysed recombination at so called *lox*P sites [83]. This process generates a large number of heavy and light chain combinations, potentially as large as the number of bacteria that have been infected by phage (phage infection is extremely effective and most *E.coli* cells in an exponentially growing culture can be infected). Griffiths *et al* [84] have constructed a library of 6.5×10^{10} clones that has yielded antibodies with affinities in the nanomolar range. Thus using naive libraries both hybridoma technology and immunization could be by-passed. Moreover, the same library can be used to select phage antibodies against a wide range of different antigens.

1.4.3.3. Selection of phage antibodies from libraries

Phage encoding the displayed antibody fragment can be affinity selected from libraries for binding to a particular antigen by passing the phage antibodies over antigen bound to a solid surface ("panning"). Several formats have been used for the affinity selection of the phage, such as antigen on a column matrix [62,70], antigen-coated plastic tubes or dishes [75], biotinylated antigen in solution followed by capture on streptavidin-coated beads [85] and whole cells [86]. Phage that display antibody fragments specific for the antigen are retained on the surface. Non-binders are removed by washing and bound phage antibodies are washed and eluted by soluble antigen (specific) [70] or acid [73] or alkali [75] (aspecific) and used to reinfect *E.coli* (Fig. 1.7). The selected phage are grown after each round of panning and selected again so that rare phage can be isolated after several rounds of panning [62]. Since the antibody fragments on top of the phage are linked to the genes encoding them, the panning procedure, that selects for antigen-binding phage, coselects for its DNA sequence simultaneously.

The power of selection is limited mainly by the efficiency at each round of selection: it seems that only a small fraction of the phage (<1%) can be recovered [87,88]. Thus to guarantee selection of at least one copy of a binding clone, it is necessary to have many copies present. By carefully optimizing selection, specific binding phage can be enriched 50-fold per round of selection leading to a 10^7-fold enrichment after four rounds of selection.

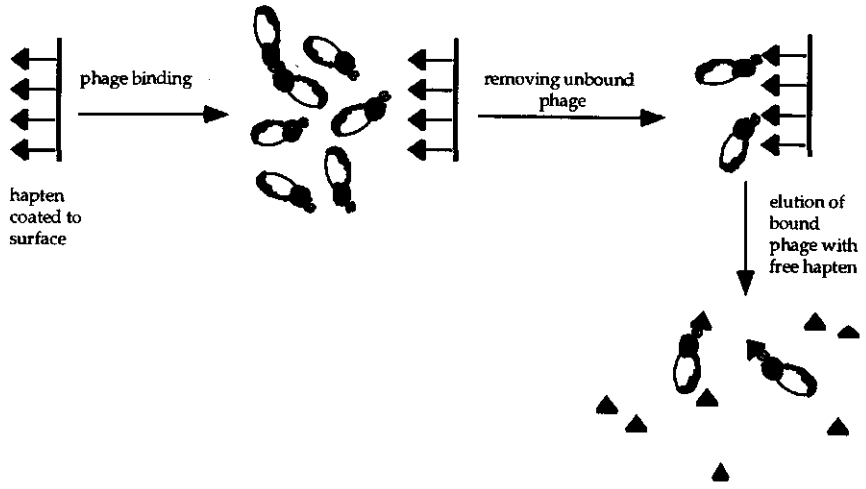


Fig. 1.7. Overview panning procedure (see text for details).

Selection efficiency can be further improved by linking antigen-recognition and phage replication [89,90]. Antibody libraries have been displayed on the surface of engineered, non-infectious phage with gene III deletions (see 1.4.3.1.). Antigens are then expressed as fusion proteins with gene III. Consequently, antigen-specific phage become infectious by binding to the gene III fusion product. Using this method one specific phage among 10^{11} non-specific particles could be recovered [89]. However, use of this system is restricted to protein and peptide antigens that can be genetically fused to gene III.

The successful expression of antibodies in *E.coli* [42,43], the use of universal PCR primers to clone repertoires of heavy and light chain V-genes [69] and the display of antibody fragments on filamentous phage [62] has led to the isolation of highly specific antibodies. Among these are human antibodies (even against self-antigens) [76] and antibodies against unstable haptens (see chapter 3 [26]) that are generated by-passing hybridoma technology and even immunization, from naive antibody phage display libraries. The construction of very large repertoires even allows the isolation of high-affinity antibodies [84]. As in the immune system, selected antibodies from a primary library can be mutated and those with improved affinities isolated [85,91].

2. *Flavins and flavoproteins*

Flavin (7,8-dimethylbenzo[g]-pteridine-2,4(3H,10H)-dione) is widely distributed in nature, free and bound to proteins. Riboflavin (Fig. 2.1), better known as vitamin B_2, is the most abundant natural flavin compound. Whereas lower organisms produce flavin biosynthetically, mammals have lost this capacity and have to take up flavin via their food. A large number of flavoproteins isolated from various biological sources have been characterized to date. These flavoproteins can be distinguished in flavoenzymes (2.2), which catalyze a wide variety of redox reactions, and flavin-binding proteins, which play a role in flavin storage and transport (2.3).

2.1. *The flavin molecule*

The most interesting feature of the versatile flavin molecule consists of its redox properties, which can be modulated by the (protein) environment. The redox active part of the flavin is the isoalloxazine ring (the three-membered ring system, Fig. 1.8) which can exist in oxidized flavoquinone, one-electron reduced flavosemiquinone and two-electron reduced flavo-1,5-dihydroquinone states. The three redox states can each exist as cationic, anionic or neutral species; protonation and deprotonation can occur at more than one atomic site . In this thesis emphasis has been laid on oxidized flavin and 1,5-dihydroflavin and therefore the properties of these molecules will be discussed further.

Oxidized and 1,5-dihydroflavin differ substantially in their charge distribution and conformation. Both oxidized and reduced flavin are flexible molecules. Oxidized flavin is most stable in the planar conformation but nevertheless highly flexible around the N(5)-N(10) axis [92]. N(10) is generally slightly out of plane, but hydrogen bonding may cause N(10) to become coplanar [92]. The pyrimidine like nucleus (ring C in Fig. 1.8) is by far the most polar portion of the isoalloxazine moiety. The most positive ring atoms are the carbonyl carbons. N(1) and N(3) are the most negative ring atoms, whereas N(5) and N(10) are nearly neutral. The benzene like ring (ring A in Fig. 1.8) shows typical aromatic charge values [93].

Reduced 1,5-dihydroflavin is bent along the N(10)-N(5) axis with typical fold angles around 155°, depending on the axial N(10) substituent [92]. N(5) substituents vary in orientation and no obvious pattern is known [92]. Upon reduction C(4a), N(5) and N(10) become more negative and N(1) less negative. In contrast to these shifts of charges, the partial charges in the O=C-NH-C=O moiety remain virtually unchanged, suggesting electronic isolation from the rest of the conjugated system. Changes in partial charge extend even into ring A [94].

The pK_a of the N(1) of reduced flavin is 6.7, implying that this nitrogen often is deprotonated. The deprotonated N(1) bears the largest share of the negative charge adopted but the carbonyl oxygens in ring C also become

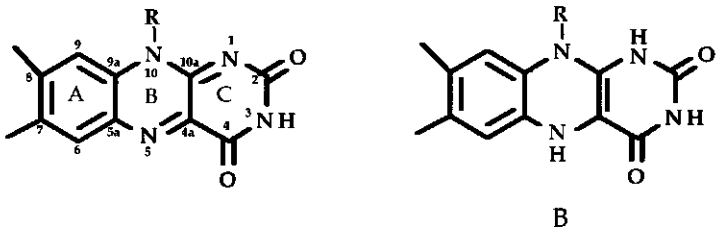


Fig. 1.8. Structures of riboflavin, FMN and FAD, both in the oxidized quinone (A) and the two electron reduced 1,5-dihydroquinone form (B). Riboflavin, R = ribityl; FMN, R = 5'-monophosphate-ribityl; FAD, R = 5'-monophosphate-ribityl-adenine.

more negative. Partial charges on the other atoms remain unchanged but, surprisingly, the C(7) and C(8) methyl groups adopt part of the negative charge [93]. The geometry of deprotonated flavin is not greatly different from the neutral molecule [92] although earlier it was proposed that anionic reduced flavin is more bent than its neutral form [95].

A prominent feature of the flavin molecule is its spectral properties. The yellow oxidized flavin has two characteristic absorption maxima at 445 nm and 367 nm, that disappear upon two electron reduction. Flavins fluoresce in the green spectral region where the position and intensity of the emission maximum is dependent on the molecular environment. For example, the fluorescence of protein-bound flavin can be rather weak which sometimes can be ascribed to energy transfer to electron-rich aromatic amino acids. The advantage of this green fluorescence is that the signal can be distinguished from the characteristic protein emission signal in the UV spectral region.

Oxidized flavin can be studied with both steady-state and time-resolved fluorescence spectroscopy. Reduced flavin, on the other hand, is only very weakly fluorescent, due to its low visible light absorption and its preference for the bent conformation that gives rise to a weak picosecond fluorescence [96]. Reduced flavin can only be studied with time-resolved fluorescence due to the sensitivity of this technique [25,96,97] or at very low temperature in rigid glasses [98]. Time-resolved polarized fluorescence also gives information about the flexibility of the flavin binding as compared to overall or segmental motions of the protein.

2.2. *Flavoproteins*

2.2.1. *Flavoenzymes*

In their function as cofactor flavins mediate a variety of chemical reactions including dehydrogenation, electron transfer, activation of molecular oxygen and photochemical reactions. For example, flavin is the photoactive chromophore in DNA repair enzymes (DNA photolyases). Theoretically, flavoproteins can make use of all three redox states during catalysis. But it turns out that they selectively use only certain redox states,

depending on their biological function. For example, in case of the DNA photolyases, two electron reduced FAD is the active form of the flavin cofactor; pyrimidine dimers are split (i.e. repaired) via donation of an electron from a photo-excited reduced flavin to the dimer, thereby forming a pyrimidine dimer anion that collapses to two monomeric pyrimidines [99].

Flavoenzymes do not use the naturally occurring riboflavin as a cofactor but use FMN or FAD instead (Fig. 1.8); apoflavoproteins generally exhibit a great specificity for one particular flavocoenzyme. Furthermore, a number of chemically modified flavins have been found in flavoproteins such as 8-hydroxy- and 6-hydroxy-FAD [95]. Flavin cofactors are in general noncovalently bound to the apoflavoproteins (in the range from mM to almost pM) although in a number of flavoproteins covalently bound flavins occur.

Flavoenzymes can be grouped into a small number of classes based on properties as the type of reaction catalyzed, the ability to use molecular oxygen as electron acceptor and the nature of auxiliary redox centres. The first itemization is in two groups: simple flavoenzymes, i.e. flavoenzymes containing no other prosthetic group than flavin, and flavoenzymes that, apart from flavin, use auxiliary redox centres [100]. The simple flavoenzymes can be subdivided in three major classes: oxidases, electron transferases and monooxygenases [100]. These subclasses will be shortly discussed since they will be referred to in this thesis.

Oxidases (such as D-amino acid oxidase, glucose oxidase, lactate oxidase) accept electrons from substrates and use oxygen as terminal electron acceptor, forming hydrogen peroxide and oxidized substrate. Oxidases shuttle between the flavoquinone and hydroquinone; flavohydroquinone is very reactive towards oxygen but reacts slowly with one-electron acceptors. In most oxidases the dihydroflavin is held in a more planar conformation than free in solution which probably makes them more reactive towards oxygen.

The electron transferases, e.g. flavodoxin, are all involved in one-electron transfers. Characteristic of this class is the slow reaction with oxygen, a process in which superoxide ($O_2^{\cdot-}$) and flavosemiquinone are formed. Flavodoxins can mediate electron transfer between other substrates or proteins, for example the strictly two electron-reacting nicotinamide nucleotides or the strictly one electron-reacting heme proteins.

Monooxygenases, like *p*-hydroxybenzoate hydroxylase and bacterial luciferase, catalyze insertion of an oxygen atom into a substrate (oxygenation). To achieve this, the enzyme is reduced by NADH or NADPH and, upon reaction with oxygen, a C(4a) hydroperoxide radical is formed. This hydroperoxide transfers an oxygen atom to the substrate, resulting in a C(4a)-hydroxyflavin, which upon dehydration returns to oxidized flavin. In absence of substrate, the hydroperoxide converts to oxidized flavin and H_2O_2 (the monooxygenase than acts like an oxidase).

2.2.2. *Non-catalytic flavin-binding proteins*

In addition to flavoenzymes there are also a number of other flavin-binding proteins, which are not catalytically active. The most well-known examples are egg white riboflavin binding protein and yolk riboflavin binding protein that play a role in the development of oocytes [101].

However, in 1985 it was shown that in man immunoglobulins (Igs) are the proteins most responsible for binding riboflavin (vitamin B_2) [102]. These Igs are thought to be responsible for the individual variations of the riboflavin levels in healthy men as well as of the abnormal processing of this vitamin in cancer patients [101]. High-affinity riboflavin-binding Igs have been isolated from the serum of two patients with multiple myeloma [103,104]. The existence of riboflavin-binding Igs may represent a response of the immune system to a stimulus by riboflavin or a flavin derivative. A possibility is that a flavoprotein containing covalently attached FMN or FAD (flavin alone is to small to be immunogenic) has elicited the production of antibodies [105], of which some bind to the flavin moiety. Whether by accident or design, the presence of these Igs has the potential to affect flavin metabolism. The multiple myeloma patient from whom riboflavin-binding monoclonal antibody was isolated did not show signs of riboflavin deficiency, but by urinary excretion the riboflavin level was decreased in this individual [106]. Since an increase in riboflavin binding levels has been noted in patients with certain types of cancer, the decreased urinary excretion that has been demonstrated in several types of cancer may be a mechanism to keep the riboflavin in circulation due to protein binding. On the other hand, the investigations of Stoppini *et al* [107] have not revealed any additional physiological function for these IgGs, so the existence of these high-affinity Igs might indeed be a coincidence.

2.3. *Flavin-protein interactions and their influence on the flavin redox potential*

The versatility of the flavin is regulated by its protein environment. This protein environment causes the binding and catalytic steps to be specific and controls the kinetics and thermodynamics of the electron transfer processes. The thermodynamics of the electron transfer are reflected in the redox potential. The redox potential indicates the tendency of a molecule to acquire electrons and become reduced or donate electrons and become oxidized. For flavin different redox potentials can be distinguished: E_1, corresponding to the quinone-semiquinone potential, E_2, corresponding to the semiquinone-hydroquinone potential and $E_0'(=E_1+E_2/2)$, that reflects the quinone-hydroquinone potential [95]. For free flavin the semiquinone form is thermodynamically destabilized which is demonstrated in a more negative E_1 and a relatively positive E_2. In oxidases and flavodoxins the semiquinone is stabilized by specific interactions with the protein surrounding it. The redox potentials of a

given flavoenzyme may give a clue in determining which molecule the enzyme can use as electron donor or acceptor in redox catalysis; variable redox potentials allow different functions for flavoproteins. However, the relation between the redox potentials of the diverse flavoproteins and their catalytic function is not yet clear.

The redox chemistry of the flavin is restricted to the isoalloxazine nucleus. Ring C of the three-membered ring system is electron-deficient and can be viewed as an "electron sink". In thermodynamic terms, any interaction which tends to lower its electron density will increase the redox potential and the other way round. The nitrogens N(5) and especially N(1) act as the redox centre of the flavin. Changes in the local environment around these two atoms may result in relatively large changes in the redox potential of the flavin. Other forces acting on the three-ring system, such as steric effects or hydrogen bonds, can also profoundly affect the flavin redox potential by hindering the folding of the molecule over the N(5)-N(10) axis. Ring A plays an indirect role in the reduction and/or reoxidation of the flavin moiety by serving as polarizability source for the changes occurring in the redox-active N(1)-C(10a)-C(4a)-N(5) region, in that way fine-tuning the modulation of the redox potential [94]. For instance, in glutathione reductase there is an Arg residue located close to the C(8) methyl group inducing polarization that could influence the rest of the isoalloxazine [108]. For oxidases, X-ray crystallographic structures show positive residues of the protein interacting with ring C. Glycolate oxidase [109] has a positively charged lysine and both glucose oxidase [110] and cholesterol oxidase [111] have histidine residues in the vicinity of ring C. In all cases the flavin is well buried in the protein, rigidly fixed by hydrogen bonds, with only the region around N(5) accessible to solvent.

Oxidases shuttle between the flavoquinone and the flavohydroquinone. However, the thermodynamics of these enzymes involves stabilization of the flavosemiquinone (reflected in an E_1 positively shifted and an E_2 negatively shifted compared to that of free flavin) which does not fit with the catalytic two-electron transfer. Stankovich and coworker have shed light on this, primarily with their studies on D-amino acid oxidase [112,113]. From structural information available on oxidases it is known (see above) that there is an amino acid side chain that forms a hydrogen bond with N(1) of the flavin ring. This hydrogen bond causes a net positive charge on the oxidized enzyme (resulting in a more positive potential) which makes the first electron easier to transfer and also makes the first electron transfer pH independent since no proton has to be transferred along with the first electron to neutralize the charge. In contrast, the transfer of the second electron is at a more normal potential for flavin and is also pH dependent since the one-electron reduced enzyme has a neutral or only slightly positive charge. For D-amino acid oxidase it has been found that binding of a substrate analogue regulates the first electron transfer by causing a negative shift of E_1 (i.e. destabilization of the semiquinone) and a positive

shift of E_2 (i.e. stabilization of the hydroquinone). Thus binding of the substrate analogue causes the conformation of the protein around the flavin to change and, according to Fitzpatrick and Massey [114], this is due to disruption of the hydrogen bond at the N(1) position of the flavin ring. Summarizing, binding of substrate causes the enzyme to be further "tuned" to a state of "active" conformation. For other oxidases, e.g. glucose oxidase and glycolic acid oxidase the protonation and redox behaviour are more complex.

In case of monooxygenases, the redox potential does not seem to influence the reactions catalyzed, nor has it yet given clear insight into the mechanism of this class of enzymes.

A distinguishing feature of flavodoxins, an important family of electron transferring flavoproteins with FMN as prosthetic group, is the very negative E_2 value resulting in stabilization of the semiquinone compared to the hydroquinone. Ring A of FMN is on the surface of the protein and in contact with bulk solvent whereas ring C is buried in a relatively apolar binding pocket. The dianion phosphate group at the end of the N(10) substituent of FMN (Fig. 1.8) is not compensated by basic residues in the protein and therefore interacts unfavorably with the negatively charged hydroquinone [115]. This feature combined with the unfavorable electrostatic environment provided by solvent-accessible surface acidic residues clustered around the FMN [116] and the apolar environment of the isoalloxazine nucleus [117] significantly determine the destabilization of the hydroquinone.

3. Catalytic antibodies

One of the major challenges for the modern biochemist is to extend the repertoire of natural biocatalysts, particularly in view of reactions for which no natural catalysts exist. At present the most successful way to design and generate a totally new biocatalyst is the generation of a catalytic antibody or abzyme (=*ab*ody en*zyme*). The immune system's capacity to produce high-affinity binding sites for virtually any ligand has been successfully exploited in recent years to create these antibodies with tailored catalytic specifications. The hypothesis that antibodies, like enzymes, could catalyze chemical reactions was proposed by Jencks in 1969 [118], surprisingly in a textbook. This concept is based on the transition state theory that states that enzymes can catalyze reactions by stabilizing the transition state of the reaction [119]. Jencks proposed that antibodies generated against such a transition state should be able to stabilize it and should therefore catalyze the reaction. The development of hybridoma technology [1], realizing the production of monoclonal antibodies, was necessary to prove Jencks' hypothesis. Immunization with stable template molecules that mimic the short-lived transition state of a chemical reaction yielded the first catalytic antibodies in 1986 [120,121].

While these first catalytic antibodies catalyzed only simple hydrolysis reactions, there are now antibodies catalyzing acyl transfers, pericyclic processes, eliminations, isomerizations (for a review see [122]), peptide synthesis [123,124] and, to a certain extent, redox reactions (see 3.2). The present generation can even catalyze reactions that are disfavored or for which no natural enzyme catalyst is known, such as the Diels-Alder reaction or a Cope rearrangement [125-128]. However, there are still a number of challenges in this field: aldol condensations, S_N2 displacements, phosphoryl and glycosyl group transfers and, most challenging, the sequence-specific cleavage of peptides and DNA are among the challenging reaction types that have not yet been catalyzed by antibodies [122].

Catalytic antibodies could potentially solve specific practical problems. Hydrolytic antibodies might be used to selectively activate prodrugs. For this purpose anti-phosphonate antibodies that converted biologically inactive esters into their bioactive forms were developed [129,130]. The antibodies produced strongly inhibited bacterial growth in *in vitro* tests. Extending this approach to the creation of bispecific antibodies derived from a catalytic antibody and a tumor-specific antibody could improve the therapeutic efficiency of many cytotoxic agents. A useful practical application of hydrolytic antibodies might be their use in the treatment of drug addiction. Catalytic antibodies that catalyze the degradation of cocaine to yield an inactive metabolite lacking cocaine's stimulating properties are a promising example in this respect [131]. In the future therapeutic approaches to the treatment of addiction or overdose might involve immunization of the host with the transition state analogue or direct administration of purified antibodies [132].

At present the crucial problem in the search for catalytic antibodies is the very small chance of selecting the desired catalyst. On one hand the available antibody pool might not be large enough, on the other hand it might be too large to successfully select the one desired antibody. Consequently, two goals should be focused on: hapten design and screening. To generate highly active catalysts in a single immunization step, it will be necessary to elucidate the rules that make one hapten better than another. Studies on hapten design are starting to shed light on this [133-135]. Screening is a very crucial step in the search for catalytic antibodies since the amount of active catalysts in a pool of antibodies can be quite low. Clearly, the larger the pool of candidates evaluated, the better the chances of identifying highly efficient catalysts and of establishing a meaningful correlation between hapten structure and catalyst function. Powerful screening methods have been developed in the past few years: sensitive immunoassays [136], labeling compounds with fluorescent tags [137] and biological selection methods that allow the catalytic activity of individual clones to be monitored directly. This last powerful screening strategy relies on bacterial molecular biology: the use of bacteria lacking an enzyme indispensable to survival; only those bacteria that express an antibody having the catalytic properties of the missing enzyme survive (for examples see [138,139]). These powerful screening techniques can be adapted for the direct selection of catalytic activities out of large phage display libraries [140].

Nevertheless, even the best catalytic antibodies so far generated are much less active than their natural enzyme counterpart. Structural and mechanistic studies [141,142] suggest that the modest activity observed in most first-generation catalytic antibodies is due to their short evolutionary history and imperfect hapten design [122]. Presumably, one-step strategies to elicit catalytic antibodies will not be sufficient to select antibodies with reasonable turnover rates, simply because the available repertoire will never be large enough. Evolutionary strategies, in which antibodies will be subjected to rounds of mutagenesis, will contribute significantly to this task [143]. Production of antibodies in bacteria (1.4.2) and the use of combinatorial libraries of antibodies displayed on phage (1.4.3) will definitely play a crucial role with respect to this. Furthermore, improved design of transition state analogues will play an important role.

3.1. Redox active catalytic antibodies

Enzymes that mediate redox reactions form an important and promising class of enzymes for practical applications. These enzymes use biological redox cofactors such as flavin (see 2) and heme. To mimic redox enzyme activity, antibodies have been generated that catalyze redox reactions. However, most of these antibodies use electron donors or acceptors different from the cofactors used in nature, for example, the periodate-dependent oxidation of sulfide to the corresponding sulfoxide, a monoxygenase reaction [144,145]. Other examples are the stereoselective

reductions of ketoamides [146] and ketones [147] for which sodium cyanoborohydride has been used as an electron-donor. These electron-donors and -acceptors can not be recycled and must therefore be added in large quantities.

In order to further expand antibody catalyzed redox reactions, strategies that allow incorporation of cofactors, that can be recycled, into the antibody binding site will have to be used. To this end antibodies have been elicited against flavin [148] and heme [149]. In an attempt to mimic peroxidase activity, Cochran and Schultz [149] generated antibodies against N-methylmesoporphyrine, a transition state analogue for porphyrine metallation. These antibodies catalyze the metallation of mesoporphyrine IX. One of these antibodies also binds the biologically relevant metalloporphyrine iron (III) mesoporphyrine. This antibody-porphyrine complex catalyzes the oxidation of several typical chromogenic peroxidase substrates [150]. However, this antibody lacks multiple binding sites for cofactor and substrate binding (which is a common feature for redox enzymes): it only binds the heme and is therefore not substrate specific. Nevertheless these studies might be a first step to the development of selective antibody-heme peroxidase catalysts.

Antibodies have also been elicited against oxidized flavin (see 2.1) [148]. One of these antibodies causes the redox potential to shift from -206 mV to -343 mV. Hence, antibody bound flavin is a stronger reducing agent than free flavin. The substrate safranine T (E_0 = 298 mV) could rapidly be reduced by the antibody-flavin complex (previously reduced by sodium dithionite), a process that did not occur with free flavin. However, this antibody again lacks the substrate binding site. Moreover, the cofactor was not catalytically recycled in the antigen-binding site.

Antibody catalysis of redox reactions is a complex matter. Strategies have emerged for the generation of catalytic antibodies that utilize "unnatural cofactors" (like sodium periodate or sodium cyanoborohydride). Redox-active heme and flavin binding antibodies have also been described. However, neither of these antibodies possesses binding sites for substrate as well as the cofactor, nor could the cofactor be catalytically reduced in the antigen-binding site.

4. *Aim of this thesis*

The goal of this thesis is two-fold. Firstly, we want to develop a redox-active antibody that can bind both a cofactor and a substrate molecule and also is capable of recycling a cofactor in its antigen-binding site. Secondly, we want to develop a model system to study how the protein-environment regulates the redox potential and compare this with the way natural flavoproteins perform this task.

To achieve the first goal we chose flavoprotein oxidases (see 2.2.1) as a model system. Using hybridoma technology antibodies have been generated against a stable reduced flavin analogue that has a benzoyl substituent covalently linked to N(5) (chapter 2). These antibodies have extensively been characterized by time-resolved polarized fluorescence spectroscopy (chapter 2). The benzoyl substituent should create a pocket in the antigen-binding site that will both accelerate cofactor recycling and serve as a substrate binding pocket. One of these antibodies has been studied for catalytical reoxidation of reduced flavin in the antigen-binding site (chapter 5).

In order to contribute to a better understanding of how protein environments regulate the flavin redox potential, scFv antibody fragments have been generated against reduced as well as oxidized flavin using a naive antibody phage display library (see 1.4.3). The direct selection of an scFv antibody against oxygen sensitive two-electron reduced flavin has been described in chapter 3. Mapping studies of anti-flavin scFvs with different flavin analogues, confirmed by molecular modelling studies (see 1.2.2.1), contribute to a better understanding of how these "artificial" protein environments regulate the flavin redox potential (chapter 4).

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Chapter 2

Monoclonal antibodies against two electron reduced riboflavin and a quantification of affinity constants for this oxygen sensitive molecule*

Abstract

In order to create a protein environment that binds preferentially to the two-electron reduced form of flavin, monoclonal antibodies have been raised against a reduced flavin derivative. Due to the low fluorescence quantum yield and visible light absorption and to the instability of reduced flavin in an aerobic environment, it is not possible to determine the affinities of these antibodies for two-electron reduced flavin using standard techniques. Because of its sensitivity, time-resolved fluorescence can be used to overcome this problem. This technique has been applied to study the binding of two antibodies, an IgG_1 and an IgM, to reduced riboflavin (1,5-dihydroriboflavin) and oxidized riboflavin (riboflavin). The affinity of the IgG_1 is more than 80 times larger for 1,5-dihydroriboflavin than for riboflavin. From analysis of the dynamical parameters of the system it is apparent that the internal motion of 1,5-dihydroriboflavin bound to IgG_1 is much more restricted than that of riboflavin. In contrast, the affinity of the IgM is only slightly higher for 1,5-dihydroriboflavin than for riboflavin and the flexibility of binding of both flavin redox states in the antigen binding site is almost similar.

2.1. Introduction

Flavins mediate a variety of chemical reactions including dehydrogenation, electron transfer, activation of molecular oxygen and photochemical reactions. This versatility sets flavoproteins apart from most other cofactor-dependent enzymes, which, in general, each catalyze a single type of reaction [1]. One of the most interesting features of the versatile flavin molecule consists of its redox properties which can be modulated by the (protein) environment. The redox active part of the flavin is the isoalloxazine ring which can exist in oxidized flavoquinone, one-electron reduced flavosemiquinone and two-electron reduced flavohydroquinone states. The redox potentials of both electron transfer steps vary largely among different flavoproteins and depend on the chemical nature of the active site in which the isoalloxazine resides. This

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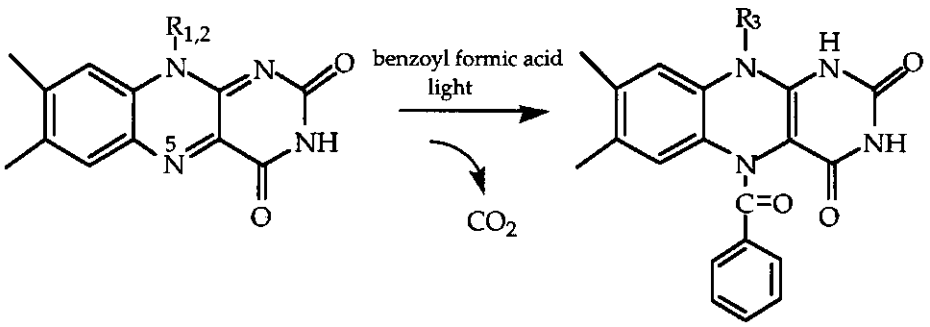
property makes the flavin suitable as an electron shuttle in very different redox reactions which explains its widespread occurrence in nature [2]. The role of the protein environment in modifying the chemical reactions and the redox properties of the flavin is not yet clear. Creating artificial protein environments with a predetermined affinity for oxidized and reduced flavin and comparing the structural and redox properties of these proteins, might contribute to gain more insight in this problem. Monoclonal antibodies (mAbs) are very useful tools for this because the flavoquinone differs from the hydroquinone in its conformation and electronic properties. This enables generation of antibodies specific for either the reduced or the oxidized form. Shokat and coworkers have indeed shown that it is possible to create an anti-flavin antibody that can influence the redox properties of flavin [3].

Here we report a strategy to obtain a protein environment which binds preferentially the 1,5-dihydroflavin and to develop a method to determine the affinity of such an antibody for this flavin. The generation of mAbs is hampered by the instability of the flavohydroquinone under aerobic conditions. This problem was circumvented through the design of a reduced flavin analogue that was not sensitive to reoxidation. Another problem is that, in contrast to its oxidized form, reduced flavin possesses a low fluorescence quantum yield and visible light absorption [4]. For this reason it is difficult to determine dissociation constants for reduced flavin using standard (fluorescence) techniques. Because of its sensitivity, however, time-resolved polarized fluorescence techniques can be used to overcome this problem. Time-resolved fluorescence is one of the very few methods to obtain quantitative information on the binding of both oxidized and reduced flavin to the antibody molecule. The excited state of a fluorophore, such as flavin, has a lifetime in the (sub)nanosecond range. Since this corresponds to the timescale of rotational and internal motion of the antigen in the antigen binding site, time-resolved fluorescence also gives information about the flexibility of the antigen binding as compared to the overall or segmental motion of the antibody.

2.2. *Experimental procedures*

2.2.1. *Synthesis of hapten and conjugates*

Riboflavin (from Sigma) dissolved in dry pyridine was refluxed for 4 hours with a fivefold excess of succinic anhydride [5]. After addition of 30% (w/v) toluene, the solvent was removed under reduced pressure. The residue was dissolved in water and purified with DEAE-Sepharose (batch wise). The succinylated riboflavin (Fig. 2.1, R=1) was eluted from this material with 1 M NaCl and was conjugated to porcine thyroglobuline (Sigma) or BSA fraction V (Boehringer Mannheim) with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Sigma) using general procedures (Fig. 2.1, R=2a or 2b, further indicated as **2a** and **2b** respectively) [6]. After conjugation, the flavin was modified on the N(5) position by irradiating



R_1 = $CH_2(CHOH)_3CH_2OCO(CH_2)_2COOH$

R_{2a} = $CH_2(CHOH)_3CH_2OCO(CH_2)_2CONH$-PTG
R_{2b} = $CH_2(CHOH)_3CH_2OCO(CH_2)_2CONH$-BSA

R_{3a} = $CH_2(CHOH)_3CH_2OCO(CH_2)_2CONH$-PTG
R_{3b} = $CH_2(CHOH)_3CH_2OCO(CH_2)_2CONH$-BSA

Figure 2.1. Synthesis of hapten and conjugates.

the flavin-protein conjugate with visible light in the presence of benzoylformic acid under anaerobic conditons yielding the N(5)-benzoyl-N(10)-ribitylsuccinimide ester flavin (Fig. 2.1, R=3a or 3b) [7]. The excess benzoylformic acid was removed by dialysis against 0.5% (w/v) ammonium bicarbonate. These hapten-carrier conjugates (indicated as **3a** and **3b** respectively) were freeze dried for storage.

2.2.2. Monoclonal antibody production, selection and purification.

Balb/c mice were immunized twice with **3a** (Fig. 2.1). Spleen cells from these mice were fused to myeloma cells (SP 2/0) [8], according to protocols described by Schots and coworkers [9]. Hybridoma clones producing antibodies against the modified flavin were selected using an Enzyme Linked Immunosorbent Assay (ELISA) against **3b** (Fig. 2.1) and BSA alone. For this purpose microtiter plates were coated with 100 µl per well of 10 µg/ml **3b** (Fig. 2.1) or BSA in PBS. The wells were blocked with 5% skimmed milk powder in PBS. Mouse antibody was detected by alkaline phosphatase conjugated rat anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc.).

Positive hybridoma clones were cloned until stability, whereafter the cultures were expanded in flasks (150 cm^2). We used Iscove's modification of Dulbecco's medium (IMDM) and low IgG fetal calf serum for these

cultures. MAbs of the IgM isotype were purified by precipitation with 8.5% polyethyleneglycol (MW 6000, Sigma) followed by gelfiltration chromatography (Superdex 200 prep grade, Pharmacia). IgG_1 antibodies were purified by thiophilic adsorption chromatography (Affi T™ thiophilic agarose, Biozym). All preparations were judged for purity by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE). Antibody concentrations were determined by measuring the absorbance at 280 nm assuming A_{280}=1.25 for 1 mg/ml and a molecular mass of 970 kDa for IgM and 150 kDa for IgG_1 [6].

2.2.3. *Sample preparation for fluorescopic measurements*

Since the fluorescence quantum yield of reduced flavin is very low, a minimal concentration of flavin is required to get a proper signal. Therefore we chose for titration of a constant amount of riboflavin or 1,5-dihydroriboflavin with antibody instead of titration of a constant antibody concentration with variable amounts of riboflavin or 1,5-dihydroriboflavin. Different amounts of antibody, up to 0.223 mg/ml for $\alpha Rf_{mod}G_1$ and to 0.816 mg/ml for $\alpha Rf_{mod}M$, were mixed with 0.1 and 0.05 μM riboflavin respectively. For measurement of 1,5-dihydroriboflavin, these solutions were deaerated by flushing with argon and riboflavin was reduced with a 30 times molar excess of sodium dithionite. All compounds were dissolved in 50 mM sodium pyrophosphate pH 8.0 containing 150 mM sodium chloride and filtered through a 0.22 μm filter (Costar).

2.2.4. *Time-resolved fluorescence and fluorescence anisotropy measurements*

Time-resolved fluorescence decays were measured using the time-correlated single photon counting set up described earlier [10]. Vertically polarized light of 444 and 450 nm was used to excite 1,5-dihydroriboflavin and riboflavin, respectively. After excitation the polarized fluorescence was monitored using a Schott (Mainz, Germany) interference filter (Schott 557,6 nm, with a full width at half maximum of 12.6 nm) in combination with a Schott cut-off filter (OG515). After each sample the background of the samples in the absence of antibody and flavin was measured at one fifth of the time of the sample acquisition time. Erythrosin B served as a reference compound to yield the dynamic instrumental response function of the set-up [11]. Erythrosin B shows a single exponential fluorescence decay time of 80 ps at 20°C which was the sample temperature used. The data were collected in a multichannel analyser (Nuclear Data model ND66) using 1024 channels per experimental decay with a time spacing of 30 ps per channel. For the registration of the fluorescence decays the excitation energy was chosen to yield a frequency of fluorescence photons of 30 kHz. In this way the signal-to-noise ratios in the fluorescence decays of riboflavin and 1,5-dihydroriboflavin were comparable.

2.2.5. Data-analysis and calculation of the affinity constant

The fluorescence decays were analyzed in terms of a continuous distribution of decay times by means of the maximum entropy method (Maximum Entropy Data Consultants Ltd., Cambridge, UK) of analysis yielding the fluorescence lifetime and rotational correlation time distributions of the free and bound riboflavin and 1,5-dihydroriboflavin [10,12,13]. This method recovers the optimal spectrum of decay times by maximizing the Shannon-Jaynes entropy and minimizing the χ^2 statistics and has the advantage that a unique solution is found with no *a priori* knowledge of the decay model.

The time-resolved fluorescence anisotropy (A(t)) of a fluorescent molecule in a protein can be adequately described by an expression which assumes two independent rotational motions: a rapid internal motion of the flavin in the antigen binding site (ϕ_{int}) and a slower motion due to the overall or segmental motion of the antibody (ϕ_∞) (the subscript is used to indicate that this time is "infinitely long" on the experimental timescale) [14]:

$$A(t) = [\beta_1 \exp(-t/\phi_{int}) + \beta_2]\exp(-t/\phi_\infty) \quad (1)$$

We can define a so-called order parameter S for a fluorescence probe attached to the protein. When there is no internal probe motion, the probe rotates together with the whole protein and S is equal to unity. Depending on the freedom of internal probe motion relative to protein rotation, S will be less than unity. The order parameter S can be obtained from (see for example [15]):

$$S = [\beta_2/(\beta_1 + \beta_2)]^{1/2} \quad (2)$$

From S we can estimate the displacement angle ψ over which the probe can move over the line of attachment to the protein (which is considered to be the local symmetry axis of the potential well in which the probe moves; a further assumption is that the emission transition moment is parallel to this symmetry axis) according to:

$$S = [1/2\cos\psi(\cos\psi + 1)]^{1/2} \quad (3)$$

The internal correlation time ϕ_{int} is inversely related to the diffusional rate constant D via:

$$D = (1 - S^2)/(6\phi_{int}) \quad (4)$$

The fraction of bound 1,5-dihydroriboflavin was calculated from anisotropy decays using a fractional analysis approach [16]. We assumed that the system is in binding equilibrium and consists of a mixture of only

two states: bound and free 1,5-dihydroriboflavin. Provided that the decay curves corresponding to free 1,5-dihydroriboflavin and of a sample where the majority of the flavin molecules is bound are known, decays measured at intermediate binding conditions can be described as a combination of both limiting decays. The fraction of bound riboflavin was calculated from the decrease of the free riboflavin contribution in the rotational correlation-time spectra. The dissociation constant (K_d) was then obtained according to the following equations (written out for riboflavin):

$$K_d = \frac{[\text{riboflavin}]_{free}[\text{binding sites}]_{free}}{[\text{binding sites}]_{occupied}} \quad (5)$$

$$[\text{binding sites}]_{free} = n[Ab] - [\text{riboflavin}]_{bound} \quad (6)$$

where n corresponds to the number of binding sites per antibody molecule.
Combination of equations 5 and 6 gives:

$$n[Ab] - [\text{riboflavin}]_{bound} = Kd.\frac{[\text{riboflavin}]_{bound}}{[\text{riboflavin}]_{free}} \quad (7)$$

Plotting $n[Ab]-[riboflavin]_{bound}$ as a function of $\frac{[\text{riboflavin}]_{bound}}{[\text{riboflavin}]_{free}}$ gives the K_d. Since riboflavin and 1,5-dihydroriboflavin are small molecules, all binding sites must be available for binding. Therefore it was assumed that n=2 for $\alpha Rf_{mod}G_1$ and n=10 for $\alpha Rf_{mod}M$ [17].

2.3. *Results*

2.3.1. *Synthesis of hapten and conjugates*

For mouse immunization we prepared the N(5) substituted reduced flavin derivative **3a** (Fig. 2.1), since the two-electron reduced flavohydroquinone quickly reoxidizes under influence of oxygen. The conjugates **2a** and **2b** (Fig. 2.1) were obtained by conjugation of the succinylated riboflavin to PTG and BSA resulting in molar ratios of 44:1 for PTG and 6:1 for BSA. After reduction in the presence of benzoyl formic acid the N(5)-benzoyl-N(10)-ribitylsuccinimide ester flavins **3a** and **3b** (Fig. 2.1) both showed an absorption spectrum that is characteristic for reduced flavin.

2.3.2. *Monoclonal antibody production, selection and purification*

After immunization of mice with conjugate **3a** and generation of mAbs the resulting hybridomas were first screened for binding to the BSA-conjugate **3b** as well as to BSA alone, and then for binding to the BSA-riboflavin conjugate **2b** (Fig. 2.1). 16 mAbs bound to **3b** and **2b** and not to BSA alone. No significant difference in binding to the conjugates **2b** and **3b** could be detected, so that at this point we had no indication whether these mAbs indeed bound the reduced flavin analogue preferentially. Two antibodies of different isotype, the IgG_1 $\alpha Rf_{mod}G_1$ and the IgM $\alpha Rf_{mod}M$, were chosen for further investigation. These hybridoma clones were grown in larger amounts and purified from tissue culture supernatants.

2.3.3. *Time-resolved total fluorescence measurements and analysis*

To study the binding of $\alpha Rf_{mod}G_1$ to riboflavin we measured 0.1 μM riboflavin incubated with antibody concentrations ranging from 0 to 1.5 μM. To investigate binding of 1,5-dihydroriboflavin to $\alpha Rf_{mod}G_1$ the riboflavin in these samples was reduced. Examples of experimental total fluorescence decays of free and antibody bound riboflavin and 1,5-dihydroriboflavin are shown in Fig. 2.2A and C. The fluorescence of antibody bound riboflavin exhibits an initial faster decay than the fluorescence of free riboflavin. The decay of free and bound 1,5-dihydroriboflavin shows much more heterogeneity. However, the fluorescence decay characteristics of free and bound 1,5-dihydroriboflavin hardly differ.

The total fluorescence decays are analysed using MEM. The lifetime distribution patterns for both free and antibody bound riboflavin and 1,5-dihydroriboflavin are shown in Fig. 2.2B and 2D. The corresponding average fluorescence lifetimes obtained are listed in Table 2.1. Free riboflavin has a major lifetime of 3.7 ns and two small contributions of 1.7 and 0.13 ns (Fig. 2.2B, broken line). The two small contributions are probably due to self-quenching as a result of a tendency of self-association of riboflavin molecules (note that riboflavin does not dissolve in water very well). This is confirmed by the fact that a lower riboflavin concentration gives a longer average fluorescence lifetime (Table 2.1). Upon addition of increasing amounts of mAb the contribution of the component at 3.7 ns decreases and two contributions around 0.89 and 0.11 ns show up (Fig. 2.2B, solid line) which are responsible for the faster initial decay.

The fluorescence lifetime distribution pattern of 1,5-dihydroriboflavin is more complex than that of riboflavin (Fig. 2.2D, broken line). This complex pattern can be explained by the presence of multiple, non-planar conformations of reduced flavin, that interconvert into each other [18]. All these substates cause a more complex lifetime distribution than

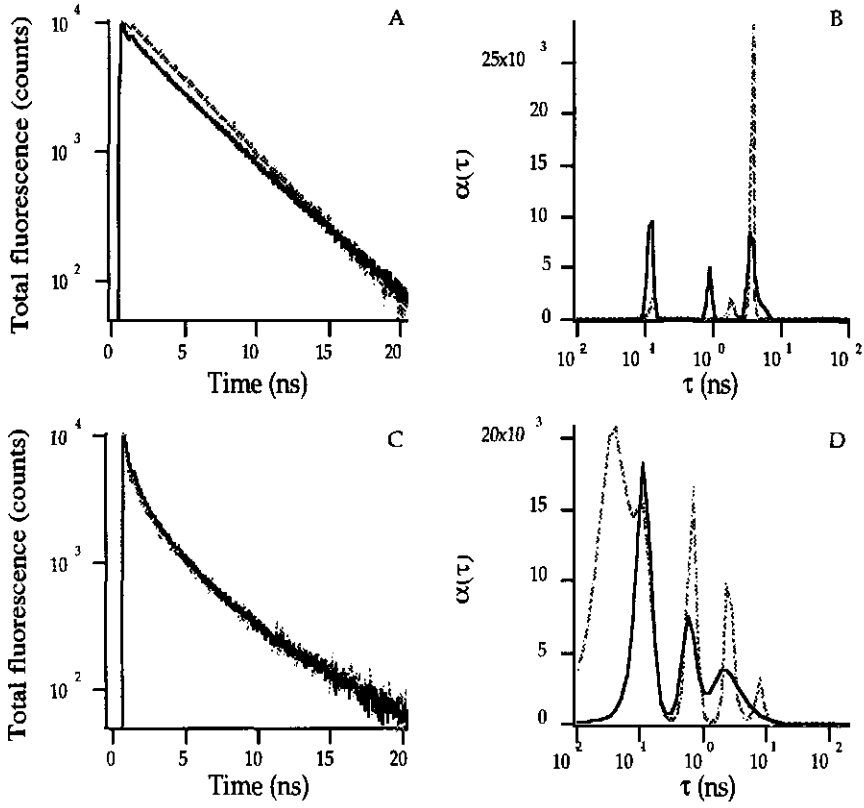


Figure 2.2. Experimental total fluorescence decays and fluorescence lifetime distributions of free and αRfmodG1 bound riboflavin and 1,5-dihydroriboflavin. In the panels A and B the broken curves reflect free riboflavin, in the panels C and D the broken curves reflect free 1,5-dihydroriboflavin. The solid curves reflect bound species in all cases. In all experiments the residuals of the fit were randomly scattered around zero, indicating an optimal fit.

riboflavin, which has a planar conformation and only shows a main nanosecond lifetime. Free 1,5-dihydroriboflavin shows a broader spectrum of fluorescence lifetimes than antibody-bound 1,5-dihydroriboflavin (Fig. 2.2D, solid line) which perhaps can be ascribed to a more restricted mobility of bound 1,5-dihydroriboflavin.

2.3.4. *Time-resolved fluorescence anisotropy and analysis*

From a fluorescence anisotropy decay one obtains information on rotational and internal motions of the fluorophore. After MEM analysis a distribution of rotational correlation times ϕ can be recovered. The experimental fluorescence anisotropy decays and corresponding rotational

	flavin concentration (μM)	riboflavin	1,5-dihydro-riboflavin
-	0.05	3.69	2.40
-	0.1	3.29	0.53
αRfmodG$_1$	0.1	2.04[a]	0.88[c]
αRfmodM	0.05	2.04[b]	0.55[c]

[a] mixture of 32% bound and 68% unbound riboflavin.
[b] mixture of 24% bound and 76% unbound riboflavin.
[c] completely bound 1,5-dihydroriboflavin.

Table 2.1. Average fluorescence lifetimes (in ns) of free and antibody bound riboflavin and 1,5-dihydroriboflavin.

correlation time spectra of free and antibody bound riboflavin and 1,5-dihydroriboflavin are shown in Fig. 2.3. The rotational diffusion of free riboflavin results in an ultrafast decay of the fluorescence anisotropy (Fig. 2.3A, broken line). Upon addition of $\alpha Rf_{mod}G_1$ the motional freedom of the riboflavin is severely restricted, indicating that binding to this antibody limits the motional freedom of riboflavin (Fig. 2.3A, solid line). In Fig. 2.3C the anisotropy decay curves of reduced 1,5-dihydroriboflavin are shown. In presence of $\alpha Rf_{mod}G_1$ the flavin is totally immobilized (solid line). Comparison of the solid curves in Fig. 2.3A and C suggests that the affinity of $\alpha Rf_{mod}G_1$ for 1,5-dihydroriboflavin is significantly higher. Intermediate antibody concentrations gave intermediate fluorescence anisotropy decays in both cases (data not shown).

The rotational correlation time distribution for free riboflavin (Fig. 2.3B, broken line) shows a main contribution at 0.14 ns which is in agreement with earlier results [19]. When antibody is added, a contribution of ϕ = 1.1 ns and one longer, non-resolved correlation time gradually arise, at the expense of the correlation time of free riboflavin (Fig. 2.3B, solid line). The contribution at 1.1 ns (ϕ_{int}) is due to restricted motion of flavin and reflects its flexible binding in the antigen binding site. Fab segmental flexibility and tumbling of the whole antibody are apparently slower than the timescale of observation [20,21] and therefore result in a contribution at infinite time (ϕ_∞).

The rotational correlation time distribution pattern of free 1,5-dihydroriboflavin is more complex than that of riboflavin (Fig. 2.3D, broken line). There is a main contribution at 0.3 ns (58%) which is in fair agreement with the value obtained for free reduced flavin mononucleotide (FMN) [10]. The other contributions are ascribed to self-association of 1,5-dihydroriboflavin molecules, since at lower 1,5-dihydroriboflavin concentrations the distribution pattern approaches that of free reduced FMN which is much better soluble in water.

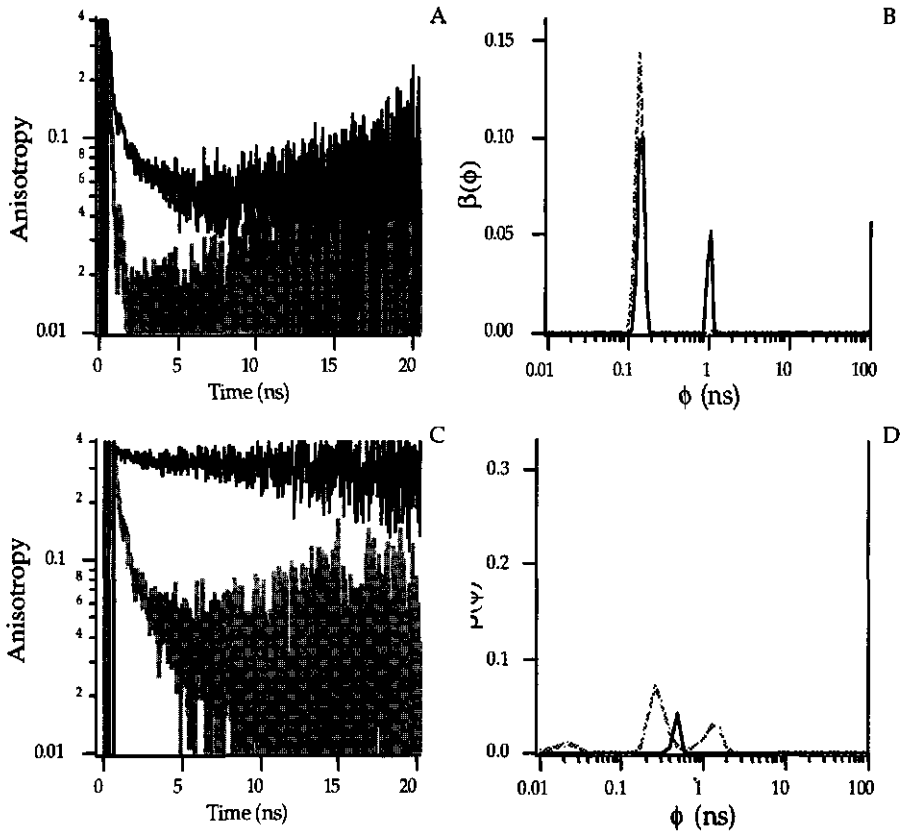


Figure 2.3. Experimental fluorescence anisotropy decays and rotational correlation time distributions of free and αRfmodG1 bound riboflavin and 1,5-dihydroriboflavin. In the panels A and B the broken curves reflect free riboflavin, in the panels C and D the broken curves reflect free 1,5-dihydroriboflavin. The solid curves reflect bound species in all cases. In all experiments the residuals of the fit were randomly scattered around zero, indicating an optimal fit. The spike at 100 ns corresponds to the infinite anisotropy and will be refered to as $\phi\infty$. For both free riboflavin and free 1,5-dihydroriboflavin no $\phi\infty$ was observed.

Upon addition of increasing amounts of antibody the rotational correlation time spectra gradually change from the initial free to the final bound situation represented by the curves in Fig. 2.3D. The peak at 0.4 ns (ϕ_{int}) reflects rapid internal motion of the flavin in the antigen binding site and the contribution at ϕ_∞ is again the result of a superposition of Fab segmental flexibility and tumbling of the whole antibody. Inspection of Figures 3 B and D reveals that at the highest antibody concentration still

antibody	antigen	S	ψ (°)	ϕ_{int}(ns)	D (ns^{-1})
αRfmodG1	riboflavin	0.59	62	1.1	0.10
αRfmodG1	1,5-dihydro-riboflavin	0.93	24	0.5	0.05
αRfmodM	riboflavin	0.70	54	1.4	0.06
αRfmodM	1,5-dihydro-riboflavin	0.77	45	1.1	0.06

Table 2.2. Geometric and dynamic analysis of fluorescence anisotropy of antibody-bound riboflavin and 1,5-dihydroriboflavin.

free riboflavin is present, whereas all 1,5-dihydroriboflavin has been bound.

For the IgM $\alpha Rf_{mod}M$ we obtained basically similar results, but a relatively higher antibody concentration was needed to bind all 1,5-dihydroriboflavin and it was not possible to bind all riboflavin.

The rotational correlation time distribution of antibody-bound flavin gives information about the motional freedom riboflavin in the antigen binding site. From the relative contribution of the internal motion (ϕ_{int}) with respect to ϕ_{∞} one can derive the so-called order parameter S, defining the angular displacement ψ of the riboflavin in the antigen binding site. The internal rotational correlation time ϕ_{int} and the order parameter S define the diffusion constant D of this reoriental motion (see Experimental Procedures). These parameters are collected in Table 2.2 for $\alpha Rf_{mod}G_1$ as well as for $\alpha Rf_{mod}M$ bound riboflavin and 1,5-dihydroriboflavin. From the order parameter S it is clear that the motion of 1,5-dihydroriboflavin bound to $\alpha Rf_{mod}G_1$ is more restricted than that of flexibly bound riboflavin. In addition, the motional dynamics of $\alpha Rf_{mod}G_1$ bound riboflavin are larger than for 1,5-dihydroriboflavin (reflected in D, see Table 2.2). In contrast, the order parameters for riboflavin and 1,5-dihydroriboflavin when bound to $\alpha Rf_{mod}M$ reveal that in this case there is only a slight difference in the flexibility of binding.

2.3.5. Determination of affinity constants

The slow anisotropy decay of 1,5-dihydroriboflavin in the presence of $\alpha Rf_{mod}G_1$ (Fig. 2.3C) strongly suggests that all reduced riboflavin is bound. The fluorescence anisotropy decay of 1,5-dihydroriboflavin in the presence of $\alpha Rf_{mod}M$ is the same, indicating that here too all 1,5-dihydroriboflavin is bound. This implies that we can assign curves corresponding to free and completely bound reduced riboflavin and therefore we are able to calculate the fractions of bound and unbound flavin using fractional

	Kd(riboflavin) (μM)	Kd(1,5-dihydro-riboflavin) (μM)
αRfmodM	27	16
αRfmodG1	5.8	0.071

Table 2.3. Dissociation constants for binding of αRfmodM and αRfmodG1 to Rf and 1,5-dihydro-riboflavin.

analysis [16]. The reduced χ^2 values obtained from these analyses indicate a very good quality of the fits ($0.99<\chi^2<1.07$ for $\alpha Rf_{mod}G_1$ and $0.99<\chi^2<1.41$ for $\alpha Rf_{mod}M$).

For riboflavin we could not define a population in which all the flavin was bound, impeding fractional analysis. The rotational correlation time spectra of free and bound riboflavin, however, are less complicated and it is therefore possible to use the relative contribution of free riboflavin as an extent of the fraction of free riboflavin present in the solution (Fig. 2.3B for $\alpha Rf_{mod}G_1$). The contribution of the rotational correlation time at 0.14 ns was normalized to the initial anisotropy of 0.38 [22]. This approach allows the determination of affinity constants for weakly bound riboflavin, circumventing the problem that saturation of binding sites could not be reached.

The dissociation constants are calculated according to equation 7 (Table 2.3). Since the error in these values is estimated to be less than 10% we indeed have succeeded in obtaining antibodies with a higher affinity for 1,5-dihydroriboflavin.

2.4. *Discussion*

The fluorescence decay characteristics of free and $\alpha Rf_{mod}G_1$ bound 1,5-dihydroriboflavin hardly differ. This is remarkable because the majority of the 1,5-dihydroriboflavin is bound. This indicates that the local environment of 1,5-dihydroriboflavin when bound is not very different from that of 1,5-dihydroriboflavin in aqueous solution.

From MEM analysis of the fluorescence of 1,5-dihydroriboflavin and riboflavin in binding equilibrium with both investigated antibodies, two rotational correlation times, a short ϕ_{int} and a long ϕ_∞, have been obtained. Because of the relatively shortfluorescence lifetimes of riboflavin and 1,5-dihydroriboflavin it is not possible to dissect the exact origin of ϕ_∞. Previously reported studies, however, on depolarizing motions of dansyl bound to IgM and IgG antibodies, ascribe this motion to wobbling of the Fab subunits (segmental flexibility) rather than tumbling of the whole antibody molecule [20,21]. Moreover, this global tumbling should have little influence on the decay of anisotropy if the rate of intramolecular flexible motion is significantly larger than that of overall

tumbling and if this motion occurs over a sufficient angular range to depolarize light considerably.

Comparison of the fluorescence anisotropy decays of $\alpha Rf_{mod}G_1$ bound riboflavin and 1,5-dihydroriboflavin already suggests that at an antibody concentration where 1,5-dihydroriboflavin is clearly totally immobilized, riboflavin is only partly bound. Further analysis reveals that both the $\alpha Rf_{mod}G_1$ and the $\alpha Rf_{mod}M$ have a higher affinity for 1,5-dihydroriboflavin than for riboflavin. Comparison of the affinities of both antibodies for 1,5-dihydroriboflavin shows an overall higher affinity of the IgG_1 $\alpha Rf_{mod}G_1$ for both riboflavin forms. The affinity of $\alpha Rf_{mod}G_1$ for 1,5-dihydroriboflavin is typical for antibodies after the second immune response. The affinities of the IgM $\alpha Rf_{mod}M$ for both flavin forms are low and typical for IgM antibodies that generally have low affinity for the antigen since they are formed prior to affinity maturation. From the dynamic and geometric parameters we conclude that binding of 1,5-dihydroriboflavin to $\alpha Rf_{mod}G_1$ is less flexible than binding of riboflavin so that not only the affinity for this reduced flavin form is higher but also the specificity. For the IgM this difference in flexibility of reduced or oxidized flavin binding is less evident. When we consider the IgM as a product of the primary immune response and the IgG_1 as a product of the secondary response, these results show that during affinity maturation not only the affinity for 1,5-dihydroriboflavin increases but also the specificity of binding to this molecule is strongly improved.

In ELISA experiments (results not shown) we can not distinguish between binding to the riboflavoquinone and the N(5)-benzoyl-N(10)-ribitylsuccinimide ester flavin that served as hapten and represents the riboflavohydroquinone form. This is not surprising because, although a powerful method, limitations and artefacts have been reported for ELISA [23-25]. Not only the fact of not being able to distinguish between both riboflavin forms is a drawback of using the ELISA method for this system, but in case of the IgM it is almost impossible to determine the, in general, weak affinities of such large, multivalent molecules on a surface. Time-resolved fluorescence allows us to obtain quantitative information on the binding of both riboflavin and 1,5-dihydroriboflavin to the antibody molecule.

2.5. *Conclusion*

Time-resolved polarized fluorescence spectroscopy proves to be an excellent technique to study binding of flavoquinone and, in particular, flavohydroquinone to these antibodies. This method not only allows us to determine the affinity for both flavin forms but also gives information about the specificity and flexibility of the binding. Moreover, because of the high dynamic range, also weak affinities can be determined from analysis of rotational motion.

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Chapter 3

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

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.

3.1. 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 pKa of the N(1) of is 6.7 (see Fig. 3.1)). Because two-electron-reduced flavin differs from oxidized flavin in its conformational and electronic properties [6,7], antibodies can discriminate between both forms [8,9], and it is possible to prepare mouse monoclonal antibodies to the two-electron-reduced flavin after immunisation with oxygen-stable analogues [8].

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

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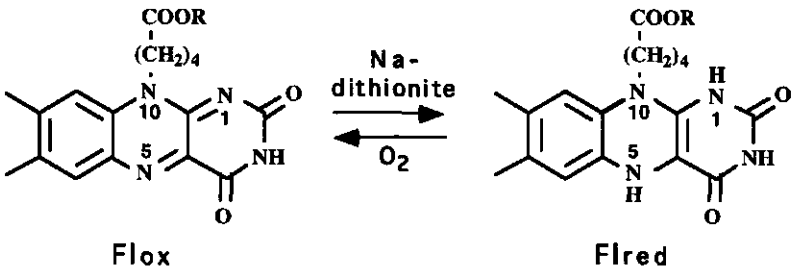

Figure 3.1. Structures of Fl_{red} and Fl_{ox}. R = H for free flavins, R = BSA for conjugates.

flavin binding to the selected fragments and to get insight in the flexibility of bound flavin [8].

3.2. Experimental procedures

3.2.1. 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 [10] (Pharmacia) and phage particles were purified using polyethylene glycol (PEG) [11].

Immunotubes (Nunc; Maxisorb, 75x12 mm) were coated overnight at room temperature with 250 μg/ml N(10)-5'-carboxybutyl-flavin (Fl_{ox}, gift of 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 hours 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., England). Fl_{ox} was reduced to N(10)-5'-carboxybutyl-1,5-dihydroflavin (Fl_{red}) by 10 mM sodiumdithionite in 100 mM sodiumphosphate, 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 sodiumdithionite and 2% skimmed milk powder. The phage were incubated in the Fl_{red}-coated tube for 2 hours 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 sodiumdithionite 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 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* [12]. 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}.

3.2.2. 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 sodiumdithionite 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 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 hour 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.

3.2.3. 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* [13].

For sequencing, clone $\alpha Fl_{red}5$ was transferred to from TG1 to 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).

3.2.4. 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 pH 7.5. 3 μM Fl_{red} was titrated anaerobically with scFv concentrations ranging over 0-5 times molar excess of scFv as compared to Fl_{red}. 0.05 μM Fl_{ox} was titrated with 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 [8]. The fractions of free and bound flavin

in solution were calculated from the decrease of the free Fl_{red} contribution in the rotational correlation time spectra. Affinity constants were obtained by fitting data to:

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

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 [14].

3.3. Results

3.3.1. 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. 3.1) 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. 3.1). 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 Materials and Methods). 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 [15], with CDR3 of 12 amino acids (GWVNVKVSKNPL). The library we used has an unmutated light chain as described by Hoogenboom *et al* [16].

3.3.2. 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 timescale. 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

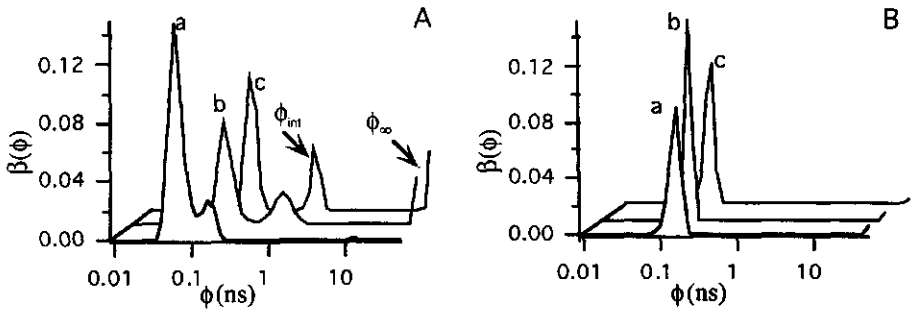

Figure 3.2. Rotational correlation time distributions of free and $\alpha Fl_{red}5$-bound Fl_{red} and Fl_{ox} at pH 6.0. A) Curve a: free Fl_{red}, curve b: Fl_{red} : $\alpha Fl_{red}5$ = 1: 0.6, curve c: Fl_{red} : $\alpha Fl_{red}5$ = 1: 2.4. B) Curve a: free Fl_{ox}, curve b: Fl_{ox} : $\alpha Fl_{red}5$ = 1: 2.3, curve c: Fl_{ox} : $\alpha Fl_{red}5$ = 1: 4.0.

(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} [17].

A typical example of a distribution of rotational correlation times, obtained after analysis of time-resolved polarized fluorescence decays, is shown in Fig. 3.2A. 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 [8]. Upon addition of $\alpha Fl_{red}5$ the motional freedom of Fl_{red} becomes more restricted as is reflected in a contribution (ϕ_{int}) of 1-3 ns. This value reflects flexible binding of Fl_{red} in the antigen binding site (examples are shown in Fig. 3.2A, curve b and c). Affinity constants for Fl_{red} were calculated from the decrease of the free Fl_{red} contribution in the rotational correlation time spectra (Table 3.1).

Tumbling of the whole scFv (ϕ_{scFv}) appears to be slower than the timescale of observation, and results in a contribution at infinitely long time (ϕ_∞) (Fig. 3.2A, curve b and c). This was unexpected as the rotational correlation time of a scFv molecule can be calculated according to Visser [18] as 10-20 ns. It appears that this discrepancy may be due association of scFv molecules by hydrophobic interactions in the presence of salt, as in experiments performed in the absence of NaCl ϕ_{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 fluorescence experiments (Fig. 3.2B), 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 3.1). 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.

pH	Kd(Flred)(μM)	Kd(Flox)(μM)	S(Flred)
7.5	0.6±0.06	100±10*	0.39±0.05
6.0	4.0±0.4		0.61±0.11

* pH 7.2

Table 3.1. Affinity constants (Kd) and order parameters (S) for binding of αFlred5 to Flred and Flox.

3.4. *Discussion*

By use of phage display technology we were able to make antibodies with high binding specificity to haptens that are unstable in 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 3.1) 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 αFl_{red} for Fl_{red} is higher at pH 7.5 (Table 3.1). 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 [7] whereas the geometry of the hapten is similar in both ionization states [6]. As indicated by the order parameters (Table 3.1), 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 anker the flavin to the antibody.

The success of this strategy to select antibodies against haptens that are unstable in 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.

3.5. References

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Chapter 4

Regulation of the flavin redox potential by flavin binding antibodies*

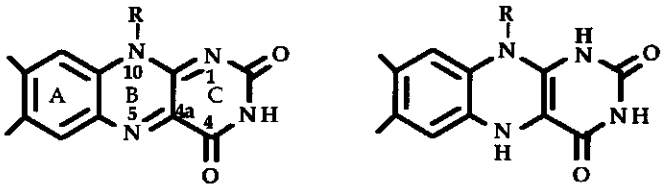
Abstract

Single chain Fv antibody fragments binding different flavin forms (αFl_{ox} and $\alpha Fl_{red}5$) have been generated from an antibody phage display library in order to study how a protein environment regulates the redox potential, starting from a protein other than a natural flavoprotein. These "flavobodies" are characterized by time-resolved and steady-state fluorescence spectroscopy as well as competitive ELISA methods (mapping of the antigen binding site) and molecular modelling. The 3-dimensional models of the antigen binding sites are in line with the experimental results. Binding of $\alpha Fl_{red}5$ to flavin increases the redox potential, mainly due to an arginine residue interacting with the flavin N(1). Thus $\alpha Fl_{red}5$ shows an "oxidase-like" redox potential behaviour, confirming the idea that positively charged residues in the vicinity of N(1) increase the redox potential. The flavobody αFl_{ox} is the most interesting result of this approach: this flavin-binding antibody, that does not resemble a natural flavoprotein, shows that when the pyrimidine like nucleus of the flavin is not involved in binding, the redox potential is not significantly affected.

4.1. Introduction

Flavin acts as cofactor in many redox enzymes and electron transferring proteins. These flavin-containing enzymes catalyze a wide variety of biochemical reactions varying from oxidase to dehydrogenase and monooxygenase type of reactions. This versatility sets flavoproteins apart from most other cofactor-dependent enzymes which, in general, only catalyze a single type of reaction [1]. The redox-active part of the flavin is the isoalloxazine ring which can exist in oxidized flavoquinone, one-electron reduced flavosemiquinone and two-electron reduced flavohydroquinone states. For flavin different redox potentials can be distinguished: E_1, corresponding to the quinone-semiquinone potential, E_2, corresponding to the semiquinone-hydroquinone potential and $E_0'(=E_1+E_2/2)$, which reflects the quinone-hydroquinone potential [2]. These redox potentials depend on the nature of the active site in which the flavin resides and vary largely among different flavoproteins [3]. The role of the protein environment of the flavin cofactor in modulating the

* This chapter has been submitted by Y.E. Bruggeman, A. Honegger, H. Kreuwel, A.J.W.G. Visser, C. Laane, A. Schots and R. Hilhorst



flavin 1,5-dihydroflavin

	R
Flox	$(CH_2)_4$-COOH
riboflavin	ribityl
FMN	ribityl-phosphate

Fig. 4.1. Structures of flavin analogues used.

redox potential is still not properly understood. Here we have investigated the structural and redox properties of monoclonal antibodies elicited against oxidized and two-electron reduced flavin and compare these with "natural" flavoproteins.

The isoalloxazine moiety of the flavin is amphipatic consisting of a hydrophobic benzene-like part (ring A, Fig. 1) and a hydrophilic pyrimidine-like part (ring C, Fig. 1). It is the N(5) atom in ring B which is particularly responsible for the redox properties of the flavin: N(5) is electron-deficient and therefore this is the site where the electrons enter the isoalloxazine [4]. The negative charge is spread over C(4a), N(5), N(10) and N(1) [5]. The degree to which this negative charge is stabilized or destabilized is an important factor governing the redox potential: a positive charge in the protein around ring C will contribute to increase the redox potential, whereas a negatively charged or hydrophobic environment will decrease it. This is clearly shown in the X-ray structures of several flavoprotein oxidases: glycolate oxidase [6] has a positively charged lysine residue, and both glucose oxidase [7] and cholesterol oxidase [8] have histidine residues in the vicinity of ring C, stabilizing the reduced flavin. In the one-electron-transferring flavodoxin, however, the flavin hydroquinone is destabilized by acidic residues in the vicinity (within 13 Å) of the N(1) atom of the flavin [9].

The apolar part of the flavin, ring A, is either in contact with bulk solvent frequently, for example in flavodoxin and cholesterol oxidase, or is bound by hydrophobic interactions as in glucose oxidase where C8 (located in ring A) is sandwiched between a tyrosine and a valine. Ring A is often not involved in the reduction and/or reoxidation of the flavin moiety and only plays an indirect role by serving as polarizability source for the changes occurring in the redox-active N(1)-C(10a)-C(4a)-N(5) region, in that way fine-tuning the modulation of the redox potential [10]. For instance, in glutathione reductase an Arg residue located close to the

C(8) methyl group induces polarization that could influence the rest of the isoalloxazine ring [11].

This illustrates that the environment of ring C mainly regulates the redox potential, suggesting that a protein only binding to ring A should hardly influence the redox potential. However, up till now such a flavoprotein has not been shown to exist. Monoclonal antibodies, or even antibody fragments consisting of the two variable (V) antigen-binding domains, offer a way to build artificial protein environments specific for different flavin redox states [12-14]. This is possible because oxidized flavin differs from reduced flavin in its conformational and electronic properties. It might even be possible to generate "unnatural" flavin-binding proteins that recognize both redox states equally well (i.e. the redox potential is not influenced) by generating antibodies that bind to ring A.

In order to contribute to a better understanding of how the protein environment regulates the flavin redox potential, we have generated scFv antibody fragments (composed of the two antigen-binding domains connected by a peptide linker [15]) against reduced [13] as well as oxidized flavin from a naive antibody phage display library [16] (in such a library an antibody repertoire is cloned on the surface of filamentous bacteriophage; antibodies can be selected by panning the library on antigen). Here we show that an antibody fragment binding mainly to ring A (αFl_{ox}) does not influence the flavin redox potential (E_0') significantly, whereas an antibody fragment binding ring C of reduced flavin ($\alpha Fl_{red}5$) causes a shift in E_0' of +65 mV.

4.2. *Experimental procedures*

4.2.1. *Phage selection*

A "single pot" human synthetic phage-antibody scFv library [16] was used for phage selection. Phage were selected for binding to oxidized N(10)-5'-carboxybutylflavin (Fl_{ox}) [13] as described by Nissim *et al* [16] and eluted with 100 mM Fl_{ox} in PBS. Clones found to give a positive ELISA signal were screened by PCR and "fingerprinted" by the frequent-cutting restriction enzyme *Bst*NI as in [17] to identify different clones. Examples of clones with different restriction patterns were selected and the 3' ends of the heavy chains were sequenced to confirm the fingerprinting as described in [13].

The selection of a phage antibody ($\alpha Fl_{red}5$) against the reduced N(10)-5'-carboxybutyl-1,5-dihydroflavin (Fl_{red}) has been described previously [13].

4.2.2. *Expression, purification and sequence analysis*

For soluble expression selected clones were subcloned into pUC119SfiNotMycHis (lacking the gene III insert but harbouring a

hexahistidine tag for purification and a cmyc tag [18] for detection) using standard recombinant DNA techniques. A 1 l culture of *E. coli* TG1 harbouring each plasmid was grown to OD_{600} of 0.9 and scFv expression was induced with β-isopropylthiogalactoside (IPTG) [19]. After induction, the culture was shaken for 3 h at 20°C. Since no scFv could be detected in the culture supernatant, scFv was purified from the cells. Cells were resuspended in 50 mM phosphate pH 7.5, 500 mM NaCl, 20 mM imidazole and 2 mM phenyl-methyl-sulfonyl fluoride (PMSF) and lysed by two passes through a French pressure cell at 18000 psi. Cell walls and insoluble material were removed from the lysate by centrifugation at 28000xg for 30 minutes. The scFv was purified by Immobilized Metal Affinity Chromatography [20] as described by Griffiths et al [21]. All purification steps were performed at 4°C. Purified scFv fragments were judged for purity by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE). The scFv concentration was determined spectrophotometrically using ε_{280} values determined from the amino acid sequences as described in [22].

For sequencing, DNA purified with Qiagen Plasmid Midiprep (Qiagen Inc.) was subjected to PCR cycle sequencing reactions with fluorescent dideoxy chain terminators (Applied Biosystems) according to the manufacturer's instructions using standard M13 primers. The sequencing reactions were analysed on an Applied Biosystems 373 Automated DNA Sequencer. Sequence analysis was performed using AutoAssembler (Applied Biosystems).

4.2.3. *Mapping of the antigen binding site with flavin (-analogues)*

The antigen binding site of αFl_{ox} was mapped (in PBS, pH 7.2) with different flavin analogues (Fig. 1) using different methods: time-resolved and steady-state fluorescence spectroscopy as well as competitive enzyme-linked-immunosorbent assay (ELISA).

Using competitive ELISA we studied binding of αFl_{ox} to Fl_{ox}, flavin mononucleotide (FMN, Boehringer Mannheim) and riboflavin (Sigma). Basically the method described by Friguet *et al* [23] was followed (omitting the pre-incubation step).

Binding of αFl_{ox} to Fl_{ox} and Fl_{red} was compared using time-resolved polarized fluorescence as described in [12]. Both Fl_{ox} and Fl_{red} were excited with light of 450 nm. Flavin was titrated with varying antibody concentrations.

As a third method we used steady state fluorescence, based on quenching of tryptophan fluorescence by bound hapten [24], to investigate binding of αFl_{ox} to Fl_{ox}, riboflavin and FMN. 0.2-0.7 μM concentrations of αFl_{ox} were titrated with flavin covering a concentration range up to 10 μM. Emission spectra (300-350 nm) were recorded after exciting at 280 nm on a SPF 500C Spectrofluorometer (SLM Aminco). Excitation and emission monochromator bandwidths were set at 4 nm.

$\alpha Fl_{red}5$ was studied for binding to Fl_{ox} and Fl_{red} using time-resolved fluorescence [13] and to Fl_{ox} [13] and riboflavin using competitive ELISA as described previously [13].

4.2.4. Calculation of the redox potential

The shift in redox potential ($\Delta E_0'$) for antibody-bound flavin compared to free flavin was calculated from Eqn. 1 [25]:

$$\Delta E_0' = E_0'_{(Ab.Fl)} - E_0'_{(Fl)} = \frac{RT}{nF} \ln \frac{K(Fl_{ox})}{K(Fl_{red})} \quad (1)$$

where R is the gas constant, T is the temperature (297 K), F is the Faraday constant and n the number of electrons transferred in the reaction (here n=2). $K(Fl_{ox})$ and $K(Fl_{red})$ are the dissociation constants for Fl_{ox} and Fl_{red}, respectively.

4.2.5. Computer modelling of the antigen binding site

Structural models of the V_H and the V_L domains of αFl_{ox} and $\alpha Fl_{red}5$ were derived by homology modelling based on sequence homology with antibody structures from the Brookhaven protein database (PDB) [26]. We used the Unix Insight II package (version 2.3.0), including the modules Biopolymer and Homology, and Discover (version 94.0) both from Biosym/MSI (San Diego). The amino acid sequences of the variable domains of the antibodies have been used as program input for the modelling of the 3-D structures.

The λ V_L domain is the same in both scFv fragments [16]. The best templates for this V_L domain were the V_L domain of antibody HIL (PDB entry 8fab, 1.8 Å resolution) with 66.7% sequence identity, the MCG light chain dimer (3mcg, 2.0 Å resolution) with 62.7% sequence identity and the V_L domain of antibody KOL (2ig2, 1.9 Å resolution, and 2fb4, 3.0 Å resolution) with 58.7% sequence identity. After structural alignment of the four potential template structures and careful analysis of the influence of local sequence differences on the structures of the the potential template molecules, the majority of the coordinates were assigned using 8fab as a template. The third complementarity determining region (CDR3), which in λ chains shows much higher structural variability than in κ chains, was modelled based on the coordinates of 2ig2.

The best templates for the V_H domain of $\alpha Fl_{red}5$ were the V_H domains of the antibody 3D6 (1dfb, 2.7 Å resolution) with 73.6% sequence identity, followed by antibody POT (1igm, 2.3Å resolution) with 72.5% identity and HIL (8fab, 1.8 Å resolution) with 70.6% sequence identity. Eventually 1dfb was used as primary template for the modelling of the V_H domain. The CDR3s of the PDB entries 1fvw, 2fvw, 8fab, 1igm and 6fab all have the same length as that of $\alpha Fl_{red}5$, but these antibodies all have the Arg H94 to

Asp H101 salt-bridge [27], which strongly influences the conformation of CDR3, while $\alpha Fl_{red}5$ lacks this salt-bridge. Therefore CDR3 was modelled using 2fvb as a template, shortening the loop by 2 residues. The lack of the Arg-Asp salt bridge suggests an "open" conformation of this CDR3, in which the loop sticks out into the solvent and is highly flexible.

For the V_H domain of αFl_{ox}, the best templates were: the antibody DB3 (2dbl, 2.9 Å, and also 1dba, 1dbb, 1dbj, 1dbk, 1dbm) with 54.2% sequence identity (but a higher similarity score than the others), followed by antibodies TE33 (1tet, 2.3 Å) with 56% sequence identity and R19.9 (1fai, 2.7 Å) with 58.7% sequence identity. The model was based primarily on the structure of 2dbl. Analogous to $\alpha Fl_{red}5$, αFl_{ox} lacks the H94-H101 salt bridge and therefore the PDB entries 1vfa, 1fdl and 1mam that have CDR3s of similar length could not be used as templates. Here CDR3 was modelled by extending the CDR3 of 1baf by two amino acids.

The relative orientation of the V_H and V_L domains was determined by structural alignment with the F_V fragments of the primary templates. After conjugate gradient energy-minimization of the models, the ligands (Fl_{ox} for αFl_{ox} and Fl_{red} for $\alpha Fl_{red}5$) were docked manually and the complexes were annealed in a 20ps molecular dynamics run. Charges were assigned consistent with the protonation states expected at pH 7.4. The forcefield used was cvff and dynamics and minimization were performed without explicit solvent, using a distance-dependent dielectric constant.

4.3. Results

4.3.1. Selection of phage antibodies and expression as scFv fragments

Phage antibodies against oxidized flavin (Fl_{ox}) were selected using standard selection techniques. As revealed by *Bst*NI fingerprinting and confirmed by sequencing, at least four different clones have been selected. The clone giving the best ELISA signal to Fl_{ox} (αFl_{ox}) was chosen for further characterization as soluble scFv fragment. The selection of the reduced flavin binding scFv $\alpha Fl_{red}5$ has been described previously [13]. The scFv fragments αFl_{ox} and $\alpha Fl_{red}5$ were purified with yields of 1.5 and 0.6 mg/l, respectively.

4.3.2. Sequence analysis

The primary structures of the variable regions of both antibodies have been determined and are shown in Fig. 2. The light chain (L) is the same for both antibodies since the library we used has an unmutated light chain (IGLV3S1, [17]) as described by Hoogenboom *et al* [28]. The homology in framework (FR) 4 and the end of FR3 of the heavy chain (H) has been imposed by the way the library was constructed [16]. αFl_{ox} comprised the V_H segment [29] DP-14 with an eight amino acid CDR3 and $\alpha Fl_{red}5$ the V_H segment DP-32 with a twelve amino acid CDR3. Interestingly, both

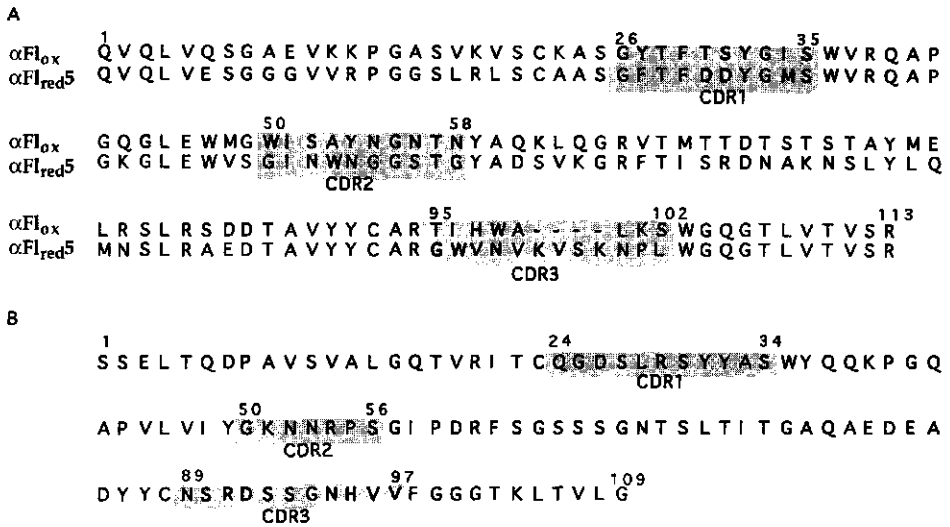


Fig. 4.2. Amino acid sequences of αFlox and αFlred5 heavy and light chain domains given in the universal one letter code. Note that the light chain is the same in both antibodies. CDR regions are shaded. Numbering is according to Kabat [45].

antibodies lack the characteristic arginine H94 to aspartate H101 salt bridge [27].

4.3.3. *Mapping of the antigen binding site*

To determine the affinities of αFl_{ox} and $\alpha Fl_{red}5$ for different flavins we had to resort to a variety of techniques. Steady state fluorescence, based on quenching of tryptophan fluorescence upon hapten binding, usually is the method of choice since the affinity can be determined directly in solution and the method is very sensitive. Competitive ELISA is another good approach but requires more antibody and hapten. We used both methods to study affinities for oxidized flavins. However, due to the low fluorescence quantum yield and the poor visible light absorption and to the instability of reduced flavin in an aerobic environment, it is not possible to determine the affinities of these antibodies for two-electron reduced flavin using these standard techniques. Because of its sensitivity, we used time-resolved fluorescence to investigate binding to reduced flavin.

4.3.3.1. *Time-resolved polarized fluorescence*

Binding of Fl_{ox} and Fl_{red} to both αFl_{ox} and $\alpha Fl_{red}5$ [13] was studied by time-resolved polarized fluorescence. The excited-state lifetime (τ) of a fluorophore in a protein is extremely sensitive to the physical properties

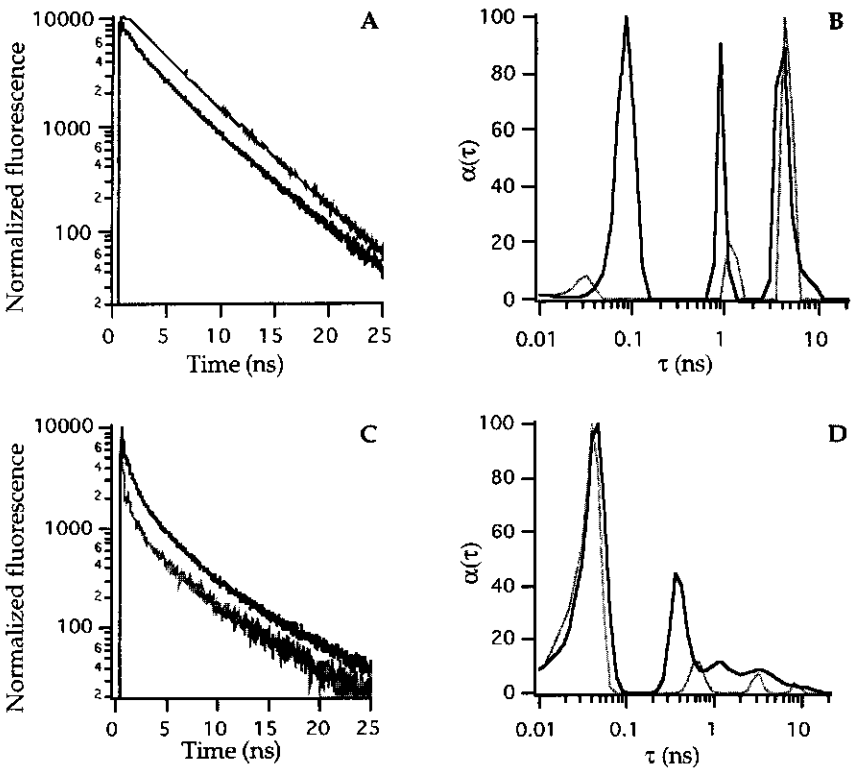


Fig. 4.3. Experimental total fluorescence decays and fluorescence lifetime distributions of free and αFl_{ox} bound Fl_{ox} and Fl_{red}. Grey curves reflect free Fl_{ox} (A, B) or free Fl_{red} (C, D). Black curves reflect bound species in all cases. In all experiments the residuals of the fit were randomly scattered around zero, indicating an optimal fit.

of its environment and therefore gives information about the (protein) environment in which the fluorescent probe resides. Using time-resolved fluorescence one can study this phenomenon at a (sub)nanosecond scale. Moreover, time-resolved polarized fluorescence gives information on the rotation of the fluorescent probe on this time scale when the rotational correlation times (ϕ) are derived from the experimental data.

The total fluorescence decays for free and αFl_{ox}-bound Fl_{ox} and Fl_{red} are shown in Fig. 3A and B, respectively. The fluorescence of antibody bound Fl_{ox} (Fig. 3A) exhibits a faster decay than the fluorescence of free Fl_{ox}. The fluorescence lifetime distributions, derived from these total fluorescence decays, reflect these observations: free Fl_{ox} (Fig. 3B) has a major lifetime of 4.2 ns and two small contributions. Upon addition of antibody the component at 4.2 ns decreases and two lifetimes at 0.9 and 0.1 ns show up.

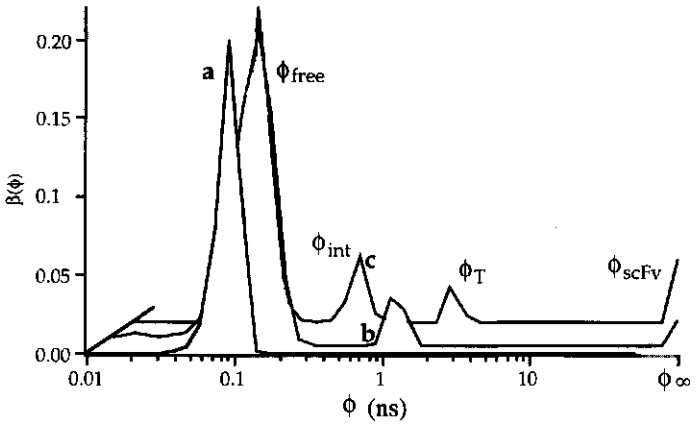


Fig. 4.4. Rotational correlation time distributions of free and αFlox bound Flox. Curve a, free Flox ; curve b, Flox : αFlox = 1:140; curve c, Flox : αFlox = 1:280 (molar ratios).

The shortening of fluorescence lifetimes indicates a dynamic quenching process.

In contrast to this, the fluorescence decay of αFl_{ox} bound Fl_{red} is slower than that of free Fl_{red} (Fig. 3C). The fluorescence lifetime distribution patterns corresponding to these curves are more complex than those of Fl_{ox} (Fig. 3D) as a result of the presence of multiple, non-planar conformations that interconvert into each other [30]. This conformational instability is also the cause of the low fluorescence quantum yield of reduced flavin [31]. The increase in fluorescence upon antibody binding, reflected in the increased contributions of longer lifetime components (among which a peak at 0.6 ns) indicates a hindered ring inversion of the flavohydroquinone due to antibody binding [31].

Apart from fluorescence lifetime distributions we also studied rotational correlation times obtained from fluorescence anisotropy decays. Examples of rotational correlation time distributions are shown in Fig. 4. Whereas free Fl_{ox} shows a single rotational correlation time (ϕ_{free} = 0.2 ns), we observe 2 more contributions upon titration of Fl_{ox} with αFl_{ox} : one that reflects the internal restricted motion of the flavin in the antigen binding site (ϕ_{int} = 1 ns) and a contribution at infinite time (not resolvable) that corresponds to tumbling of the scFv itself (ϕ_{scFv}). However, when more antibody is added a fourth contribution at 2.2 ns emerges. We ascribe this peak (ϕ_T) to homo-energy transfer (i.e. energy transfer between two identical flavin chromophores). This can be explained by assuming dimer formation: scFv fragments secreted from bacteria are often present as both monomers and dimers [32], suggesting that the V_H and V_L domains of different chains can pair. The distance between the two antigen binding sites of the dimer, i.e. the distance between the two bound flavins, can be calculated from the Förster equation (Eqn. 2, see for example [33]):

$$k_T = 8.71 * 10^{17} R^{-6} \kappa^2 n^{-4} k_r J \quad (2)$$

where R is the distance (in nm) between the two flavins, κ is the orientation factor describing the relative orientation of donor and acceptor transition dipoles (here κ=3.29, estimation based on data on lipoamide dehydrogenase, taken from [34]), n the refractive index of the intervening medium (here n=1.4), k_r the radiative rate constant of the donor (0.0556 ns^{-1}) and J the overlap integral determined from absorption and emission spectra ($5.53.10^{-15}$ $M^{-1}cm^3$) (n, k_r and J are taken from Bastiaens *et al* [35]). The rate of transfer (k_T) is calculated from the correlation time associated with energy transfer (ϕ_T) (Eqn. 3):

$$k_T = \frac{1}{2\phi_T} \quad (3)$$

Moreover, from the relative contributions (β_{int}, β_T and β_{scFv}) of each antibody-associated peak (ϕ_{int}, ϕ_T and ϕ_{scFv}, respectively) to the rotational correlation time distribution, the intermolecular angle (θ) between the flavins (more exactly the angle between the symmetry axes of the potential wells in which the flavins move) can be determined according to Eqn. 4 [35]:

$$\frac{\beta_{scFv} - \beta_T}{\beta_T + \beta_{scFv}} = \frac{3}{2}\cos^2\theta - \frac{1}{2} \quad (4)$$

For the dimer form of the scFv αFl_{ox} we determined a centre-to-centre distance (R) of 32 Å between the two antigen binding sites at an angle (θ) of 143±1.3°. From the crystal structure of a dimerized antibody with a 5 residue flexible linker a distance of 65 Å between the antigen binding sites has been determined [36]. However, αFl_{ox} has a 15 residue $(Gly_4Ser)_3$ linker and hence there will be less constraint between the two subunits of the dimer so that they can approach each other more closely.

The affinity constants of αFl_{ox} for Fl_{ox} and Fl_{red} were calculated from the decrease of the free flavin contribution in the rotational correlation times as described in [13] (Table 1). The affinity constants of $\alpha Fl_{red}5$ for Fl_{red} (both protonated and deprotonated at N(1)) have been published elsewhere [13] but have been included in Table 1 for comparison.

4.3.3.2. Steady state fluorescence

We also investigated binding of αFl_{ox} to Fl_{ox}, riboflavin and FMN using steady state fluorescence. Fig. 5 shows the quenching of the tryptophan (Trp) fluorescence upon titrating with Fl_{ox} and riboflavin. The titration curves shows a striking biphasic pattern that could not be fitted to a function describing the hyperbolic curve characteristic for antibody-antigen binding (see for example [13]). The large initial decrease could not

antibody	flavin	Kd (μM)	shift in Eo' (mV)
αFlox	Flox	43(1,3) 15,5 (2,3)	+8(3)
	Flred	23(1,3)	
	Rf	43(2,3)	
	FMN	42(2,3)	
αFlred5	Flox	100(2,3)	+41(4)
	Flred	4.0(1,4) 0.6(1,5)	+65(5)
	Rf	>100(2,3)	

(1) determined by time-resolved fluorescence
(2) determined by ELISA
(3) determined at pH 7.2
(4) determined at pH 6.0
(5) determined at pH 7.5

Table 4.1. Summary of dissociation constants (Kd) and redox potential shifts (ΔE).

be due to photobleaching of the Trp since in the absence of flavin and under continuous excitation (for 20 minutes) there is only a fast 6% decrease in Trp fluorescence followed by stabilization of the Trp emission. Moreover, the decrease was not dependent on the light intensity.

However, it has been known for a long time that flavins can sensitize the destructive photooxidation of a large variety of substrates, such as amino acids, in the presence of oxygen [37]. The products of flavin sensitized photooxidations have not been clearly identified. Our results indicate that one or more Trp residues in the αFl_{ox} binding site are sensitive to photooxidation, explaining the large initial fluorescence decrease in the titration curve. However, the fact that after the breakpoint in the curve the Trp fluorescence still slowly decreases suggests that there are also Trp residues involved in binding that are not sensitive to photooxidation. The crippled antibody that remains after photooxidation is still capable of binding flavin although with a decreased affinity (reflected in the flatter part of the titration curve). Fl_{ox} and riboflavin both have the same effect on photooxidation since the titration curves overlap. Therefore it is not possible to pronounce upon differences in affinity between two flavin analogues.

The breakpoint in the titration curves is located around 0.5 μM flavin. Since it has been known that part of the purified antibody might be

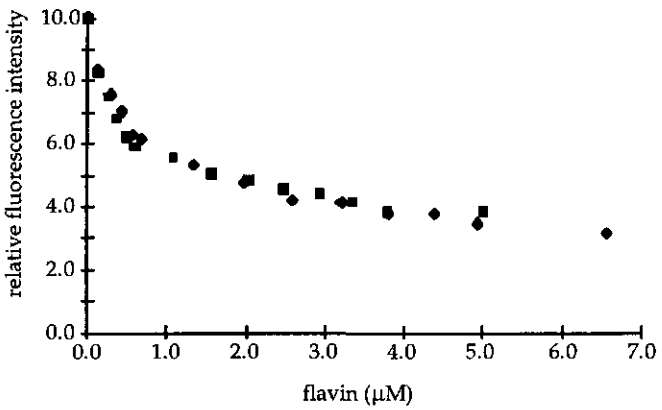


Fig. 4.5. Tryptophan quenching in αFl_{ox} (0.7 μM): ◆, αFl_{ox} /Fl_{ox}; ■, αFl_{ox} /riboflavin.

incorrectly folded [38], we assume that this is the concentration of correctly folded antibody (we assume that only correctly folded antibody binds flavin). This implies that only one Trp residue is close enough to the flavin to be photooxidized since this is less than equimolar to the antibody concentration (0.7 μM). From this it can be estimated that 70% of the purified scFv has been properly folded.

We tried to determine the affinity of αFl_{ox} for Fl_{red} using this approach. This was, however, not possible since it appeared that dithionite, used to reduce flavin, quenches the Trp fluorescence. Furthermore we could not study binding of Fl_{ox} (and other flavin derivatives) to $\alpha Fl_{red}5$ due to the weak binding of this scFv to Fl_{ox} [13]: the amount of flavin that has to be added to quench the Trp is so large that flavin will absorb part of the excitation light.

4.3.3.3. Competitive ELISA

Competitive ELISA experiments with αFl_{ox} have been performed using Fl_{ox}, riboflavin and the well-soluble FMN as competitors. We basically followed the method described by Friguet *et al* [23] except that we did not pre-incubate antibody and antigen. In our opinion, this step only is useful when the equilibrium between bound and free antibodies is slowly attained. Based on knowledge of k_{on} and k_{off} rates for antibody-antigen binding this is only true for high affinity antibodies (see for example [24]). In our experiments the ELISA plate was coated with a BSA-Flox conjugate whereas different flavins served as competitive antigens in solution. Therefore, the competition is not strictly fair in case riboflavin and FMN are used as competitors, since it might be expected that the affinity for Fl_{ox} (coated to the plate) is higher than for these flavin derivatives. Therefore the dissociation constants determined must be considered as relative

values which can very well be used to rank the strength of binding of αFl_{ox} to different flavins.

The results obtained are shown in Table 1 and indicate that the substituent on N(10) plays a role in binding: riboflavin and FMN bind worse than Fl_{ox}, to which the antibody was selected. Apparently, the extra phosphate group that FMN possesses compared to riboflavin does not influence binding, neither sterical nor electrostatical.

Competitive ELISA was also used to study binding of $\alpha Fl_{red}5$ to Fl_{ox} (described in [13]) and riboflavin. In the latter case we could not determine the apparent binding constant for riboflavin exactly because the situation that antibody binding to the ELISA plate is fully inhibited by the competing antigen, could not be reached. Therefore we must conclude that binding to riboflavin is at least several orders of magnitude weaker than to Fl_{ox} (Table 1).

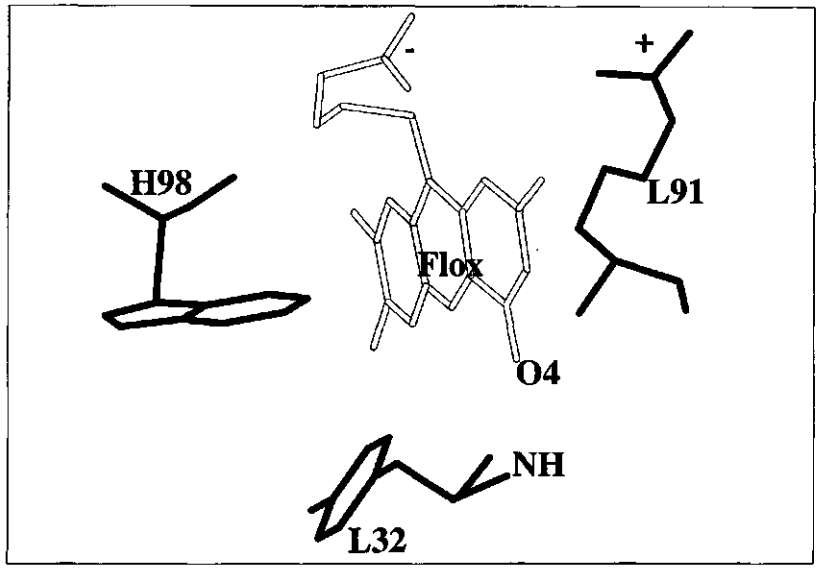
4.3.4. Redox potentials

The flavin redox potential indicates the tendency of the flavin molecule to aquire or donate electrons. The shift in redox potential ($\Delta E_0'$) upon binding of antibody to flavin shows how the antibody affects this. $\Delta E_0'$ values have been calculated for both scFv fragments for the Fl_{ox}/Fl_{red} couple from the dissociation constants obtained from time-resolved fluorescence data (Table 1). For αFl_{ox} we only observe a minor shift, but $\alpha Fl_{red}5$ causes a +41 to +65 mV shift in E_0' dependent on pH. Intuitively, these redox potential shifts indicate that αFl_{ox} binds more to ring A of the flavin or to the N(10) substituent and that αFl_{red} is more directed against ring C.

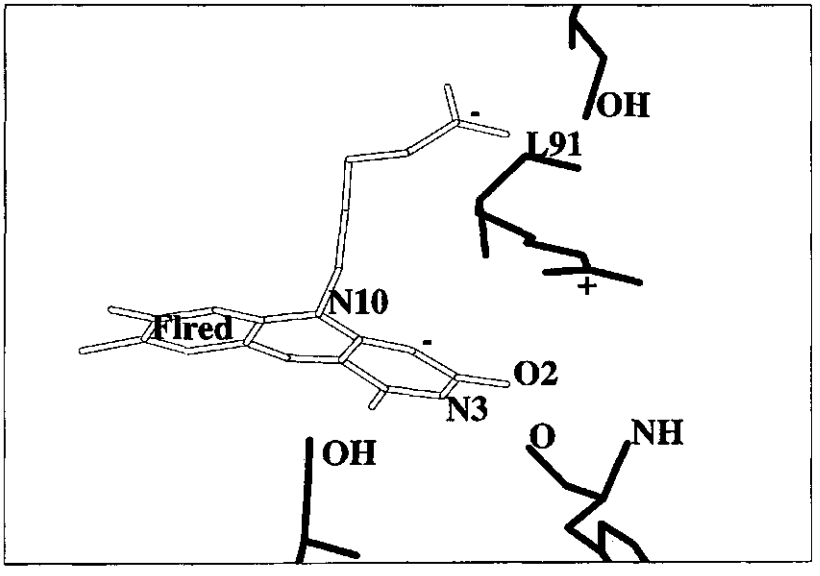
4.3.5. Modelling of the antigen binding site

To understand the molecular basis of the regulation of the flavin redox potential by the antibody, we took advantage of the highly conserved and well-studied structures of antibody V domains and generated models of the 3-dimensional structures of the Fv domains of αFl_{ox} and $\alpha Fl_{red}5$, based on sequence homology with antibody structures from the Brookhaven database.

A fragment of the three dimensional model of the Fl_{ox}-αFl_{ox} complex is shown in figure 6A. Fl_{ox} is positioned in a cavity in the αFl_{ox} binding site that on the bottom side is closed by Trp H47 (±7 Å from ring A of the flavin) while another Trp residue, H50, is also much involved in shaping the binding site (Trp H47 and H50 are not shown in this model). These two Trp residues, however, are not directly involved in binding, in contrast to another Trp (H98). Fl_{ox} is bound mainly by hydrophobic interaction of this residue with ring A of the flavin. The N(1)/C2=O part of ring C and the main part of the "tail" (except for the terminal carboxyl group that points back to the protein) stick out of the antigen binding site into the solvent. There is a hydrogen bond between flavin C(4)=O and the



A



B

Fig. 4.6. Fragment sof the 3-dimensional models of Flox bound to αFlox (A) and of Flred (deprotonated at N(1)) bound to αFlred5 (drawn with the graphical program O [46]).

backbone NH of Tyr L32. Another important factor governing the flavin binding is an electrostatic interaction between Arg L91 and the terminal carboxyl group of the flavin N(10) substituent (±4 Å).

Figure 6B shows a fragment of the model of Fl_{red}-$\alpha Fl_{red}5$ in the situation that N(1) is negatively charged. Fl_{red} is well-embedded in the antigen binding site. Ring C of the flavin is bound by two hydrogen bonds: between flavin C(2)=O and the backbone NH of Tyr L32 and between flavin N(3)H and the backbone C=O of Tyr L32. In addition there is a hydrogen bond between flavin N(10) and Ser H100B. The N(10) substituent of the flavin ("tail") plays an important role in binding since the terminal carboxyl group forms a hydrogen bond with Ser L93 and a salt bridge with Arg L91 (surprisingly this is the same residue that is involved in "tail" binding in αFl_{ox}). This flexible arginine residue can move from the terminal carboxyl group to the negatively charged N(1) which explains why negatively charged Fl_{red} is bound more strongly than its neutral form.

Analysis of the hypervariable loops involved in binding reveals that αFl_{ox} uses 4 CDRs (L1, L3, H2 and H3) to bind the flavin. H2 is involved in shaping the binding site but not directly in binding the flavin. The binding site of $\alpha Fl_{red}5$ is predominantly shaped by L1, L3, H1, H2 and H3 and residues of L1, L3 and H3 are directly involved in binding. According to Wilson and Stanfield [39] the minimum number of CDRs used in binding is four and H3 and L3 are always used whereas there is a lack of interaction between L2 and smaller antigens or haptens. Here too we see this absence of L2 in hapten binding for both our antibodies.

The light chain is in both cases much involved in binding although the binding sites are differently shaped. Surprisingly, Arg L91 and Tyr L32 are both used for binding in αFl_{ox} as well as in $\alpha Fl_{red}5$. Since the light chain in both antibodies is the same, this illustrates once more that not only the primary sequence of the V domain determines the structure of the binding site, but that interaction between both domains is of the utmost importance [40].

4.4. Discussion

scFv antibody fragments binding different flavin forms (αFl_{ox} and $\alpha Fl_{red}5$) have been selected from a phage antibody library and are characterized by experimental mapping and molecular modelling in order to study how a protein regulates the redox potential, but here we use a protein other than natural occurring flavoenzymes. Modelling of the antigen binding site is a good alternative to understand the molecular basis of protein-ligand interactions when no X-ray crystallographic or NMR structure is available. The system we investigated lends itself very well to this approach since the flavin ligands docked in the antigen binding site are relatively rigid (compared to peptide and protein antigens) and, in particular, since the result of the modelling can be verified with experimental data.

4.4.1. *Comparison of data obtained by experimental mapping methods*

Binding of αFl_{ox} to Fl_{ox} was investigated by competitive ELISA and both steady-state and time-resolved fluorescence spectroscopy. The latter technique is, due to its sensitivity, the preferred method to quantify binding to the oxygen sensitive, colourless and low-fluorescent Fl_{red} [12]. The dissociation constant for Fl_{ox} determined from time-resolved fluorescence anisotropy data differs from that determined by competitive ELISA that is about three fold lower.

This difference might originate from the fact that the equilibrium between antibody and antigen in solution, in the ELISA plate is affected by the antigen coated on the surface of the plate. This antigen withdrawes part of the antibody from the solution. This might be amplified by the fact that Fl_{ox} tends to dimerize so that not only affinity but also avidity plays a role in binding to the flavin coated to the ELISA plate, resulting in a stronger binding to surface coated antigen than to antigen in solution. This implies that less antigen is needed to satisfy the antibody left in solution, resulting in a lower dissociation constant.

Comparison of steady state fluorescence spectroscopy data, based on quenching of the Trp fluorescence by Fl_{ox} binding, to data obtained by time-resolved fluorescence and competitive ELISA is impossible due to the interference of photooxidation of Trp in the former experiment. However, it is clear that the affinity constant of αFl_{ox} for Fl_{ox} is low (in the 10^{-5} M range) and data obtained by the same method can be compared to each other. For this reason, the redox potential for the Fl_{ox}/Fl_{red} couple was calculated from the dissociation constants obtained from time-resolved fluorescence anisotropy results.

αFl_{ox} binds Fl_{ox} and Fl_{red} approximately equally well indicating that either ring A of the flavin nucleus or the N(10) substituent is involved in binding, since these parts are the same for both Fl_{ox} and Fl_{red} (in contrast to ring C that accepts the electrons and adopts a new electron density distribution upon reduction). Competitive ELISA experiments with Fl_{ox}, riboflavin and FMN, flavin analogues that only differ in their N(10) substituent, reveal that binding to this substituent indeed plays a role (Table 1).

αFl_{red} binds significantly better to Fl_{red} than to Fl_{ox} as determined by time-resolved fluorescence spectroscopy [13], suggesting that ring C of the flavin is involved in binding. This is supported by the fact that the affinity for Fl_{red} is higher when N(1), located in ring C, is deprotonated [13]. Since riboflavin binds worse than Fl_{ox} (Table 1), binding to the N(10) substituent must also play a role.

These experimental data give a first outline of the way flavin is bound to αFl_{ox} and $\alpha Fl_{red}5$ and serve as a basis to judge the validity of the 3-dimensional molecular models.

4.4.2. Validity of the models

The 3-dimensional model of the αFl_{ox} binding site is in good agreement with the experimental results. The interaction between ring A of the flavin and Trp H98 is the main interaction causing binding of αFl_{ox} to Fl_{ox} or Fl_{red} which is in agreement with experimental data. This Trp residue is most likely the residue sensitive to photooxidation which fits in our assumption that only one Trp residue is involved in photooxidation, as determined from the breakpoint in the titration curve monitoring the quenching of the Trp fluorescence (Fig. 5). From the slower decreasing second part of this titration curve we concluded that there are also other Trp residues involved in binding that are not sensitive to photooxidation but quench the Trp fluorescence via radiationless energy transfer. We assume that Trp H47 and H50 are the residues involved in this process; they are probably too remote or not well-positioned for photooxidation. When flavin is excited (as in time-resolved fluorescence experiments) we noticed quenching of the flavin fluorescence which might be ascribed to Trp H98 as well as H47 and H50, or partly to the hydrogen bond between flavin C(4)=O and Tyr L32 [31].

The electrostatic interaction between the negatively charged carboxyl group at the terminus of the flavin N(10) substituent and the positively charged Arg L91 explains the lower affinity for FMN and riboflavin compared to Fl_{ox}. The presence of this Arg residue is unexpected since there was no negative charge present in the N(10) substituent of the original hapten (instead of the carboxyl group there was a peptide bond linking the hapten to a protein carrier).

The model reveals that the N(10) substituent is exposed to the solvent which explains why riboflavin and FMN are bound equally well. The terminal phosphate group of the N(10) substituent of FMN can apparently not form a salt bridge with Arg L91, probably due to torsion or sterical effects (the distance between N(10)-phosphate in FMN is larger than that of N(10)-COO^- in Fl_{ox}, moreover a phosphate group is more bulky than a carboxyl group). Considering this model the low affinity of αFl_{ox} for Fl_{ox} is not surprising, since there are few amino acid residues (only Trp H98, Arg L91 and Tyr L32) directly involved in binding and the flavin is not well-embedded in the antigen binding site.

The 3-dimensional model of $\alpha Fl_{red}5$ is also in good agreement with the experimental results. As in αFl_{ox} the flavin is partly bound via the carboxyl group at the terminus of the N(10) substituent by electrostatic interaction with Arg L91 and also by a hydrogen bond to Ser L93 which explains that Fl_{ox} is bound better than riboflavin. Surprisingly, this positively charged Arg residue is flexible enough to move to N(1) of the flavin ring when this atom acquires a negative charge. This is obviously the explanation for the difference in binding between protonated and deprotonated Fl_{red} (changes in conformation cannot be the cause here, for the geometry of Fl_{red} in both ionization states is not greatly different [41]).

The arginine shift is undoubtedly a matter of induced fit since $\alpha Fl_{red}5$ was selected against protonated Fl_{red}.

Furthermore the isoalloxazine nucleus itself is bound by three hydrogen bonds: between flavin N(10) and Ser H100B, C(2)=O and Tyr L32, N(3)H and Tyr L32. These hydrogen bonds (in particular the one to N(10) [42]) and the constraint the binding pocket imposes on Fl_{red} explain the increased fluorescence upon antibody binding because the rapid interconversions of the multiple, non-planar conformations of reduced flavin are damped [30,31].

4.4.3. *Comparison with other flavoproteins*

αFl_{ox} hardly influences the flavin redox potential. From previous studies (see introduction) it has been known that binding to the redox active ring C of the flavin ring predominantly regulates the redox potential. Here we have shown that the antibody αFl_{ox}, binding predominantly to ring A, barely changes the redox potential. The small change in redox potential (+ 8 mV) might be due to a polarizing effect Trp H98 has on ring A of the flavin [10]. Ring C of the flavin is mainly exposed to the solvent which makes αFl_{ox} a "reversed flavodoxin". In flavodoxin the flavin, FMN, is also situated on a scaffold of loops as in the antigen binding site of an antibody. However, in this case ring A of the flavin sticks out to the solvent instead of ring C .

Positively charged residues in the vicinity of ring C are found to increase the flavin redox potential in flavoproteins [6-8]. For flavodoxin it has been explicitly suggested that in particular the interaction with the flavin N(1) nucleus is important for the regulation of the redox potential [43]; the unfavorable electrostatic interaction between the phosphate dianion and the negatively charged flavohydroquinone has been proposed as a partial explanation for the low redox potential of flavodoxins [44]. For oxidases it has been shown that positively charged residues in the vicinity of N(1) contribute to increase the redox potential (see introduction). Our model indicates that $\alpha Fl_{red}5$ has a positively charged arginine residue (L91) that not only shields the negatively charged carboxyl group at the end of the N(10) substituent of Fl_{red}, but also interacts with N(1) when deprotonated. We assume that these interactions are mainly responsible for the 65 mV increase in the redox potential when $\alpha Fl_{red}5$ binds to deprotonated Fl_{red}. When Fl_{red} is protonated at N(1), there is a smaller, +41 mV, shift in redox potential, that we ascribe mainly to the favorable electrostatic interaction between the carboxyl group and Arg L91.

The hydrogen bonds to flavin N(10), C(2)=O and N(3)H probably also contribute to the positive shift in redox potential by increasing the activation barrier for the transition from the bent to the planar conformation [42].

In the future these models needs to be further verified by mutagenesis studies. This will be a comprehensive study since the redox potential is not only regulated by amino acids having a close contact with the flavin,

but also by charged residues at a greater distance of the flavin nucleus [9]. In the present study of our models we only judged those residues that were in immediate contact with the flavin.

4.5. Conclusion

Our approach using non-natural flavin binding proteins to investigate the way a protein environment regulates the flavin redox potential supports observations on natural flavoproteins. These flavin binding antibodies ("flavobodies") form tailored protein environments that can be generated against any flavin of interest within a period of a few weeks, even if they are not stable under physiological conditions [13]. $\alpha Fl_{red}5$ shows an "oxidase like" redox potential behaviour confirming the idea that positively charged residues in the vicinity of N(1) increase the redox potential. Furthermore this antibody indeed shows that interaction with the flavin N(1) directly influences the redox potential. However, the flavobody αFl_{ox} is the most remarkable result of this approach: this flavin binding antibody, that does not resemble a natural flavoprotein, shows that when ring C of the flavin is not involved in binding, the redox potential in turn is not significantly affected.

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Chapter 5

Accelerated oxidation of dihydroflavoquinone by a flavin-binding antibody*

5.1. Introduction

To date antibodies have been reported to catalyze a wide variety of chemical reactions, including redox reactions (for a review see [1]). However, in these antibodies the oxidation/reduction is directly performed by highly reactive compounds (such as hydrogen peroxide, sodium periodate or sodium borohydride) without the assistance of redox active groups in the antibody. Shokat *et al* [2] have shown that a reduced flavin-antibody complex was a more powerful reductant than free reduced flavin due to a shift to a more negative flavin redox potential upon antibody binding. The flavin, however, was not catalytically reduced in the antigen binding site. The goal of this work is to develop an antibody that can recycle a cofactor in its antigen binding site. As a first step we investigated the oxidation of reduced flavin in an antigen binding site. Additionally, we attempted to catalytically reduce the flavin in the antigen binding site.

Reduced flavin is very reactive towards molecular oxygen (O_2). The initial reaction probably proceeds by a one-electron reduction of O_2 (to superoxide) followed by a radical recombination reaction yielding the C(4a)-hydroperoxy-4a,5-dihydroflavin as an intermediate, which in turn disintegrates by either a homolytic or a heterolytic reaction of the C(4a)-OOH bond [3]. Flavoprotein oxidases and monooxygenases accelerate the oxidation of reduced flavin by a factor 100-1000 [4] suggesting stabilization of the hydroperoxide, for example by hydrogen bonds from the flavin N(5)H or the peroxide to amino acids, or by creating a hydrophobic environment for the flavin hydroperoxide [5].

5.2. Experimental procedures

5.2.1. Oxidation of antibody-bound reduced flavin

To investigate the influence of reduced flavin binding antibodies on the reoxidation rate of reduced flavin, we studied the production of superoxide (O_2^-) which is formed by a one electron transfer from reduced flavin to O_2. O_2^- is captured by nitro blue tetrazolium (NBT) to form blue formazan. 1 μM riboflavin (from Sigma) (for $\alpha Rf_{mod}G_1$) or N(10)-5'-carboxybutyl-flavin (for $\alpha Fl_{red}5$), 10 mM EDTA, 20 mM NBT (from

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Boehringer Mannheim) and various antibody concentrations in air-saturated buffer were illuminated with white light to reduce the flavin [6] during several time intervals of 2 minutes. After each period of light exposure, blue formazan was detected at its absorption maximum (ε_{560} = 10,4 $mM^{-1}cm^{-1}$, [7]). Antibody concentrations were chosen so that the percentage of bound reduced flavin varied from 0 to nearly 100%. At room temperature, experiments with $\alpha Rf_{mod}G_1$ were performed in 50 mM phosphate/150 mM NaCl pH 7.0, whereas experiments with $\alpha Fl_{red}5$ were performed in 100 mM phosphate/100 mM NaCl pH 7.5.

5.2.2. *Reduction of antibody-bound oxidized flavin*

To investigate the reduction of antibody bound flavin by NAD(P)H, 1 μM riboflavin was incubated with 50μM NAD(P)H (from Boehringer Mannheim) and 3 μM $\alpha Rf_{mod}G_1$ (sites) (more than 90% of the flavin is bound under these conditions) in PBS. To prevent reoxidation of reduced flavin, the solutions were deaerated by flushing with argon. Traces of oxygen were removed by adding 2 nM glucose oxidase (Boehringer Mannheim) and 0.1 mM glucose. Reduction of the flavin was followed, after excitation at 450 nm, on a SPF 500C Spectrofluorometer (SLM Aminco) at its emission maximum (525 nm).

To investigate the reduction of antibody bound flavin by benzoyl formic acid (see Fig.1, chapter 2), the experiment was set up as described under 5.2.1 with the exception that NBT was replaced by 10 mM benzoyl formic acid (from Aldrich).

5.3. *Results and discussion*

5.3.1. *Reoxidation of flavin in the antigen binding site (cofactor recycling)*

Starting-point to investigate the capability of antibodies in catalyzing cofactor recycling (reoxidation of reduced flavin in the antigen binding site) is the reactivity of reduced flavin with O_2. Monoclonal antibodies have been generated against reduced flavin: $\alpha Rf_{mod}G_1$ (against the hapten N(5)-benzoyl-N(10)-(ribityl succinamide ester) flavin [8]) and $\alpha Fl_{red}5$ (against 1,5-dihydro-N(10)-5'-carboxybutyl flavin [9]) (see Fig. 5.1). $\alpha Rf_{mod}G_1$ is a complete antibody (IgG_1 isotype) obtained via mouse immunization. On the contrary $\alpha Fl_{red}5$ is a single chain Fv antibody fragment [10], composed of the two antigen-binding domains connected by a peptide linker, generated entirely *in vitro* by antibody phage display technology. Binding of these antibodies to oxidized and reduced flavin was characterized by time-resolved fluorescence [8,9]: affinities of $\alpha Rf_{mod}G_1$ and $\alpha Fl_{red}5$ for reduced flavin are 0.07 and 0.6 μM and for oxidized flavin 6 and 100 μM, respectively.

To determine whether the protein environment of these antibodies can accelerate the oxidation of reduced flavin by O_2, superoxide (O_2^-), formed by electron transfer from reduced flavin to O_2, was trapped with nitro blue

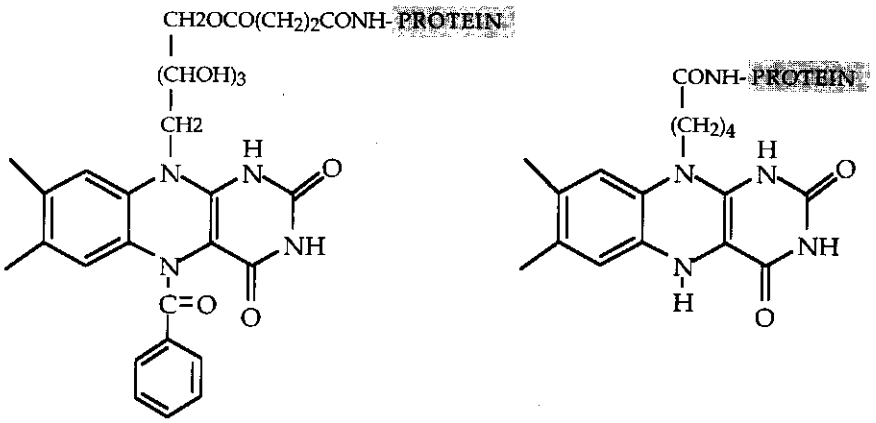

Fig. 5.1. Haptens for the antibodies $\alpha Rf_{mod}G_1$ (A) and $\alpha Fl_{red}5$ (B).

tetrazolium (NBT) resulting in the formation of blue formazan. For $\alpha Rf_{mod}G_1$, the O_2^- production rate (v) steeply increases at low antibody concentrations (Fig. 5.2A). However, at higher concentrations, the O_2^- production rate gradually declines. This suggests two competing processes: on the one hand antibody-bound reduced flavin is reoxidized faster than free reduced flavin, but on the other hand antibody-bound oxidized flavin can not be reduced by EDTA and light any more and is therefore withdrawn from the reaction mixture (Fig. 5.3). Indeed, in an experiment in which all flavin was bound to antibody in a fully anaerobic environment, the antibody-flavin complex could not be reduced as monitored by steady-state fluorescence. This is feasible because flavin and EDTA have to form an adduct to achieve reduction of the flavin [6]. Apparently antibody-bound flavin is not accessible for EDTA as is the case for many flavoproteins [6]. The acceleration factor (k_{bound}/k_{free}) for oxidation of antibody-bound reduced flavin compared to free reduced flavin was determined from:

$$v = k_{free} * Fl_{free} + k_{bound} * Fl_{bound}$$

where v is the superoxide production rate (in $nmol.min^{-1}$), k_{bound} and k_{free} are rate constants (min^{-1}) and Fl_{free} and Fl_{bound} are the amounts of free and antibody-bound reduced flavin present in solution (in nmol). The acceleration factor was determined for those points where the fraction bound oxidized flavin could be neglected, allowing determination of the amounts of free and bound reduced flavin from the dissociation constant

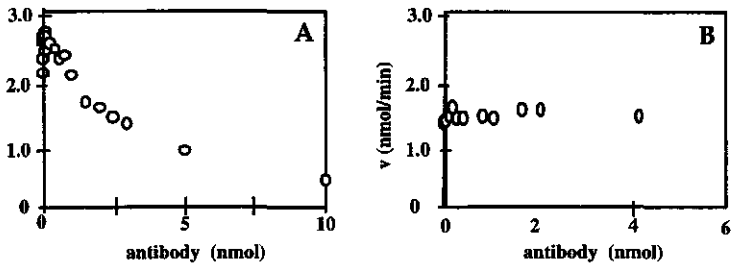

Fig. 5.2. Effect of reduced flavin binding antibodies on the rate of O_2^- production for 1 nmol flavin . A, $\alpha Rf_{mod}G_1$; B: $\alpha Fl_{red}5$.

of $\alpha Rf_{mod}G_1$ for reduced flavin. We found that oxidation of $\alpha Rf_{mod}G_1$ bound flavin is 12 times faster than that of free reduced flavin.

Figure 5.2B, showing the effect of $\alpha Fl_{red}5$, reveals a different curve: the O_2^- production rate is independent of the antibody concentration. Within this antibody concentration range we should have noticed an eventual acceleration and, given the K_d of $\alpha Fl_{red}5$ for oxidized flavin (100 μM), withdrawal of oxidized flavin does not play a role here. The acceleration of O_2^- production rate was $\alpha Rf_{mod}G_1$ specific, since an aspecific antibody (αBSA) did not show an effect on the O_2^- production rate in the range where $\alpha Rf_{mod}G_1$ showed a sharp increase.

Since the reaction rate strongly depends on the oxygen concentration if this is > 0.2 mM [3], small differences in temperature and atmospheric conditions influence the reaction rate considerably. This most likely is the explanation for the higher rate of O_2^- production in absence of antibody in Fig. 5.2A (compared to 2B) rather than small differences in pH or N(10) substituent of the flavin. N(10)-5'-carboxybutyl flavin (Fig. 5.2B) shows a two times higher O_2^- production compared to riboflavin.

The shift in redox potential (ΔE) upon binding of the flavin to the antibody reflects the flavin redox state stabilized by the antibody. ΔE values for two-electron transfer were calculated based on thermodynamic criteria [11] (see also Chapter 4) from the dissociation constants for oxidized and reduced flavin [8,9]. Both antibodies stabilize the flavohydroquinone: +56 mV and +65 mV shifts were determined, due to binding to $\alpha Rf_{mod}G_1$ and $\alpha Fl_{red}5$, respectively.

The antibody $\alpha Rf_{mod}G_1$ accelerates the O_2^- production rate 12 times. This is not the result of the redox potential shift since $\alpha Fl_{red}5$, that causes a comparable shift, does not influence the O_2^- production rate. Consequently, the rate acceleration must be due to the protein environment; this could either be an apolar pocket around N(5) or hydrogen bonds to N(5) or the hydroperoxide that stabilize the C4a-hydroperoxydihydroflavin and were induced by the N(5) benzoyl substituent in the hapten. This hypothesis is supported by the fact that the antibody $\alpha Fl_{red}5$, generated against 1,5-dihydroflavoquinone, hardly showed an effect on the O_2^- production rate. The latter is in agreement

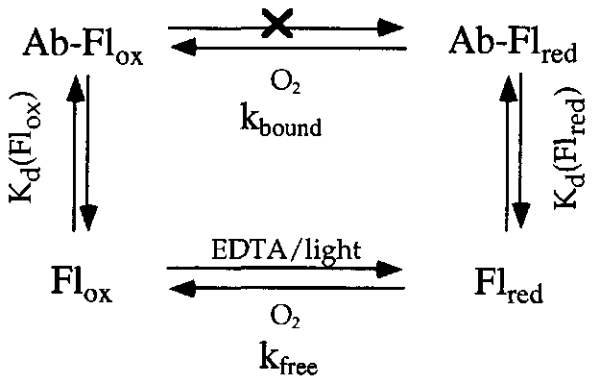

Fig. 5.3. Reaction scheme.

with the 3-dimensional model of the antigen binding site of $\alpha Fl_{red}5$ (see Chapter 4). From this model it is clear that there are no hydrogen bonds to the flavin N(5) or C(4a), neither are there hydrophobic residues in the vicinity of these two atoms.

The rate acceleration found for $\alpha Rf_{mod}G_1$ is 10-100 fold lower than for most oxidases. This could be related to the flavin conformation, i.e. planar versus bent. For example, in flavodoxins flavohydroquinone is forced into a planar conformation which probably makes it a more powerful reducing agent. In most oxidases the flavo-hydroquinone is also held in a flat conformation. Antibodies, however, are adapted to the conformation the flavin possesses during antibody selection. Therefore the flavohydroquinone must be in the bent conformation when bound to $\alpha Rf_{mod}G_1$ or $\alpha Fl_{red}5$.

5.3.2. *Reduction of antibody-bound flavin*

Now that we have proven that $\alpha Rf_{mod}G_1$ can accelerate the oxidation of reduced flavin, we have tried to reduce flavin specifically in the antigen binding site. However, flavin could not be reduced in the antigen binding site in the presence of benzoyl formic acid and light, NADH or NADPH.

5.4. *Conclusion*

Here, we have shown that antibodies are able to accelerate oxidation of reduced flavin within the antigen binding site. Comparison of two antibodies that bring about a comparable shift in redox potential, indicates that the reoxidation rate of reduced flavin is not dependent on the redox potential. However, the flavin could not be catalytically reduced in the antigen binding site. Therefore we must conclude that at present this flavobody cannot accomplish a catalytic redox cycle. On the one hand, screening more substrates might yield a compound that can reduce antibody bound flavin and so complete the cycle. On the other hand, it

might be wiser to do a step backwards and to redesign the hapten. Modulation of the flavin conformation via hapten design could yield a next generation of more efficient flavobodies and shed more light on the role of flavin conformation and redox potential in catalysis.

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Chapter 6

Summarizing discussion

6.1. Introduction

Nature provides a pool of thousands of different enzymes. These highly evolved catalysts selectively accelerate chemical reactions. Natural biocatalysts, however, are often not stable under the more extreme conditions under which chemical processes are performed, such as high temperature or pH. Moreover, there are many reactions for which no natural enzyme is known. Therefore, the development of artificial enzymes has been given serious attention the last decade.

These artificial enzymes can be divided in three categories: synzymes, molecular imprinted polymers (MIPs) and catalytic antibodies (or abzymes). The first category comprises supramolecular systems such as cyclodextrines and porphyrins that are designed to form a substrate-binding pocket containing catalytically active groups. The second category consists of polymers formed in the presence of a target molecule that resembles a transition state of a reaction and acts as a template for polymerization; upon removal of the template, a specific cavity is created. The third category, catalytic antibodies, has been extensively described in 1.3 of this thesis. Compared to natural enzymes, that accelerate reactions up to 10^{17} fold [1], artificial enzymes are slow. Catalytic antibodies are generally more efficient than synzymes or MIPs; the best catalytic antibodies give rate accelerations of 10^6 fold whereas synzymes at their best give 10^3-10^4 fold accelerations and MIPs are even slower with 10-100 fold rate enhancements [2,3]. Thus catalytic antibodies are at present the best artificial enzyme system, which is not surprising since catalytic antibodies use the same building blocks as enzymes (i.e. amino acids). These highly versatile building blocks differ in size, shape, charge, polarity and chemical reactivity and thus form a reservoir of chemical reactivity that can be applied to build tailored biocatalysts. Moreover, catalytic antibodies are, compared to the other artificial enzymes, relatively easy to generate and to modify.

For these reasons, we chose the catalytic antibody system to attain our goal: the development of an artificial enzyme mimicking a redox enzyme (discussed below).

6.2. Design and development of a redox-active catalytic antibody

To extend antibody catalysis to cofactor-dependent redox reactions, we chose to mimic flavoprotein oxidases. To achieve this goal the antibody must bind flavin ("flavobody") and thus be elicited against a flavin (analogue). We designed a flavin-based hapten, that served a threefold goal. Firstly, it should be in a reduced flavin conformation since in

oxidases the flavin cofactor is reduced upon substrate oxidation. Secondly, it should also introduce a substrate binding pocket in the antigen binding site. Thirdly, it must introduce a hydrophobic pocket adjacent to N(5) or hydrogen bonds to the N(5) or the C4a position of the flavin, since this will facilitate reoxidation of the flavin in the antigen binding site. The hapten N(5)-benzoyl-N(10)-(ribityl succinimide) ester flavin seems to serve this threefold goal. The N(5) substituent both stabilizes the reduced conformation of the flavin (since 1,5-dihydro reduced flavin is not stable) and will induce a hydrophobic pocket adjacent to N(5) that could both serve as a substrate binding pocket and facilitate reoxidation of the flavin.

The antibody $\alpha Rf_{mod}G_1$, generated against this hapten, using traditional hybridoma technology (see 1.4.1), stabilizes the reduced flavin conformation as was proven by time-resolved polarized fluorescence binding studies with oxidized and reduced flavin (Chapter 2). A +56 mV shift in redox potential compared to free flavin ($\Delta E_0'$) for the two-electron reduction, was calculated from the dissociation constants for reduced and oxidized flavin (0.07 and 6 μM, respectively). $\alpha Rf_{mod}G_1$ accelerates the reoxidation of reduced flavin 12 times, which is only 10-100 times less than for most oxidases (chapter 5). We ascribed this difference with oxidases to the flavin conformation, i.e. planar versus bent. For example, in flavodoxins flavohydroquinone is forced into an energetically less favorable planar conformation which probably makes it a more powerful reducing agent. In most oxidases the flavohydroquinone is also held in a flat conformation. Antibodies, however, are adapted to the conformation the flavin possesses during antibody selection. Therefore the flavohydroquinone must be in the bent conformation when bound to $\alpha Rf_{mod}G_1$.

In contrast, the scFv antibody fragment $\alpha Fl_{red}5$, elicited via an alternative strategy against (reduced) 1,5-dihydroflavin (Chapter 3), did not show an effect on the reoxidation rate of reduced flavin. This antibody causes a comparable shift in E_0'. Consequently, the rate acceleration must be due to the specific protein environment induced by the N(5) benzoyl substituent in the hapten.

Unfortunately, $\alpha Rf_{mod}G_1$ bound flavin could not be reduced by NADH, NAD(P)H or benzoyl formic acid. Therefore we must conclude that at present this flavobody cannot accomplish a catalytic redox cycle. On the one hand, screening more substrates might yield a compound that can reduce antibody bound flavin and so complete the cycle. On the other hand, it might be wiser to do a step backward and to redesign the hapten. For example, a C(5) substituted 5-deazaflavin will have the advantage of a flat conformation which will probably enhance the flavin reoxidation rate. Moreover, the N(5) substituent should be more carefully designed with a direct relation to the substrate to be used and preferentially possess some features of the transition state conformation of the substrate (for example a imine like N(5) substituent to mimic alanine oxidation).

6.3. Antibodies against an unstable hapten

Antibody technology has made a great stride forward since the development of hybridoma technology in 1975 [4]. The possibility to display functional antibody fragments at the tip of filamentous phage has allowed the development of antibody phage libraries. At present antibodies can be generated totally *in vitro* from naive (i.e. derived from an unimmunized source) phage display libraries [5]. With this technique animal cell culture and even mouse immunization are by-passed. Moreover, we demonstrated that antibodies can even be generated against the unstable hapten 1,5-dihydroflavin. This reduced flavin rapidly reoxidizes under aerobic conditions and can therefore not be used directly for the generation of antibodies in (the aerobic environment of) a mouse. However, using a naive antibody scFv phage display library, we could perform the selection under anaerobic and reducing conditions and were able to select an antibody, $\alpha Fl_{red}5$, that specifically binds 1,5 dihydro-reduced flavin (Chapter 3).

6.4. Regulation of the flavin redox potential by flavobodies

The role of the protein environment of the flavin cofactor in modulating its redox potential is still not thoroughly understood. Up till now the most comprehensive studies have been performed on flavodoxins, but other flavoproteins such as D-amino acid oxidase have been investigated too in this respect, although not as thoroughly [6]. In order to contribute to a better understanding of this issue, we have generated scFv antibody fragments against reduced (see chapter 3) and oxidized flavin (see chapter 4). These flavobodies are characterized by time-resolved and steady-state fluorescence spectroscopy and ELISA methods (mapping) and molecular modelling. The 3-dimensional models of the antigen binding sites were in good agreement with the experimental results. Binding of $\alpha Fl_{red}5$ to flavin increases the redox potential, mainly due to an arginine residue interacting with the flavin N(1). This was in agreement with literature data on oxidases claiming that positive residues in the vicinity of ring C of the flavin (see fig. 1.2, chapter 1) increase the flavin redox potential. However, the flavobody αFl_{ox} is the most remarkable result of this approach: this flavin binding antibody, that does not resemble a natural flavoprotein, shows that when the electron deficient ring C of the flavin is not involved in binding, the redox potential is not significantly affected (chapter 4).

In conclusion, we like to state that flavobodies form tailored protein environments that can be selected from antibody phage display libraries against different flavin analogues, even if they are not stable under physiological conditions, within a period of a few weeks. For these reasons, antibody fragments are novel tools, additional to natural proteins, to study the effect of different protein environments on the flavin redox potential. Moreover, when expressed in *E.coli*, primary

sequence information can easily be obtained and mutations can readily be performed which could give additional information on the effect of a specific amino acid on the flavin redox potential.

6.5. *The future of redox-active flavobodies*

We have shown that it is possible to generate antibodies that influence the flavin redox potential, even in a fashion comparable with natural flavoproteins (chapter 4). We have also shown that antibodies are capable of catalyzing the reoxidation of reduced flavin, like oxidases (Chapter 5). However, we failed to develop an antibody that catalyzes flavin reduction by substrate binding. The results described here taught us that the development of a redox active flavobody is an ambitious project. First of all a better hapten should be designed (and synthesized) that will lead to an antibody that can bind both the flavin cofactor and the substrate in the correct conformation and orientation. Moreover, it should induce those amino acids that regulate the flavin redox potential in the desired way (based on current knowledge) and which activate the substrate when placed in the antigen binding site.

The second step is the generation of antibodies against such a hapten. This can be performed either *in vivo* or *in vitro*. *In vitro* strategies have the advantage that the hapten does not necessarily have to be stable under physiological conditions (Chapter 3). Moreover, different elution strategies can be applied in the panning procedure as described in Chapter 3. Here, our goal was to select antibodies specific for reduced flavin. In order to remove cross-reactive antibodies (i.e. antibodies that also bind oxidized flavin), we performed a pre-elution step with oxididized flavin prior to eluting the (specific) reduced flavin binding antibodies with reduced flavin. This strategy yielded an antibody population enriched for reduced flavin binding. This approach might be extended, for example, if one wants to select for antibodies binding ring C of the flavin. In that case pre-elution with a flavin analogue substituted on ring C (thus eluting ring A binders) might be useful. On the other hand, with *in vitro* selection of antibodies from naive antibody phage display libraries, the chance of success is dependent on the library size and diversity: large and diverse libraries give a higher chance of selecting antibodies with the desired properties (here we used a library with a repertoire size of 10^8, at present, however, repertoires of $>10^{10}$ are available).

These improved selection strategies, however, are related to selection for binding and not for catalysis. For this much more powerful selection strategies are required. For enzymes a very elegant selection strategy, based on phage display (see 1.4.3), has been developed to select for catalytic activity [7]. In this approach enzymes displayed on phage are selected by their catalytic activity using a suicide inhibitor linked to a biotin. Active phage enzymes were covalently attached to the suicide inhibitor and could be captured with streptavidin-coated beads. For flavoenzymes suicide inhibitors have been reported [8] that inactivate D-amino acid oxidase via

modification of an active site histidine. Using such an inhibitor, one can select for the presence of a histidine in the antigen binding site. If, based on such inhibitors, a selection system for redox active flavobodies could be developed, this would greatly enhance the chances of selecting a catalytic antibody. The best strategy would be first to select a large and diverse repertoire of phage antibodies against an optimally designed hapten, and then to randomly mutate these first generation flavobodies and select them for covalent binding to suicide inhibitors.

6.6. Conclusion: the future of catalytic antibodies in general

Although scientists have been quite successful in developing catalytic antibodies for a wide variety of chemical reactions, most catalytic antibodies are modest catalysts at best. This is not surprising since the evolution of enzymes has been going on for millions of years whereas catalytic antibodies are only in the first stage of their "evolution". During their evolution enzymes have been taught not only to bind selectively the transition states of their substrates but also to participate directly in bond making and breaking. Most catalytic antibodies only have been selected for binding a transition state analogue, although a few strategies for direct selection for catalysis. The principle of this approach is that a catalytic antibody is expressed in bacteria lacking an enzyme that is crucial to survive; only those bacteria that express a catalytic antibody that catalyzes the same reaction as the missing enzyme will survive. Improving catalysis by mutagenesis ("evolution") has been described (for example [9]).

One of the problems in selecting catalytic antibodies is to "fish" a weak antibody catalyst out of a pool of enzymes simultaneously produced by the hybridoma or bacterial cell. An enzyme contamination of less than 0.1% in the purified antibody sample can catalyze substrate conversion and insinuate that it is the antibody that is catalytically active. We have been working on a catalytic antibody, elicited against the hapten N(5)-benzoyl-N(10)-(ribityl succinimide) ester flavin, that seems to show phosphodiesterase activity to several carbohydrate-phosphate esters. In a way this is not surprising since these antibodies bind carbohydrates due to the carbohydrate substituent at the N(10) of the flavin hapten. However, we found that the hybridoma cells producing this antibody also produce several phosphodiesterases that could stick to the antibody during the purification steps and confuse the measurements for catalytic activity.

It remains to be seen if it is worth the effort to develop catalytic antibodies for reactions for which there are also enzyme equivalents. In this case the only benefit could be that the process of designing and developing these antibodies teaches us more about how enzymes work [2]. However, there are other cases where catalytic antibodies, despite the efforts it costs to construct them, may be useful: for synthesis of compounds for which no enzyme alternative exists (for example [10,11]) and for prodrug activation used in therapy [12,13]. In the latter case the present situation is that enzymes are linked to antibodies recognizing

tumor epitopes; the enzymes activate prodrugs for efficient tumor killing. The problem is that most enzymes are of non-human origin and therefore give rise to an immune response of the patient against the enzyme. Humanized catalytic antibodies may replace these enzymes and thus circumvent this problem. In conclusion, there are practical applications for catalytic antibodies that are worth the efforts and costs to generate them. In other cases the scientific challenge is to learn more about enzymes by mimicking their evolution in an (relatively) extremely short period of time (i.e. decades).

6.7. References

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Abbreviations

BSA	bovine serum albumine
CDR	complementarity determining region
ELISA	enzyme-linked immunosorbent assay
F_{ab}	a light chain dimerized to V_H-C_H1
FMN	flavin mononucleotide
Fl_{ox}	N(10)-5'-carboxybutyl-flavin (see chapter 3)
Fl_{red}	N(10)-5'-carboxybutyl-1,5-dihydroflavin (see chapter 3)
FR region	framework region
MEM	maximum entropy method
MIP	Molecular Imprinted Polymer
PBS	phosphate-buffered saline
scFv	single chain Fv fragment
V domain	antibody variable domain
αFl_{ox}	scFv against N(10)-5'-carboxybutyl-flavin
αFl_{red}	scFv against N(10)-5'-carboxybutyl-1,5-dihydroflavin
$\alpha Rf_{mod}G_1$	IgG_1 against the modified riboflavin 3a (see chapter 2)
$\alpha Rf_{mod}M$	IgM against the modified riboflavin 3a (see chapter 2)

Samenvatting voor niet-vakgenoten

In ons dagelijks leven spelen enzymen een vitale rol. Enzymen zijn eiwitten die in staat zijn chemische reakties selectief en veelal meer dan een miljard keer te versnellen. In ons lichaam verlopen bijna alle processen met behulp van enzymen. Kenmerkend voor enzymen is dat ze in het algemeen onder milde reaktiecondities werken en minder ongewenste bijprodukten geven dan "klassieke" chemische processen. Vanwege deze redenen is ook de industrie in toenemende mate geïnteresseerd in het gebruik van deze "schone" biokatalysatoren. Een bekend voorbeeld zijn de proteases (eiwit splitsende enzymen) en lipases (vetsplitsende enzymen) in wasmiddelen die eiwit- en vetvlekken uit textiel kunnen verwijderen. Een ander voorbeeld is het gebruik van het pectinase om de helderheid van vruchtesappen en wijn te verbeteren.

Enzymen hebben echter als nadeel dat de milde reaktiecondities waaronder ze werken soms ook ongunstig kunnen zijn: als het enzym traag werkt onder de gegeven condities, kan dit bijna niet verbeterd worden door bv. de temperatuur te verhogen of de pH (zuurgraad) te veranderen, omdat dit vaak invloed heeft op de stabiliteit van het enzym. Bovendien zijn er ook tal van reakties te bedenken waarvoor geen natuurlijk voorkomend enzym beschikbaar is. Dit heeft de laatste jaren geleid tot een toenemende interesse in het ontwikkelen van artificiële enzymen (ook wel synzymen genoemd), die de bestaande verzameling van natuurlijke enzymen moeten uitbreiden.

Een inmiddels beroemde klasse van artificiële enzymen zijn de katalytische antilichamen (ook wel abzymen genoemd), die het onderwerp van dit proefschrift zijn. Om te begrijpen hoe deze katalytische antilichamen werken, moet we eerst wat meer in detail kijken naar hun natuurlijke tegenhangers, de enzymen. In een enzym vindt de reaktie plaats in een holte van het eiwitmolekuul, het actieve centrum. Hier worden de substraten (= stoffen die door het enzym omgezet worden) gebonden en zijn katalytische groepen aanwezig die ervoor zorgen dat de reaktie verloopt. De katalytische groepen in het actieve centrum zijn zodanig gerangschikt, dat selectief een bepaalde groep van het substraat wordt omgezet.

Het principe van katalytische antilichamen is nu dat in het antilichaam het actieve centrum van een enzym nagebootst wordt. Antilichamen (ook wel antistoffen of afweerstoffen genoemd) zijn, net als enzymen, grote eiwitmolekulen. Ieder mens of dier heeft antilichamen in zijn bloed, die als taak hebben om ervoor te zorgen dat lichaamsvreemde stoffen onschadelijk gemaakt worden. Dit gebeurt door deze stoffen te binden en af te voeren. Men kan zo'n proces bewust opwekken door een proefdier, meestal een muis, in te spuiten (immuniseren) met een lichaamsvreemde stof (antigeen). Als men nu dit antigeen zodanig kiest dat het precies past in het actieve centrum van een enzym, dan zal de antigeenbindingsplaats van het antilichaam opgewekt tegen dit molekuul, op zijn beurt weer veel moeten lijken op het actieve centrum van het

overeenkomstige enzym. Op deze manier kan men dus een katalytisch antilichaam maken. Alvorens hier dieper op in te gaan, zullen eerst de verschillende methoden om antilichamen te maken nader uitgelegd worden omdat zij een belangrijke rol hebben gespeeld in het tot stand komen van dit proefschrift.

Antilichaamtechnologie

Zoals al eerder genoemd, kunnen antilichamen opgewekt worden in muizen door immunisatie met een antigeen. Na immunisatie is het essentieel om de lichaamscellen van de muis die de antilichamen produceren in handen te krijgen en wel op een zodanige manier dat ze buiten de muis (*in vitro*) verder kunnen groeien. Meestal worden hiervoor de miltcellen uit de muis geïsoleerd. De milt is een orgaan dat rijk is aan antilichaamproducerende cellen. Deze miltcellen kunnen echter niet zomaar verder groeien in bv. een reageerbuis. Onderzoekers uit Engeland bedachten in 1975 een oplossing voor dit probleem. Zij fuseerden de miltcellen met myelomacellen. Myelomacellen zijn kankercellen d.w.z. cellen die zich oneindig blijven delen en dus het vermogen hebben om *in vitro* te groeien. In de gefuseerde cel, de hybridomacel, wordt het antilichaam producerende vermogen van de miltcel gecombineerd met het vermogen om *in vitro* te groeien van de myelomacel, waardoor er een klein antilichaamfabriekje gevormd wordt. Elke afzonderlijke hybridomacel produceert een specifiek (monoklonaal) antilichaam. De ontwikkeling van deze hybridomatechniek betekende een belangrijke wetenschappelijke doorbraak waarvoor de uitvinders zelfs de Nobel prijs ontvingen. In hoofdstuk 2 is beschreven hoe we met behulp van deze techniek antilichamen hebben gemaakt.

Hoe mooi bovenstaande techniek ook is, er kleven toch nadelen aan. Zo moet de eerste stap in het antilichaamgeneratie proces toch door een proefdier, de muis, gedaan worden. Bovendien is de uiteindelijke antilichaamproducent, de hybridomacel, nog steeds van dierlijke origine en daardoor veel lastiger te kweken en genetisch te manipuleren dan bijvoorbeeld een bacteriële cel. Genetische manipulatie (d.w.z. het aanbrengen van veranderingen in het erfelijk materiaal dat codeert voor een eiwit) is met name van belang als men de eigenschappen van een antilichaam wil verbeteren.

De laatste jaren is hard gewerkt aan een methode om antilichamen volledig *in vitro* te maken m.b.v. een bacterieel systeem. In deze benadering wordt het immuunsysteem als het ware nagebouwd in een reageerbuis. Het systeem maakt gebruik van bacteriofagen (virussen die bacteriën als gastheer gebruiken) en stukjes erfelijk materiaal (DNA), die de genetische code bevatten om antilichamen te produceren. Bacteriofagen zijn niets anders dan een stukje DNA met een eiwitmantel eromheen. Wanneer antilichaam DNA op de juiste plaats in het genetische materiaal van de bacteriofaag wordt gebracht, ontstaan er fagen met antilichamen op hun oppervlak.

De volgende stap is dan om in elke bacteriofaag een verschillend stukje antilichaam te zetten. Zo ontstaat er een grote verzameling fagen met allemaal een ander antilichaam op hun oppervlak, een zogenaamde antilichaam bibliotheek. Uit deze bibliotheek kunnen vervolgens die antilichamen geselecteerd worden die binden aan een bepaald antigeen. Dit gebeurt door het antigeen te immobiliseren op een oppervlak (bijvoorbeeld de wand van een plastic buisje) en de faag-antilichamen hier aan toe te voegen. Sommige antilichamen kunnen het antigeen binden en blijven dan ook aan het oppervlak plakken. De niet bindende fagen worden weggespoeld. Het is hierbij belangrijk om te bedenken dat uit één bibliotheek antilichamen tegen verschillende antigenen geselecteerd kunnen worden.

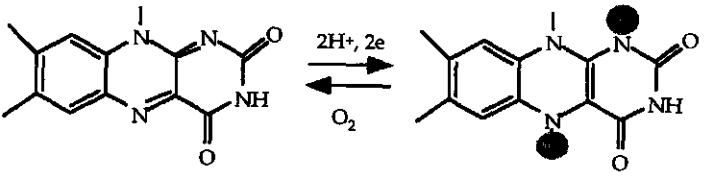
Een geheel nieuwe toepassing van deze techniek is beschreven in hoofdstuk 3. Wij hebben een faag-antilichaam bibliotheek (gekregen van één van de grondleggers van deze techniek, Dr. Greg Winter uit Cambridge, Engeland) gebruikt om antilichamen te selecteren tegen een instabiel antigeen, gereduceerd flavine (zie figuur 1A). Dit gereduceerde flavine is zeer gevoelig voor zuurstof, onder invloed hiervan oxideert het zeer snel. Het is duidelijk dat tegen dit molekuul nooit antilichamen gemaakt hadden kunnen worden via muis immunisatie: het is immers onmogelijk om een muis in een zuurstofvrij hokje te plaatsen. Fagen zijn echter niet afhankelijk van zuurstof en daarom kan een faag-antilichaam bibliotheek prima gebruikt worden om antilichamen te maken tegen gereduceerd flavine. Wij hebben het hele selectieproces simpelweg onder zuurstofvrije (anaerobe) condities uitgevoerd en op deze manier antilichamen verkregen die zeer specifiek binden aan gereduceerd flavine.

Katalytische antilichamen met redox eigenschappen

Wij hebben bovenstaande technieken gebruikt om een katalytisch antilichaam te bouwen dat redox reakties uit kan voeren. Een redox reaktie is een reaktie waarbij electronen overgedragen worden: als de electronen worden onttrokken aan een verbinding spreken we over oxidatie, als de electronen worden toegevoegd over reductie. Redox-enzymen maken voor dit soort reakties gebruik van een extra redox-actieve groep. Een bepaalde klasse redoxenzymen, de flavoenzymen, gebruikt daarvoor flavine. Ons doel was om zo'n flavoenzym na te bouwen en wel zodanig het een oxidatie reaktie uit kan voeren.

Als een substraat geoxideerd wordt, worden er electronen aan onttrokken. Het zal duidelijk zijn dat deze electronen ergens naar toe moeten. Flavine is erg geschikt als electronen acceptor. Het flavine molekuul wordt daarbij wel zelf gereduceerd en zal dus vervolgens weer geoxideerd (ofwel gerecycled) moeten worden om opnieuw betrokken te kunnen worden bij substraatoxidatie. Een zeer geschikt molekuul om de electronen van flavine over te nemen is zuurstof (waarbij waterstofperoxide gevormd wordt). De strukturen van geoxideerd en gereduceerd flavine zijn weergegeven in figuur 1A.

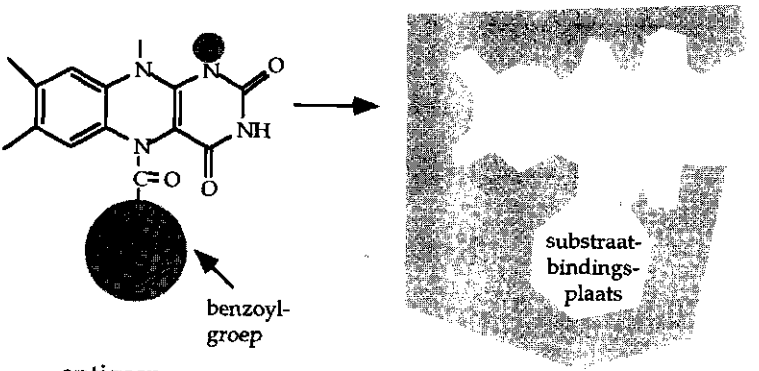
A:



geoxideerd flavine
(vlak)

gereduceerd flavine
(gebogen)
(instabiel)

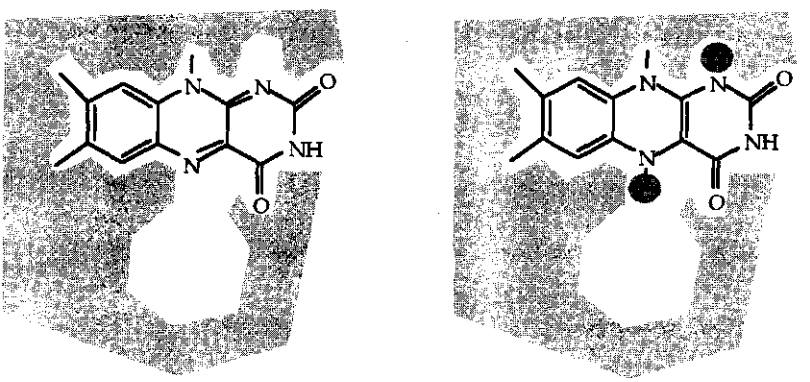
B:



antigeen
(gebogen)
(stabiel)

antilichaam

C:



antilichaam + geoxideerd flavine

antilichaam + gereduceerd flavine

Figuur 1

De antilichamen die dit proces moeten nabootsen, moeten dus in staat zijn om flavine te binden en vervolgens om dit flavine te reduceren, waarbij dan een substraat molekuul geoxideerd wordt. De drijvende kracht achter deze reaktie moet dan zijn dat het gereduceerde flavine beter in de antigeenbindingsplaats past dan geoxideerd flavine (gereduceerd en geoxideerd flavine hebben verschillende conformaties). Anderzijds moet het ook weer niet te goed passen want het flavine moet ook weer opnieuw geoxideerd kunnen worden. Bovendien moet er in de antigeen-bindingsplaats naast ruimte om het flavine te binden, ook ruimte zijn om het substraat te binden en wel zodanig dat het dicht bij het flavine komt te zitten zodat de electronen gemakkelijk overgedragen kunnen worden.

Om aan al deze voorwaarden te kunnen voldoen, hebben we antilichamen opgewekt tegen het antigeen dat is weergegeven in figuur 1B. Dit antigeen lijkt op gereduceerd flavine, maar is in tegenstelling tot echt gereduceerd flavine, niet gevoelig voor zuurstof. Dit komt door de stabiliserende werking van de benzoyl groep. Een antilichaam opgewekt tegen het gebogen gereduceerd flavine antigeen, zal een antigeen-bindingsplaats hebben waar het vlakke geoxideerde flavine veel minder goed in past (zie figuur 1C). Het vlakke geoxideerde flavine zal zich dus graag willen buigen, waarvoor het natuurlijk wel electronen nodig heeft, die geleverd moeten worden door een substraat. De extra benzoylgroep die aan het flavine-antigeen gezet is, heeft niet alleen een stabiliserende invloed, maar creëert ook een holte in de antigeen-bindingsplaats die ruimte biedt aan het substraat (zie figuur 1C). Bovendien zal de benzoylgroep ook een dusdanige eiwitomgeving moeten creëren dat het gebogen flavine ook weer gemakkelijk zal moeten kunnen reoxideren tot de vlakke structuur. In hoofdstuk 2 is beschreven hoe dit veelzijdige antigeen gesynthetiseerd is en hoe antilichamen hier tegen zijn opgewekt. Naderhand is bestudeerd hoe deze antilichamen binden aan gereduceerd en geoxideerd flavine.

In hoofdstuk 5 staat beschreven hoe we het katalyse proces bestudeerd hebben. Het blijkt dat een van de antilichamen, opgewekt tegen het hierboven beschreven antigeen, het flavinereoxidatie proces meer dan 10 keer versnelt. Vergelijkbare enzymen versnellen deze reaktie 100-1000 keer. De reductie van geoxideerd flavine kan echter niet in de antigeenbindingsplaats plaatsvinden, waardoor de katalytische cirkel dus nog niet rond is. Wat dat betreft kunnen we dus stellen dat het gelukt is om een antilichaam op te wekken dat als het ware voor de helft katalytisch actief is.

De moleculaire basis van de flavine binding door antilichamen

Naast het ontwikkelen van een redox actief antilichaam, hebben we ook onderzocht hoe nu precies geoxideerd en gereduceerd flavine op moleculair niveau gebonden worden in de antigeenbindingsplaats. Anders gezegd: waarom bindt het ene antilichaam beter aan gereduceerd flavine, het andere antilichaam aan geoxideerd flavine en bindt weer een

ander antilichaam beide redoxtoestanden even goed. Het verschil in binding tussen de verschillende flavine redoxtoestanden wordt uitgedrukt in de redoxpotentiaal. Deze redoxpotentiaal is bepaald voor twee antilichamen, die beide verkregen zijn uit een faag-antilichaam bibliotheek. Deze antilichamen zijn vervolgens uitgebreid bestudeerd voor wat betreft binding aan verschillende flavines, onder andere met behulp van fluorescentie spectroscopie. Daarnaast is van deze antilichamen de DNA sequentie (genetische code) bepaald en op basis hiervan zijn 3-dimensionale modellen van het antilichaam gebouwd (in nauwe samenwerking met Dr. Annemarie Honnegger uit de groep van Prof. Andreas Plückthun (Universität Zürich). Deze modellen zijn vergeleken met de experimentele gegevens en een en ander blijkt goed met elkaar te kloppen. Dit werk staat uitgebreid beschreven in hoofdstuk 4.

Tot slot wordt het onderzoek in een bredere context geplaatst en worden de (on)mogelijkheden van katalytische antilichamen bediscussieerd (hoofdstuk 6).

Curriculum vitae

Yvonne Evelien Bruggeman werd op 28 oktober 1967 geboren in Almelo. In 1986 behaalde zij het VWO diploma aan het Twents Carmellyceum te Oldenzaal en begon zij de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit in Wageningen. De doctoraalfase omvatte twee afstudeervakken aan de Landbouwuniversiteit (Organische Chemie en Biochemie) en een stage aan het Nederlands Kanker Instituut te Amsterdam (Immunologie). In maart 1992 studeerde zij cum laude af en trad als assistent in opleiding in dienst van de vakgroep Biochemie van de Landbouwuniversiteit Wageningen. Vanaf augustus 1996 is zij werkzaam op het Unilever Research Laboratorium te Vlaardingen.